J. Physiol. (1966), **185**, *pp.* 224–238
With 2 plates and 7 text-figures
Printed in Great Britain

ENTRY OF FLUORESCENT DYES INTO THE SARCOTUBULAR SYSTEM OF THE FROG MUSCLE

BY M. ENDO*

From the Department of Physiology, University College London

(*Received* 4 *January* 1966)

SUMMARY

1. A fluorescence microscope was used in order to detect whether fluorescent dyes can quickly diffuse into and out of certain parts of single muscle fibres of the frog.

2. Several fluorescent dyes entered a system arranged at the centre of each I-band without entering the main part of the fibre.

3. The dyes quickly diffused out of the fibres on washing, becoming invisible within a few minutes.

4. In a hypertonic solution the fluorescent striations were more conspicuous and faded more slowly.

5. It is concluded that the dyes have access to some component of the triads.

6. The 'dye space' measured with Lissamine Rhodamine B 200 was 1–2 % of the fibre volume, and the implication of this value is discussed.

7. From an analysis of the time course of entry or exit of the dye it is suggested that the principal resistance to radial diffusion of the dye is distributed along the tubules rather than at the mouths of the tubules.

INTRODUCTION

A. F. Huxley & Taylor (1955, 1958) have shown that a moderate depolarization of small areas of frog muscle membrane caused a localized contraction only if the micropipette that was used as a current electrode was placed opposite a Z-line. This was the place where the triad system is situated in frog muscle (Porter & Palade, 1957), and they suggested that the first step of the link between excitation and contraction might be a conduction of depolarization from the surface to the interior through some component of the triad system. This would require that the electrical resistance between lumen of the triad system and extracellular space should be low. Since then, several electrophysiological phenomena have also

* Present address: Department of Pharmacology, Faculty of Medicine, University of Tokyo, Japan.

been interpreted in terms of effective continuity between sarcotubular system and extracellular space. Thus, Hodgkin & Horowicz (1960*a*) found that potassium ions in an external solution acted more slowly in altering the membrane potential of muscle fibres than chloride ions. They interpreted this observation by assuming that the permeability to potassium ions was rather selectively located in the tubular membrane while the permeability to chloride ions was in the surface membrane, and hence the effect of changing potassium concentration had a latent period necessary to equilibrate the concentration of the ion inside the tubules with that in the external solution. Adrian & Freygang (1962) showed that when a constant hyperpolarizing current was applied to the muscle fibre membrane, the apparent membrane conductance fell slowly, with a time constant of the order of several hundred milliseconds. They suggested that this was due to the decrease in the concentration of potassium in the tubules whose wall was again assumed to be the only site permeable to potassium ions. Fatt (1964) measured the transverse impedance of muscle fibres over a wide range of frequencies, and found that it was successfully analysed by assuming two components one of which was regarded as the contribution of the tubular system. Freygang, Goldstein & Hellam (1964) and Freygang, Goldstein, Hellam & Peachey (1964) showed that in the negative after-potential of frog fibres there is a long-duration component whose magnitude is proportional to the number of impulses of tetanic activity, and they interpreted this as due to an accumulation in the tubular system of potassium ions which left the fibre during the action potentials.

All these phenomena suggest strongly that the lumen of the tubular system is continuous with the extracellular space. Actual openings that would provide such continuity have not been seen with the electron microscope in muscle from adult frogs, although they have been clearly shown in mammalian heart muscle (Simpson & Oertelis, 1961; Nelson & Benson, 1963) and in muscle from a fish and a tadpole (Franzini-Armstrong & Porter, 1964). However, the possibility remains that even if there is a membrane at the mouth of the tubular system of frog fibres, its permeability to ions may be very high, resulting in effective continuity, or that there may be openings in the living state which are obliterated during fixation or other preparatory procedures for electron microscopy.

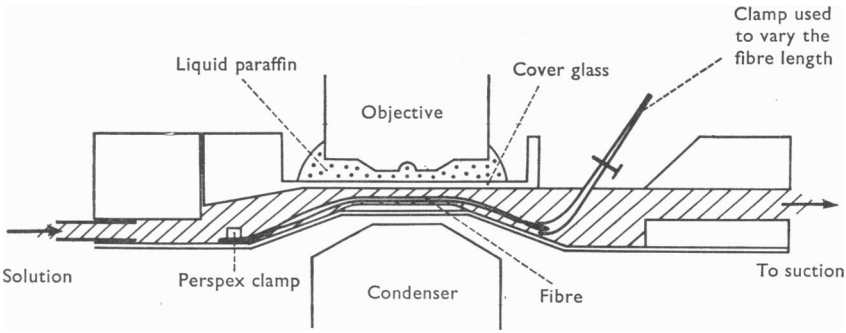
An experiment to test this possibility, first proposed by Professor A. L. Hodgkin, would be to immerse a fibre in a solution of a fluorescent dye, wash quickly with a dye-free solution, and use a fluorescence microscope to find whether the dye is present at the position of the triads. This has been done in the work described in this paper, and a positive result was obtained, as has already been briefly reported (Endo, 1964*a*, *b*). Since this

work was completed, independent evidence along the same lines, but using electron microscopy or autoradiography to detect the material entering the tubules, has appeared (H. E. Huxley, 1964; Page, 1964; D. K. Hill, 1964).

METHODS

Material and general procedure. Single muscle fibres were isolated from the semitendinosus muscle of frogs (*Rana temporaria*). Care was taken to remove connective tissue as far as possible, because any remaining pieces were strongly stained with the fluorescent dyes and interfered with observation of structures within the muscle fibres.

Fibres were tightly fixed in a trough mounted on the stage of a microscope, as shown in Text-fig. 1, so that they should not move away from the field of view of the microscope when a solution was perfused quickly through the trough. Fibres were in contact with the bottom of the trough. The maximum flow rate during perfusion was about 1·6 ml./sec, the dead space of the system being less than 0·3 ml. Ringer solution contained (mM) NaCl 115, KCl 2·5, $CaCl_2$ 1·8, Na_2HPO_4 2·15, and NaH_2PO_4 0·85.



Text-fig. 1. Arrangements for holding the fibre under the microscope and changing the solution around the fibre quickly. The trough is made of Perspex and glass. The drawing is approximately to scale except for the depth of the trough, which is exaggerated.

Microscopy. The fluorescence microscope consisted of a high-pressure mercury discharge lamp and an ordinary light microscope, with the filter system described below.

The objective was an oil immersion of focal length 3·75 mm, N.A. 0·95, modified by the manufacturers (Cooke, Troughton & Simms, Ltd.) to have a concave front surface with centre of curvature in the object plane. Hence, the image-forming rays always entered the lens perpendicular to the surface, and the image remained well corrected whatever the refractive index of the immersion medium. A cover glass had to be used in order to prevent flooding during rapid flow of solutions; the spherical aberration introduced by the difference of refractive index between the Ringer solution and the cover glass was neutralized by using, between the cover and the objective, a liquid of intermediate refractive index (paraffin oil). The effective N.A. of the objective (proportional to refractive index of immersion fluid) was then 0·89. The focal length of the condenser used was 7 mm and its N.A. 0·95.

The microscope had to be easily converted to a polarizing microscope so as to relate the fluorescent image of the fibre to the striation patterns. This was achieved by the system shown in Text-fig. 2, in which turning a mirror was the only necessary action.

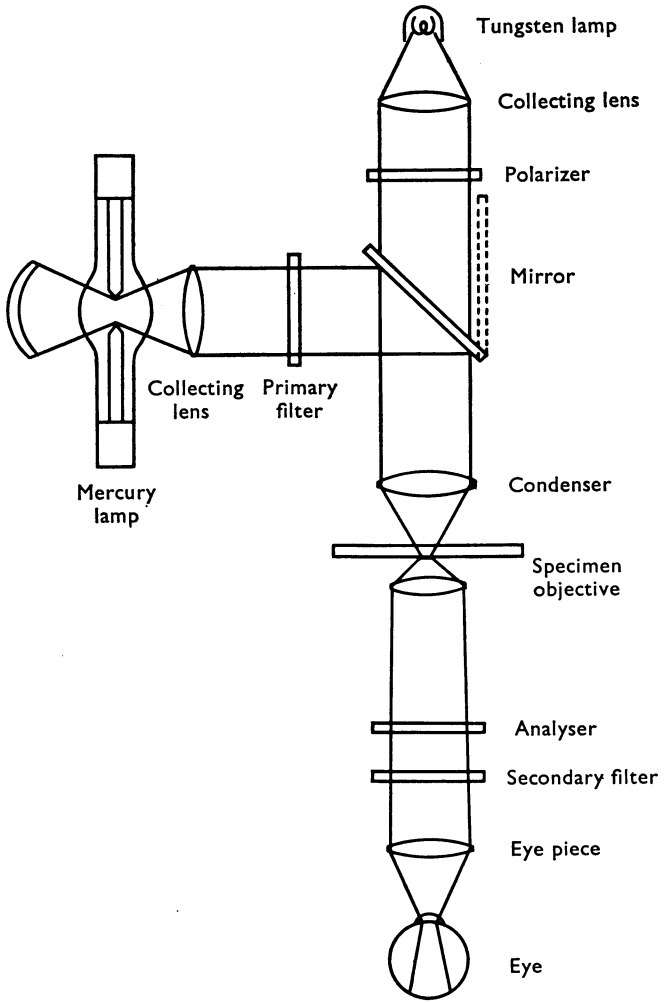
The mercury lamp used was an A.E.I. extra-high pressure mercury discharge prefocus

lamp (type ME/D, 250 W) which emitted an intense light of mean horizontal luminous intensity 1300 cd.

Fluorescent dyes. A dye had to be chosen according to the following criteria.

(i) The dye must not easily enter the sarcoplasm, otherwise the whole fibre will stain diffusely and no internal structure can be seen. Most of the dyes tried did stain the fibre diffusely and were therefore unsuitable. Several sulphonated dyes were good in this respect, possibly because of the low pK of this group, and dyes of this type were used in the experiments.

(ii) The dye should not be toxic to fibres even in high concentration. Many positive ionic dyes produced an irreversible contracture in the concentrations necessary to get a sufficient intensity of fluorescence.



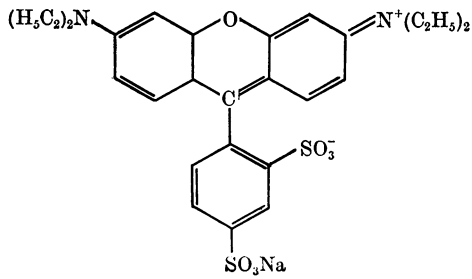
Text-fig. 2. Diagram of the microscope arranged for quick switching over between fluorescence and polarizing microscopy.

(iii) The fluorescence of the dye should not fade appreciably while it is irradiated with the exciting light, as is the case with many fluorescent dyes. Otherwise, it would be difficult to differentiate fading of the dye from its exit from the fibre by washing.

(iv) A dye with an excitation band in the visible region is to be preferred because intense irradiation with ultraviolet light causes irreversible muscle contracture (Azuma, 1927).

(v) Finally, the dye should be strongly fluorescent and easily soluble in water.

Lissamine Rhodamine B 200 (Imperial Chemical Industries, Dyestuffs Division; Colour Index reference Acid Red 52) was the only dye found which fulfilled all of the above requirements, and it was mainly used in this work. As is shown in Text-fig. 3, this dye carries one positive and two negative charges per molecule in solution, and its molecular weight is about 558. The first excitation band of the dye is in the green region and the corresponding emission band is in the red. The concentration used was 0·5–2 mg/ml., usually 1 mg/ml.; higher concentrations tended to reduce fluorescence intensity by quenching. Some other sulphonated dyes were used in the experiments to confirm the main results obtained with Lissamine Rhodamine B 200. The dyes were simply dissolved in Ringer solution. The pH of the solution of Lissamine Rhodamine B 200 was checked with a pH meter and found to be unchanged.



Text-fig. 3. The formula of Lissamine Rhodamine B 200 (C.I. Acid Red 52).

Filter system. The absorption and emission bands of Lissamine Rhodamine B 200 are close together. For this dye, therefore, two interference filters transmitting the 546 mμ mercury line were used as the main primary filter to provide a sharp cut-off toward the region of the emission band. These filters transmitted small amounts of deep red and ultraviolet light which were eliminated by a $CuSO_4$ solution and a yellow filter (Kodak no. 8) respectively; the latter also prevented fluorescence in the interference filters themselves. The secondary filter consisted of two red filters (Kodak no. 25) and additionally in some experiments a light red filter (Kodak no. 23).

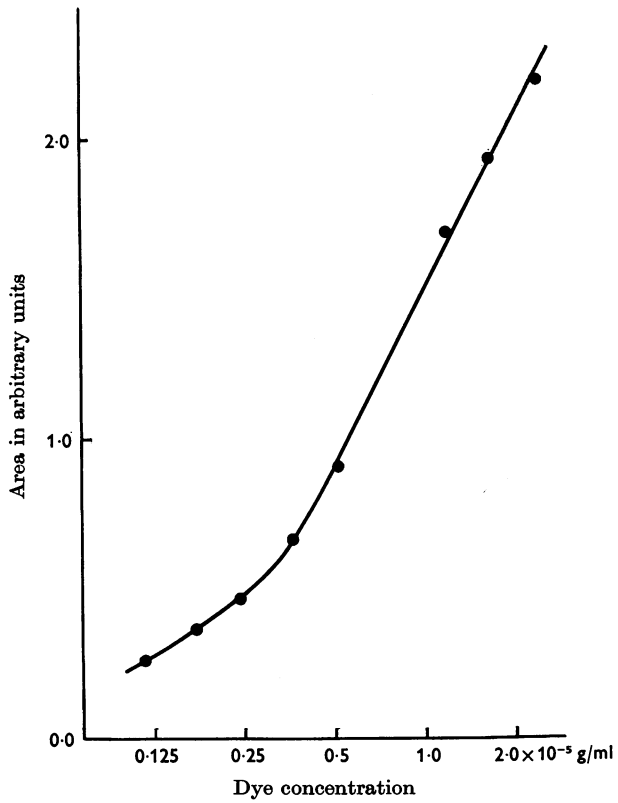
For other dyes, an ordinary filter system consisting of an ultraviolet transmitting and an ultraviolet absorbing filter was used.

Photography. The microscopical images were recorded with an ordinary 35 mm camera. Kodak Tri-X film was used. To obtain images of good quality, a magnification of about 100 times on the film was chosen and a fine-grain developer (Microdol, Kodak Ltd.) was used. An exposure time of several seconds was necessary. To obtain a series of pictures for quantitative studies during exit of the dye from the fibre, magnification was set at about 50 times and a fast developer was used (D–76, Kodak, Ltd.). An exposure of 0·5 sec was sufficient in this case; the image on the film was very grainy but good enough for the purpose.

Measurement of fluorescence intensity. The density of photographic negatives was measured with a densitometer. Using a slit whose width was equivalent to about 0·2 μ on the fibre, the density of the fibre image was scanned in the direction perpendicular to the fibre axis. Since the length of the slit was equivalent to about 80 μ on the fibre, the non-uniformity of the fluorescence due to striation patterns was completely averaged. Calibration of the fluorescence intensity was done as follows. After pictures of a fibre were taken,

glass capillaries whose inner diameter was about the same as the fibre width were filled with dye solutions of various concentrations and mounted in place of the fibre. Photography and densitometry were carried out under exactly the same conditions and in the same way as with the fibre itself. Areas under the densitometer tracings were plotted against concentration of the dye; Text-fig. 4 gives an example. Using this graph as a calibration curve, the area under the densitometer tracing of each fibre photograph was converted to mean dye concentration within the fibre.

The width of fibre measured microscopically will be called 'fibre diameter'.



Text-fig. 4. An example of calibration curves for measuring fluorescence intensity of fibres. Each point was obtained from a microphotographic negative of a glass capillary filled with a dye solution of the stated concentration. (Further explanation in text.)

RESULTS

Localized entry of fluorescent dye. A single muscle fibre was mounted under the microscope as shown in Text-fig. 1, and a Ringer solution containing Lissamine Rhodamine B 200 was applied to the fibre for several minutes. Dye-free Ringer solution was then made to flow through the trough. Plate 1, figs. 1–4, show a typical observation. As soon as the dye

was removed from around the fibre by the dye-free solution, striations were clearly seen in the fibre by fluorescence microscopy, with a periodicity equal to that of the A- or I-bands. Since the depth of focus of the microscope was only about 1 μ and the fibre thickness was usually near 100 μ, the striated appearance could not be due to a periodic adsorption of the dye at the fibre surface only, but indicates that within the fibre there is a periodic structure which is much more easily accessible to the dye than other parts of the interior of the fibre.

Location of dye. As shown in Pl. 1, fig. 5, the position of the fluorescent bands containing the dye was at the centre of the bands which appeared dark under polarized light, i.e. the I-bands. This was established by visual observations during repeated switching over between fluorescence and polarizing optics, and also by many pairs of pictures similar to that shown in Pl. 1, fig. 5.

The bands containing the dye were definitely narrower than the whole I-bands. For example, in Pl. 1, fig. 1, the width of each bright band is not more than a quarter of the striation spacing, while the I-band width is over 0·6 of the striation spacing at this degree of stretch (sarcomere length 4·2 μ). A definite figure for the width of the band containing the dye cannot be given because many factors must tend to exaggerate the width (limited resolving power; optical aberrations; parts of the band above and below the plane of focus; obliquity of the bands, etc.). Although the apparent width was usually between 0·5 and 1 μ, it does not seem safe to conclude that the real width was of this order and not, say, 0·1 μ or less.

These results suggest strongly that the dye enters some part of the triad system, which is located at the centre of the I-band in frog muscle.

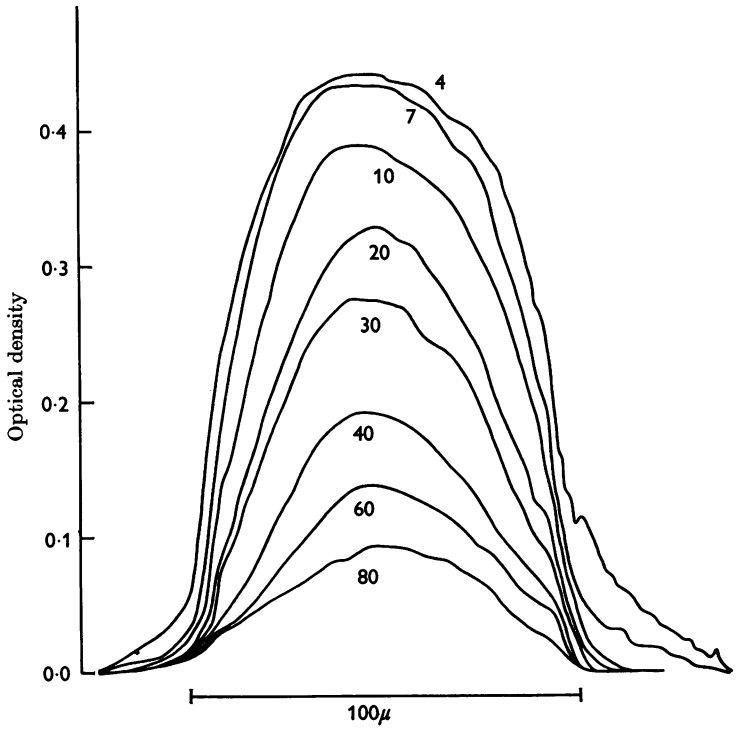
Speed of entry and exit. During washing with a dye-free solution, the intensity of the fluorescence decreased quickly, and after 1 or 2 min from the start of washing the fluorescent bands were not visible any more (Pl. 1, fig. 4; Text-fig. 5). Since the dye does not fade as a result of irradiation with the exciting light, the above finding shows that the dye diffused out of the fibre in 1 or 2 min. A similar time course was observed for the entry of the dye. After the dye had been applied for only a few seconds, striations were clearly seen. However, the fluorescence intensity of the striations increased with the increase in the duration of application of the dye up to 1–2 min, beyond which no further change was noticeable.

Other effects of the dye. The experiments could be repeated many times with similar results except some tendency toward deterioration of the fibre, probably due to the intense irradiation with light.

The dye itself seemed to have no effect on the fibres; fibres were able to respond with normal propagated twitches after repeating the experiments several times or after a prolonged immersion in a dye solution without

irradiation, and isotonic twitches of sartorius muscles recorded on a smoked drum were not changed by application of the dye.

Experiments with other dyes. As mentioned under Methods, a large number of dyes were tried and nearly all were found either to penetrate into the sarcoplasm or to damage the muscle fibres. Successful experiments were carried out with several sulphonated dyes in addition to Lissamine

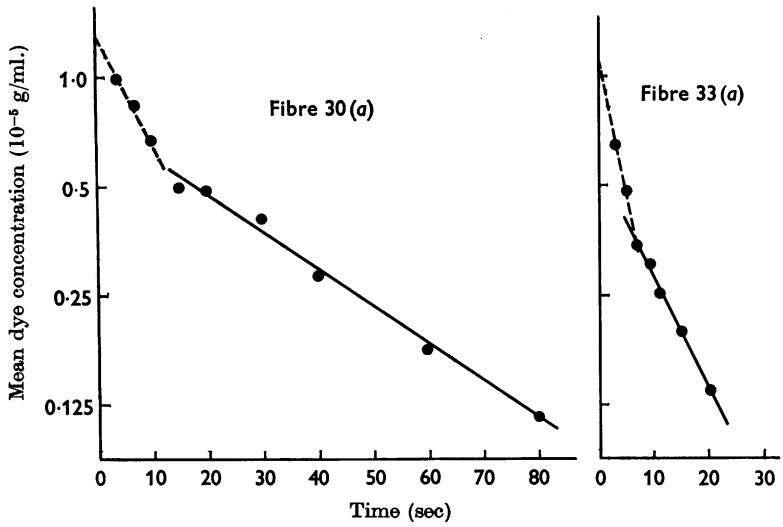


Text-fig. 5. Densitometer tracings of a series of fluorescence photomicrographs of a fibre. Fibre 30(*a*). Ordinate: optical density of photographic negative. Abscissa: distance across the image of the fibre. The number by each trace gives the time in seconds from the start of washing. Lissamine Rhodamine B 200 2 mg/ml. was applied for 2 min.

Rhodamine B 200, namely Lissamine Rhodamine GS (I.C.I. Ltd.; Colour Index reference Acid Red 50), Lissamine Flavin FFS (I.C.I. Ltd.; C.I. Acid Yellow 7) Primulin A (I.C.I. Ltd.; C.I. Direct Yellow 59) and Fluorite HWP (I.C.I. Ltd.; a stilbene derivative). In each case, fluorescence was seen at the centre of the I-bands.

Quantitative observations

Dye space. To estimate the amount of dye that entered the fibre, series of fluorescent photographs taken during washing were analysed by the densitometry procedure described under Methods to give the mean concentration of dye within the fibre. This quantity was plotted against time since the start of washing; an example is shown in Text-fig. 6. Extrapolating the mean dye concentration to zero time and comparing this value with the concentration in the applied solution gave a 'dye space' of 1–2% of the fibre volume (Table 1).



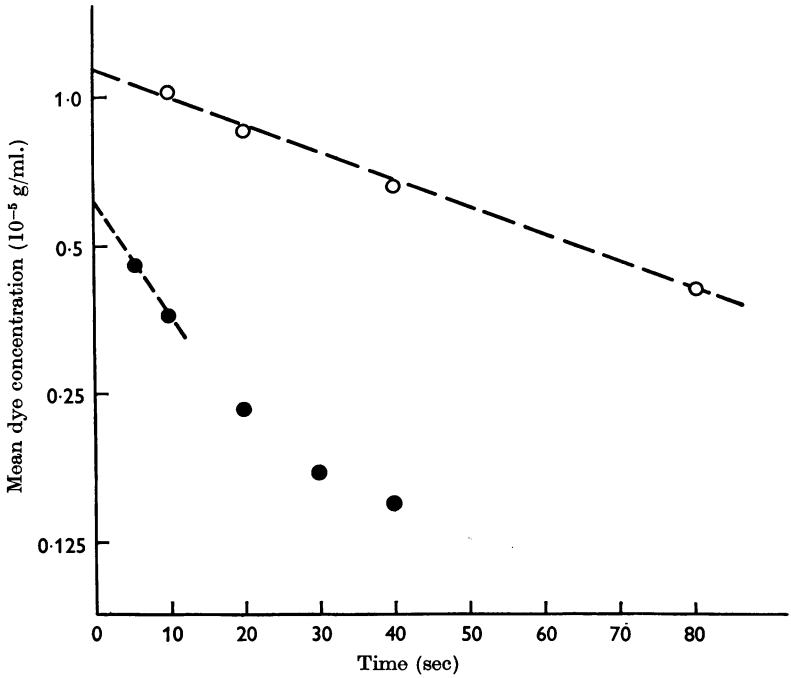
Text-fig. 6. Time courses of the mean dye concentration during washing in two fibres of different size. Each point was obtained from the corresponding photograph taken in series (see Methods). The concentration of the dye applied to the fibres was 1 mg/ml.

TABLE 1. Estimation of 'dye space' and of half-times of the exit of the dye

Fibre (experiment) reference	Fibre diameter (μ)	Concentration of the dye used (mg/ml.)	'Dye space' (%)	Initial half-time (sec)	Half-time of the slower phase (sec)
30(*a*)	93	1·0	1·3	10	29
30(*b*)	93	0·5	1·1	—	—
32	75	1·0	2·0	12	22
31(*a*)	54	1·0	1·25	12	17
31(*b*)	54	0·5	1·3	—	—
33(*a*)	37	1·0	1·1	4	10
33(*b*)	37	0·5	0·93	—	—

The dye used was Lissamine Rhodamine B200. 'Dye space' is expressed as a percentage of the fibre volume.

Time course. Text-fig. 6 also shows the time course with which the dye was washed out of a large and a small fibre. In most cases, the curves were of this type, with an initial rapid phase of loss followed by a slower exponentially declining phase. The time for the fluorescent intensity to drop to half of its initial value (initial half-time) as well as the half-time of the slower phase was measured and the values found are given in Table 1. It is seen that the loss of dye is more rapid in small fibres than large, the time constant of the slower phase being roughly proportional to diameter although the dependence of initial half-time on fibre diameter is not clear.



Text-fig. 7. Time courses of the mean dye concentration during washing. Fibre 41. Open circles (○) were from an experiment in a 2·5 × hypertonic solution made by adding sucrose. Closed circles (●) were obtained from the same fibre in normal Ringer solution.

Effect of hypertonic solutions. H. E. Huxley, Page & Wilkie (1963) found that when a frog muscle was soaked in a Ringer solution made hypertonic with sucrose, the triad system swelled while the fibres as a whole shrank. This suggested that if the fluorescent dye is indeed entering some part of the triads, then immersion in a hypertonic solution should increase the intensity of the fluorescence. This was found to be the case. Text-fig. 7 shows the time course of the fluorescence in two experiments on the same fibre, one using solutions of normal osmotic pressure and the other with

the osmotic pressure of both the dye solution and the washing solution increased by a factor of 2·5 by addition of sucrose. In the hypertonic experiment, both the initial dye space and the time constant for loss of the dye were greatly increased.

Dye distribution across fibre diameter

If the resistance to entry or exit of the dye is entirely located at the mouths of the tubules (either because of a membrane, or because the openings are very small), the dye concentration inside the tubules should rise or fall with the same time course at all distances from the fibre surface, the concentration being uniform across the fibre diameter at each instant. If, however, the resistance is distributed along the length of the tubules, the dye concentration should increase or decrease more rapidly in the outer parts of the fibre than near the centre. The second of these possibilities was found experimentally to be correct.

As regards loss of dye during washing, the series of densitometer traces in Text-fig. 5 show that the decline is more rapid near the edges of the fibre. The difference is, however, more clearly seen during entry of the dye. In Pl. 2, figs. 6–9, show the wash-out the dye after a 10 sec application and figs. 10–13 show the wash-out after a 10 min application. In fig. 6, early after the short application, it is clear that the outer parts of the fibre were fluorescing more strongly than the central parts.

DISCUSSION

It is clear from the results described in the present paper that there is a periodically arranged system in frog fibres into and out of which several fluorescent dyes can easily diffuse without entering the main part of the fibre. The system was situated at the level of the Z-line, and is very likely to be some component of the triads. The results in a hypertonic solution strongly support this idea. H. E. Huxley (1964) and Page (1964) have also shown by electron microscopy that the central element of the triad system of frog fibres is accessible to particles as large as ferritin. These results together give the first direct evidence for continuity between the lumen of the triad tubules and the extracellular space in frog skeletal muscle. Thus, the suggestion about excitation contraction coupling made by A. F. Huxley & Taylor (1958) and the interpretation of several electrophysiological phenomena (see Introduction) have now obtained strong support.

Size of dye space

The 'dye space' measured with Lissamine Rhodamine B200 amounted to 1–2% of the fibre volume. This value is not easy to interpret. Estimation of the volume of central element of triad system on electron micro-

graphs gives 0·2–0·3 % (Page, 1964; Peachey, 1965), which is in very good agreement with estimates based on electrophysiological data (Hodgkin & Horowicz, 1960*a*; Adrian & Freygang, 1962). On the other hand the figure for the whole triad system including lateral elements would be of the order of 5 % (Page, 1964; Peachey, 1965). The values obtained in the present paper are midway between the two groups of the figures. They are also bigger than the figure for the space accessible to albumin molecules in toad fibres (Hill, 1964), which included some space other than the central element of the triad.

There are several factors which may have introduced errors in the estimates of dye space, for example: (1) The fibres are not truly cylindrical. (2) The fraction of fluorescent light scattered or absorbed may be larger in the fibres than in the calibration tubes. (3) The non-linearity of the relation between dye concentration and densitometer readings will introduce errors when the dye concentration is not uniform throughout the cross-section of the fibre.

It is not practicable to make detailed allowance for such factors but it seems most unlikely that they can account for any large part of the differences that are under discussion.

The following are some possible explanations for the relatively large size of the space measured in these experiments.

(1) The relative volume of the central element of the triads may be underestimated by electron microscopy because of shrinkage during preparation. In this case, however, the discrepancy between the dye space and the spaces found by electrophysiological methods remains unexplained.

(2) The dye may become adsorbed to the walls of the tubules or to the surface membrane.

(3) Fixed charges in the lumen of triad tubules might cause an increase in dye concentration, though the polarity required is the reverse of that proposed by Fatt (1964).

(4) The dye may gain access to other structures, e.g. the lateral elements of the triads, but not necessarily at as high a concentration as in the applied solution.

(5) The dye may cause the central element to swell.

The present evidence does not allow a decision to be made between these possibilities.

Half-time of escape of dye

If one takes the half-time of the slower phase as a measure of the time course with which the dye leaves the fibre, it is very much slower than the half-time of a few seconds for entry or exit of K^+ or small anions (Hodgkin & Horowicz, 1960*a*, *b*). The time for the fluorescent intensity to drop to half its initial value was, however, mostly determined by the fast phase, and

Table 1 shows that this half-time ranged from 4 sec in a small fibre to more than 10 sec in larger fibres. Even these values are considerably larger than the half-times estimated for K^+, Cl^- and NO_3^-: for K escape in a Cl-free medium, Hodgkin & Horowicz (1960*a*, Table 1, Group III) found a half-time averaging 3·1 sec for fibres of mean diameter 142 μ, which would correspond to not more than 2 sec in a fibre of 90–100 μ, and for the rise in twitch tension on replacing Cl^- by NO_3^- Hodgkin & Horowicz (1960*b*, p. 378) found time constants of 2·0 and 1·5 sec for the on and off effects respectively, corresponding to half-times of about 1·4 and 1·0 sec. An allowance for the lower diffusion coefficient of the dye (not more than a factor of 2) would bring the expected half-time up to about 3 sec, which is still less than one-third of the value found for fibres of comparable size in the present work. The half-times of the effects described by Adrian & Freygang (1962) and by Freygang, Goldstein & Hallam (1964) are shorter even than Hodgkin & Horowicz's values.

Even the most rapid phase of dye exit is thus very substantially slower than the other physiological phenomena which have been attributed to movement of solutes to and from a tubule space. The discrepancy is of the same order of magnitude as that which exists as regards the size of the space, so it is possible that the slower time course is simply due to the larger space, the resistance for access to the space being greater only in proportion to the lower diffusion coefficient of the dye.

Location of resistance to escape of dye

The resistance to radial diffusion might be assumed to be located (*a*) at the mouths of the tubules, (*b*) along the length of the tubules or (*c*) at the points of access from the tubules to additional spaces accessible to the dye, e.g. adsorption sites or outer elements of the triads. The consequences of the simplest forms of these assumptions are given in Table 2.

TABLE 2. The consequences of assumptions about location of resistance to escape of dye

Resistance located	Half-time proportional to	Distribution over fibre cross-section	Time course of dye loss
(*a*) At mouths of tubules	Diameter	Uniform	Exponential
(*b*) Along the tubules	(Diameter)^2	More rapid near surface	Rapid early phase
(*c*) At access from tubules to extra spaces	Independent of fibre size	Uniform	Exponential

The results are qualitatively in agreement with all three of the expectations based on the assumption that the resistance is distributed along the tubules, but it should be mentioned (1) that the half-time of the slower

phase (which shows clearer dependence on fibre diameter than that of the fast phase) was proportional to the first power of diameter rather than to the square and (2) that the size of the rapid component in the wash-out of the dye (Text-fig. 6) was larger than the value (30 % of the total content) expected for uniform resistance. It seems probable that most, but perhaps not all, of the resistance is distributed along the length of the tubules.

Accessibility of the tubules to anions

Adrian & Freygang (1962) assumed that the mouths of the tubules were impermeable to anions. The present results, and also those of H. E. Huxley (1964) and Page (1964) using ferritin which also carries a negative charge, show that this is not the case. Most of Adrian & Freygang's theory is however not affected by this conclusion.

I should like to thank Professor A. F. Huxley for suggesting this problem to me and for much helpful advice and discussion throughout this work and preparation of the manuscript. I am also grateful to Dr J. H. Callomon for permission to use the densitometer; and to Imperial Chemical Industries, Ltd., for providing the fluorescent dyes.

This work was done during the tenure of a Rockefeller fellowship which the author gratefully acknowledges.

REFERENCES

ADRIAN, R. H. & FREYGANG, W. H. (1962). The potassium and chloride conductance of frog muscle membrane. *J. Physiol.* **163**, 61–103.

AZUMA, Y. (1927). Effects of ultra-violet rays upon skeletal muscle. *Proc. R. Soc.* B, **101**, 24–29.

ENDO, M. (1964*a*). Staining of a single muscle fibre with fluorescent dyes. *J. Physiol.* **172**, 11*P*.

ENDO, M. (1964*b*). Entry of a dye into the sarcotubular system of muscle. *Nature, Lond.*, **202**, 1115–1116.

FATT, P. (1964). An analysis of the transverse electrical impedance of striated muscle. *Proc. R. Soc.* B, **159**, 606–651.

FRANZINI-ARMSTRONG, C. & PORTER, K. R. (1964). Sarcolemmal invaginations and the T-system in fish skeletal muscle. *Nature, Lond.*, **202**, 355–357.

FREYGANG, W. H., Jr., GOLDSTEIN, D. A. & HELLAM, D. C. (1964). The after-potential that follows trains of impulses in frog muscle fibers. *J. gen. Physiol.* **47**, 929–952.

FREYGANG, W. H., Jr., GOLDSTEIN, D. A., HELLAM, D. C. & PEACHEY, L. D. (1964). The relation between the late after-potential and the size of the transverse tubular system of frog muscle. *J. gen. Physiol.* **48**, 235–263.

HILL, D. K. (1964). The space accessible to albumin within the striated muscle fibre of the toad. *J. Physiol.* **175**, 275–294.

HODGKIN, A. L. & HOROWICZ, P. (1960*a*). The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. *J. Physiol.* **153**, 370–385.

HODGKIN, A. L. & HOROWICZ, P. (1960*b*). The effect of nitrate and other anions on the mechanical response of single muscle fibres. *J. Physiol.* **153**, 404–412.

HUXLEY, A. F. & TAYLOR, R. E. (1955). Function of Krause's membrane. *Nature, Lond.*, **176**, 1068.

HUXLEY, A. F. & TAYLOR, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

HUXLEY, H. E. (1964). Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle. *Nature, Lond.*, **202**, 1067–1071.

HUXLEY, H. E., PAGE, S. & WILKIE, D. R. (1963). An electron-microscopic study of muscle in hypertonic solutions. Appendix of DYDYNSKA, M. & WILKIE, D. R. *J. Physiol.* **169**, 312–329.

NELSON, D. A. & BENSON, E. S. (1963). On the structural continuities of the transverse tubular system of rabbit and human myocardial cells. *J. cell Biol.* **16**, 297–313.

PAGE, S. (1964). The organization of the sarcoplasmic reticulum in frog muscle. *J. Physiol.* **175**, 10–11*P*.

PEACHEY, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. cell Biol.* **25**, no. 3, part 2, 209–231.

PORTER, K. R. & PALADE, G. E. (1957). Studies on the endoplasmic reticulum III. Its form and distribution in striated muscle cells. *J. biophys. biochem. Cytol.* **3**, 269–300.

SIMPSON, F. O. & OERTELIS, S. J. (1961). Relationship of the sarcoplasmic reticulum to sarcolemma in sheep cardiac muscle. *Nature, Lond.*, **189**, 758–759.

EXPLANATION OF PLATES

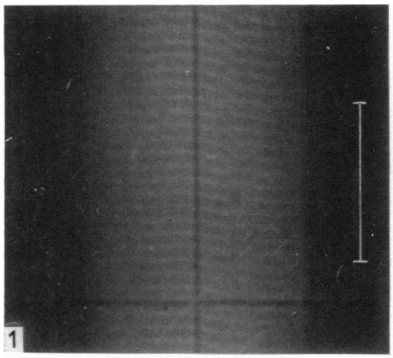
PLATE 1

Figs. 1–4. A series of fluorescence photomicrographs of a single muscle fibre of the frog. A Ringer solution containing Lissamine Rhodamine B 200 2 mg/ml. was applied to the fibre and then the fibre was washed with a dye-free solution. Fibre 40(*c*). The exposures were made during 9–17 sec (Fig. 1), 20–28 sec (Fig. 2), 40–48 sec (Fig. 3), and 60–68 sec (Fig. 4) after the beginning of washing. Scale bar, 50 μ.

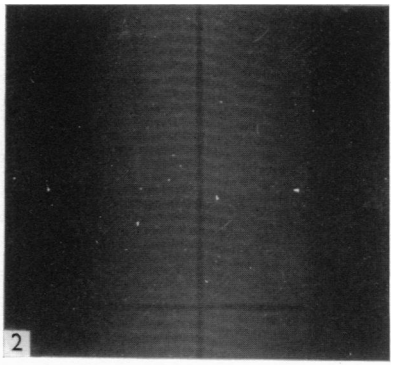
Fig. 5. Comparison of two photomicrographs of the same fibre. Left half: fluorescent image of the fibre as in Fig. 1. Right half: image under polarized light. The print of the right half was made by turning the negative upside down, so that the part of the fibre in this half is the same as in the other half. It is clear that fluorescent bands are situated in the dark bands under polarized light, i.e. I-bands. Fibre 40(*d*). Scale bar, 50 μ.

PLATE 2

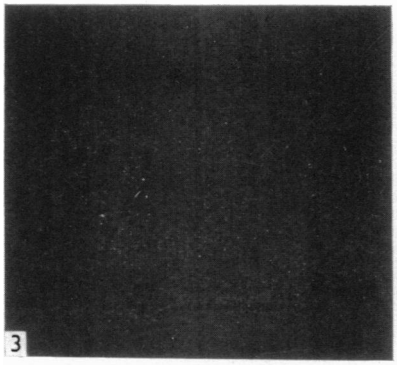
Figs. 6–13. Two series of fluorescence micrographs of the same part of a fibre (fibre 43). A solution containing 2 mg/ml. Lissamine Rhodamine B 200 was applied to the fibre for 10 sec in one series (Figs. 6–9) and for 10 min in the other (Figs. 10–13). The time in seconds given under each figure is the time at which the exposure (0·5 sec) was made, measured from the start of washing with a dye-free solution. Scale bar, 50 μ.



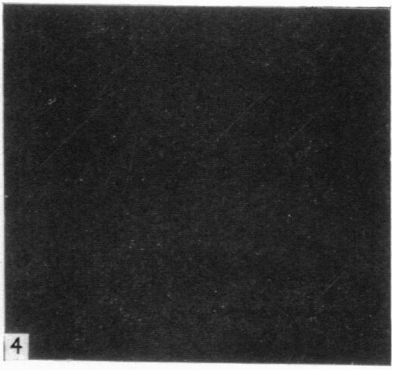
9–17 sec



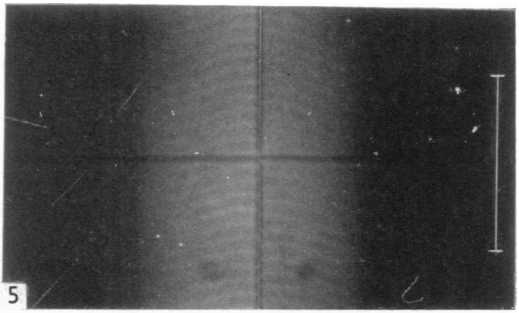
20–28 sec



40–48 sec



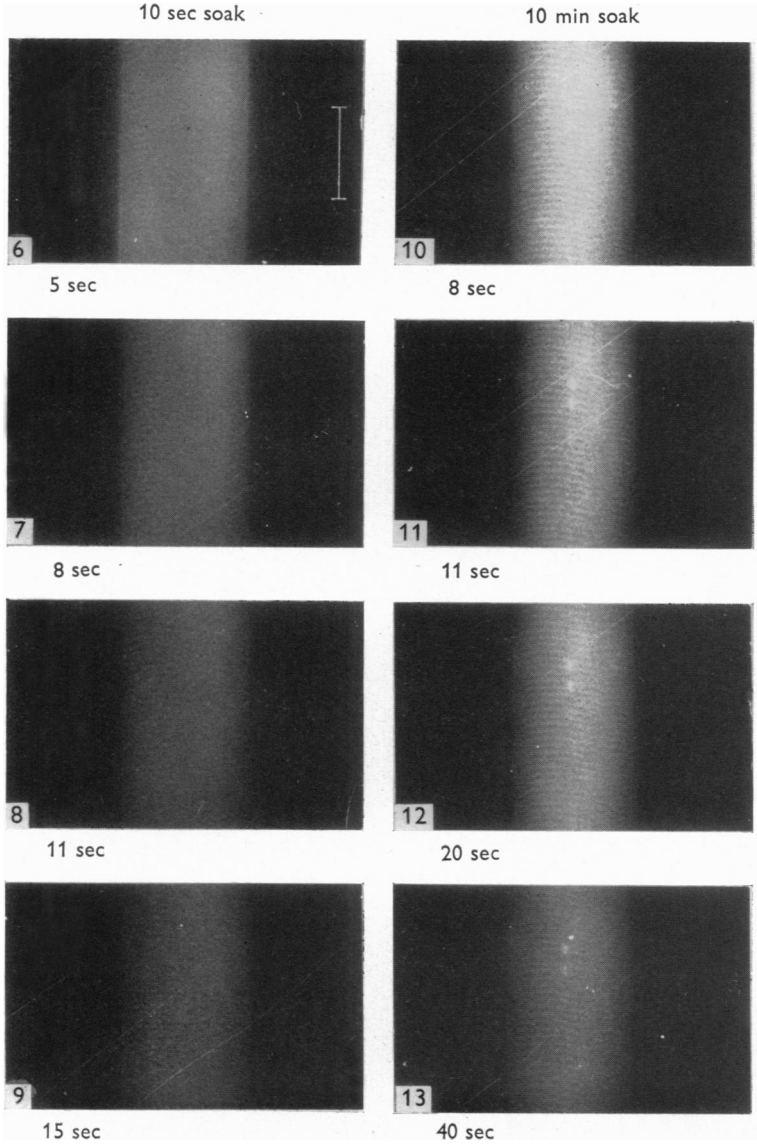
60–68 sec



Fluorescent Polarized

M. ENDO

(*Facing p.* 238)


10 sec soak
10 min soak
6
5 sec
10
8 sec
7
8 sec
11
11 sec
8
11 sec
12
20 sec
9
15 sec
13
40 sec