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THE SPACE ACCESSIBLE TO ALBUMIN WITHIN THE STRIATED MUSCLE FIBRE OF THE TOAD

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For some years evidence has been accumulating (see, for instance, a review by Huxley, 1964) which has made it fairly certain that the central elements (intermediary vesicles) of the triads of striated muscle are linked together to form a transverse network of tubules, the so-called 'T-system', which passes right across the fibre, and makes connexion with the extracellular space through the sarcolemma. The continuity of the T-system, and the existence of definite 'holes' in the sarcolemma, have recently been demonstrated convincingly in certain fish muscles (*Mollienesia* sp.) and in muscle of the tadpole (*Rana pipiens*) by Franzini-Armstrong & Porter (1964); they have also been seen in mammalian cardiac muscle (Simpson & Oertelis, 1962; Nelson & Benson, 1963).

The central elements of the triads are well preserved when glutaraldehyde is used as the fixative, and in frog or toad muscle electron micrographs show that the space within the vesicles is not less than about 250 Å in diameter (p. 287). The vesicles should be capable, if indeed there is a through connexion with the extracellular space, of admitting very large molecules. It was thought that serum albumin, for instance, with molecules about 80 Å in diameter, might be capable of entering the muscle through this system. This has been tested, and the technique and results of the investigation are the subject of this paper. The sartorius muscle of the toad (*Bufo bufo*) was soaked in a solution containing radioactive albumin. The muscle was then fixed and embedded, and autoradiography was used to determine the volume and the location of the space accessible to the albumin. It will be seen that an albumin-accessible space does indeed exist at the centre of the I band (along the line of the T-system), and that its volume is about what might be expected. But in addition, and somewhat surprisingly, a second space appears to be present; it has about half the volume of the first, and lies near the A–I boundary.

The autoradiographs have incidentally provided a means of observing the inter-fibre spaces in a muscle, and of measuring the *in vivo* spacing of the fibres from one another. This was found to be very variable. A point of interest is that a considerable proportion of the inter-spaces are

found to be less than 1000 Å across, which is much less than the spacing to be expected if the inter-space volume were uniformly distributed.

Before proceeding to a description of the method it is necessary to discuss two matters which have a bearing on the design of the experiments. One is concerned with the rate of diffusion of albumin, and the second with the effects of radiation on the muscle.

The diffusion of albumin into the muscle

Diffusion along the inter-fibre spaces. The diffusion of albumin into the muscle may be thought of as taking place in two stages, first, along the inter-fibre spaces and secondly into the fibres. Consider, first, the rate of penetration into the inter-spaces. According to Svedberg & Pedersen (1940) the diffusion coefficient for horse serum albumin is $7{\cdot}2 \times 10^{-7}$ cm²/sec at 25° C. At 0° C it would be about half this value (Hitchcock, 1946), that is $3{\cdot}6 \times 10^{-7}$ cm²/sec. Hill & Macpherson (1954) have shown that, in order to allow for the 'roundabout journey' through the inter-spaces of a muscle, the diffusion constant which should be used in calculations based on the assumption of straight-line diffusion is about one-quarter of the value for diffusion in free solution. For albumin at 0° C the effective diffusion coefficient is therefore $0{\cdot}9 \times 10^{-7}$ cm²/sec. The rate of penetration can then be calculated with the aid of Hill & Macpherson's Table I, which is applicable to the case of diffusion into a sartorius muscle exposed at both surfaces. The muscle is taken as divided into ten equal zones, 0 to $0{\cdot}2b$, $0{\cdot}2b$ to $0{\cdot}4b$, . . ., $1{\cdot}8b$ to $2{\cdot}0b$, where $2b$ is the thickness of the muscle. The toad's sartorius shown in Pl. 1 is typical of the muscles used in the present experiments. Its thickness is taken as 0·35 mm, but allowing for shrinkage in preparation (p. 282) the thickness of the living muscle should be taken as 0·50 mm. A further correction must be made to allow for the fact that during the period in the albumin solution the muscle was not stretched, and it was about 25% shorter, or 0·56 mm thick, at that stage. In Hill & Macpherson's Table I the constant b should be taken as 0·028 cm, with $k = 0{\cdot}9 \times 10^{-7}$ cm²/sec. The values for the percentage concentrations in the different zones of the muscle for a series of times from 3·5 to 413 min are shown in Table 1. The minimum duration of exposure in the experiments described was 60 min. This would be expected to give a degree of saturation ranging from 92 to 54% in the different zones of the muscle, with an average of 70%.

Diffusion within the fibre. The rate of diffusion of the albumin from the inter-spaces into the interior of the fibres is not so easily calculated. The difficulty is due to the lack of any well-established theory for predicting the restriction to free diffusion in channels of molecular dimensions. The problem is discussed by Pappenheimer (1953) in connexion with the passage of molecules through capillary walls.

The molecular weight of serum albumin is 73,000, and for a partial specific volume of 0·75 (a value generally valid for proteins) the equivalent sphere would have a diameter of 81 Å. The tubules within the fibres are elliptical in section, with a minimum internal diameter of about 250 Å (p. 287). According to the only experimental data known to Pappenheimer (1953, p. 397) diffusion would be expected to be very severely restricted, for it appears that the diffusion rates of sucrose or urea are reduced by 50% even when the diameter of the pores through which diffusion has to take place is as much as 20 times larger than the diffusing molecules.

The rate of diffusion of the albumin in the internal tubules of the muscle fibre would probably be at least 10 times, but possibly as much as 100 times, slower than in free solution. A calculation can be made to find what this would mean in terms of the degree of saturation in the fibre for different times of exposure. The kinetics of diffusion for a cylinder are given by Hill (1928, pp. 70–71). The largest fibres in the muscles used in the present experiments had a diameter of about 45 μ when embedded, and after allowing for shrinkage in preparation, and for the greater diameter at the shorter length during exposure to the albumin

solution, the figure is 70 μ. Allowing, as before, for the roundabout path of diffusion, by taking the effective diffusion constant as one-quarter of the value in free solution ($3{\cdot}6 \times 10^{-7}$ cm^2/sec), and then further reducing it by a factor of one hundred, to allow for the maximum likely degree of restriction in the narrow tubules, the value arrived at is $0{\cdot}9 \times 10^{-9}$ cm^2/sec. The cylinder has a radius of 0·0035 cm. The average degree of saturation for different times may then be calculated from Hill's Table IV (1928, p. 71). The results are given in Table 2. It is seen that in spite of the assumed 100-fold reduction in the diffusion constant the average degree of saturation is as much as 70 % for the least time of exposure (60 min) used in the present experiments.

TABLE 1. Calculation of concentration (percentage of final) reached by diffusion in five zones of muscle inter-fibre spaces

Time (min)	0 to 0·2	0·2 to 0·4	0·4 to 0·6	0·6 to 0·8	0·8 to 1·0
3·5	67	18	3	0	0
4·7	71	25	5	1	0
5·9	74	33	9	2	0
8·8	77	40	15	5	1
11·7	80	46	22	8	3
17·7	84	54	32	16	9
29·5	88	65	46	31	24
41	90	71	55	43	37
59	92	79	67	58	54
88	95	87	80	75	72
118	97	92	88	84	83
147	98	95	92	90	90
177	99	97	95	94	94
236	99	99	98	98	97
295	100	99	99	99	99
354	100	100	100	100	99
413	100	100	100	100	100

TABLE 2. Average degree of saturation (percentage of the final) reached by diffusion in a single muscle fibre

Time (min)	Saturation (%)	Time (min)	Saturation (%)
0·7	13	23	61
1·2	16	35	71
1·6	18	46	78
2·3	22	70	88
4·6	30	93	93
7·0	36	116	96
9·3	41	162	99
12	45	232	100
16	52		

This result is not so surprising when it is remembered that in the kinetics of diffusion the time constant is inversely related to the *square* of the linear dimensions. In the present case the restriction due to the assumed reduction in the value of the diffusion constant is compensated by the shortness of the distance which has to be traversed.

The entry into the fibres is, of course, delayed while the albumin first penetrates the inter-spaces. The over-all time course of diffusion, taking both stages into account, has not been calculated. The values given in Tables 1 and 2 do, however, make it probable that such a calculation would predict a failure to reach equilibrium during the shorter exposure times employed. In actual fact the results show that after 60 min exposure the penetration of the albumin, even into the deeper fibres, is not far from complete. The restriction factor for diffusion within the fibre is probably therefore not so much as 100.

The relative immobility of the albumin, as compared with the more rapid diffusion of the osmium tetroxide used for fixation, is an essential feature of the method. The diffusion coefficient of OsO_4 in the inter-spaces is probably about 10 times that of the albumin, and in the internal tubules it is perhaps 100–1000 times greater. At all events there is no evidence from the autoradiographs of any redistribution of material which could be accounted for by outward diffusion of the albumin during fixation.

Radiation damage

It will be seen that only 1–3 parts in 1000 of the volume of the muscle fibre is accessible to albumin. This meant that satisfactory autoradiographs could be obtained only by using extremely concentrated radioactivity in the external soaking solution; so high, in fact, that the effects of radiation on the muscle could not be neglected. The susceptibility of muscles to damage from this cause has been discussed previously (Hill, 1959). In the present experiments the concentration used was either 4260 or 30,900 μc/ml. The lower concentration was only 4 times higher than that previously used for an exposure of several days to tritiated adenine (Hill, 1959), and in the present experiments there was no sign of any damage after 22 hr exposure. Though this concentration was perhaps just adequate for the present work, a higher rate of grain production in the autoradiographs was desirable and two experiments were made using the higher concentration. This was much greater than had previously been considered safe and, as will be seen, there is evidence that damage was done to some of the fibres, which became abnormally permeable to the albumin even after 1–5 hr exposure.

The radiation from tritium is mostly absorbed in passing through as little as 1 μ of water, so only the extreme surface of the fibre and of the regions immediately bordering the internal spaces occupied by the albumin would be affected by the radiation. It may be for this reason that the muscle was largely able to survive the exposure to the high concentration.

The muscles were exposed to the radioactive solution at a temperature of 0° C. It is worth recalling (Hill, 1959) that a muscle is much less susceptible to radiation damage at 0° C than it is at 20° C; it seems likely that at the higher temperature a muscle exposed at 30,900 μc/ml. would have become seriously damaged.

METHODS

Preparation of tritiated albumin

Crystallized bovine plasma albumin (Armour) was labelled with tritium by two methods.

Method 1. *Catalytic gas exposure*

Albumin 0·5 g was treated by the standard catalytic gas exposure method (TR 4) at the Radiochemical Centre (Amersham, Buckinghamshire), using 7 c of tritium gas for a period of 5 days. This procedure leads to a general replacement of a proportion of all the hydrogen atoms present in the molecule, but only the tritium which becomes covalently bonded to carbon is stable, and 'labile' tritium (i.e. that attached by non-covalent bonds to oxygen, nitrogen or sulphur) was removed in two stages, first by dissolving the product in water and freeze-drying, and secondly by dialysis in Visking cellophane tubing against distilled water, for 24 hr at room temperature, followed by freeze-drying.

The albumin thus prepared had a specific activity of 56 μc/mg, as measured in a Packard Tri-Carb scintillation spectrometer. The radiochemical purity was examined by electrophoresis on cellulose acetate in buffer at pH 8. The activity was found to be almost entirely associated with the main protein fraction. There was no sign of any highly active minor constituent.

Method 2. Acetylation with tritiated acetic anhydride

The tritiation of the albumin in this case involved an additive rather than a replacement procedure, and was done by acetylation of a proportion of the free amino groups of the lysine and arginine. (This method of labelling the albumin with tritium was suggested by Dr J. H. Humphrey, who kindly supplied details of a suitable procedure.) It is known that acetylation does not affect the solubility of albumin (Putnam, 1953). It involves a comparatively mild reaction which would not be likely to produce active break-down products, and yields a stable product. It seemed that for the present application there could be no objection to this as a procedure for labelling albumin with tritium.

Procedure for acetylation. The albumin was dissolved in a strong solution of sodium acetate (200 mg in 1·5 ml. 1 M-sodium acetate), which was required to neutralize the acetic acid liberated by the reaction. The solution was cooled to 0° C. Tritiated acetic anhydride 100 mc (2·2 c/m-mole, 4·64 mg: the Radiochemical Centre) was condensed to the end of its ampoule by immersing the tip in solid CO_2/acetone for 30 min. The ampoule was opened, and 0·8 ml. 1 M-sodium acetate was introduced. The temperature was raised to 0° C and 1·2 ml. albumin solution (200 mg albumin) was added. The mixture was stirred vigorously for 5 min while the acetic anhydride dissolved. After a further 15 min standing the mixture was diluted with 20 ml. water, and was then dialysed in Visking cellophane tubing, first for 6 hr against running tap water, and secondly against distilled water at 10° C for a further 20 hr. It was then freeze-dried.

The reactive amino groups number 59 per molecule (Porter, 1948) and were present in excess; a maximum of 50% of the activity present could therefore have become incorporated in the albumin. The activity of the product was 134 μc/mg, which is 54% of this theoretical maximum. The proportion of reactive amino groups acetylated was 14·5%. The final yield was 161 mg (21 mc). It was not thought necessary to test for the presence of highly active contaminants by electrophoresis.

Further purification of the albumin prepared by either of the above methods was not considered to be necessary. It was not thought likely that a significant amount of any impurity likely to be present would be retained during fixation and dehydration. The consistency of the results achieved using albumin tritiated by the two entirely different procedures makes it seem that the assumption was justified.

Experimental procedure

English toads (*Bufo bufo*) were used, because very small specimens were obtainable from the dealers, and their sartorius muscles, which weighed only 8–10 mg, were lighter and thinner than those of the frogs which are normally available. Small muscles were required because very small volumes of radioactive solution were available, and also because it was desirable to minimize diffusion distances.

The radioactive albumin solutions. The sartorius was dissected with a small portion of the pelvic bone left attached, and it was then given a preliminary soaking for 30 min in Ringer's solution at 0° C (composition (mM): NaCl, 96; KCl, 5·0; $CaCl_2$, 4·0). Four experiments are reported here, referred to as *A*, *B*, *C* and *D*, and two different soaking solutions were used, made up as follows:

Solution 1 (Expts. *A*, *B*): 161 mg ^{3}H-albumin (134 μc/mg), 0·322 ml. 0·1 N-NaOH, 0·193 ml. modified Ringer's solution. (Total weight, 0·676 g; activity, 31,900 μc/g.)

Solution 2 (Expts. *C*, *D*): 111 mg ^{3}H-albumin (56 μc/mg), 139 mg non-radioactive albumin, 0·50 ml. 0·1 N-NaOH, 0·30 ml. modified Ringer's solution. (Total weight, 1·05 g; activity, 5900 μc/g.)

The concentration of protein in both solutions was 23·8% (wt./wt.). The amount of NaOH was chosen to give pH = 7·3.

The modified Ringer's solution had the following composition (mM): NaCl, 192; KCl, 6·65; $NaHCO_3$, 6·35; $CaCl_2$, 5·3. This gives normal K and Ca concentrations, and the mixture is

isotonic with ordinary Ringer's solution. The Na concentration is high, but this is more or less inevitable since the protein possesses a considerable negative charge which has to be neutralized.

Baker (1958) gives some figures to indicate a lower limit for the concentration of albumin which must be present in a solution if immobilization by gelling in osmium tetroxide fixative is to occur; he says that 12 % serum albumin is gelled, but 8 % is not. In solution 1 the required activity could be obtained only by using the radioactive protein at a concentration of 24 % (wt./wt.), and though this is an unnecessarily high value to be certain of adequate fixation it was thought worth while, in solution 2, to add non-radioactive albumin to bring the total concentration up to the same value.

Exposure of the muscles to the radioactive albumin solution. Solution 1 required the use of the entire stock of tritiated albumin prepared by method 2; even then only 0·6 ml. solution was available. In order to immerse the muscle in such a small volume it was not practicable to have it fixed to a holder; the muscle was therefore soaked, unattached, in a pool of the fluid in the bottom of a 1 in. (2·5 cm) round-bottom tube. It was lowered into the solution by a length of cotton tied to the tendon. In Expts. *A* and *B*, with short exposure to the radioactive solution, the tube was not filled with oxygen since it was possible to keep the solution stirred at regular intervals throughout, so that the muscles would obtain sufficient oxygen from the air. In Expts. *C* and *D*, the same method for immersion was used, but since the exposure was much longer than in *A* and *B* the tube was filled with oxygen and closed with a cork.

The solutions were used at 0° C. The exposure times were as follows: solution 1: *A*, 1 hr; *B*, 5 hr; solution 2: *C*, 22 hr; *D*, 22 hr. In Expts. *A* and *B* the muscles were immersed together, that for *A* being removed first: similarly, the muscles used in Expts. *C* and *D* were immersed together. The radioactivity was diluted by the water of the muscle (and pelvic bone) and the activities after equilibration were measured by withdrawing and weighing a few mg of the solution, which was then assayed. The final activities were: solution 1: 29,500 μc/g (30,900 μc/ml.); solution 2: 4060 μc/g (4260 μc/ml.). (The specific gravity of the solution was taken to be 1·05.)

Fixation, dehydration and embedding. After the allotted exposure time a muscle was quickly slipped on to a glass holder and stretched by about 10 % of the extended length in the body. It was then fixed.

In Expts. *A*, *B* and *C* the muscle was plunged into buffered osmium tetroxide fixative at 0° C, and left for 30 min. (Composition: 200 mg OsO_4 (1 %); 118 mg Na barbitone; 78 mg Na acetate; 4·5 ml. 0·1 N-HCl; 15·5 ml. water; pH 7·4.)

In Expt. *D* the muscle was fixed by freeze-drying. It was frozen in Arcton 6 at −160° C, and dried at −60° C for 3 hr in a Speedivac-Pearse Tissue Dryer (Edwards High Vacuum Ltd).

To avoid any possibility of the albumin re-dissolving, the muscles of Expts. *A*, *B* and *C* were not passed through the usual alcohol series after fixation, but they were dehydrated in pure ethanol at 0° C for 30 min. The freeze-dried muscle was also transferred to ethanol after drying.

In Expts. *A* and *B* the muscles were infiltrated with methacrylate monomer (10 % methyl-, 90 % butyl-methacrylates) for 2·5 hr, and embedded at 65° C in this mixture, with 1 % dichloro-benzoyl peroxide as activator. In Expts. *C* and *D* the muscles were infiltrated with Araldite (30 parts resin CY 212, 30 parts hardener HY 964, 1·5 parts dibutyl-phthalate, vol./vol.) for 12 hr at 58° C, and embedded in Araldite with activator (50 parts above mixture, 1 part accelerator DY 064, vol./vol.) at 73° C for 20 hr.

In each experiment one or more pieces of the muscle (which was cut up immediately before embedding) was oriented in an 'end-on' manner in the capsule to allow cross-sections to be cut.

Autoradiography. Sections were cut with a Huxley Ultra-Microtome (Cambridge Instrument Co.) at a thickness of 0·6 μ for transverse sections, or 0·45 μ for longitudinal sections.

Autoradiographs were made with Kodak A.R. 10 stripping film. During exposure (up to 62 days) the slides were stored over a desiccant (silica gel) to minimize latent image fading. The autoradiographs were developed with Kodak D. 76 fine-grain developer, for 4 min at 20° C; they were then fixed and washed.

Autoradiographs of transverse sections were mostly not stained. Euparal was used as the mounting medium except in those cases (Expts. *C*, *D*) where the grain count was so low as to be comparable with the background. For the latter it was found better to use water (with 25 % glycerol to prevent evaporation) under the cover-glass. In this aqueous mountant the emulsion swells and, at high power, the majority of the background grains are out of focus at the plane, in contact with the section, where the tritium-produced silver grains lie. The background correction is thereby much reduced. There is some loss of visual clarity.

Longitudinal sections, used for the location analysis, had to be stained to show the striations. This was done, after processing and drying the autoradiograph, by immersing it in an alkaline solution of basic fuchsin in 50 % acetone, for 5 min (composition: 0·5 % basic fuchsin, 50 % acetone, 50 % water, the mixture saturated with sodium borate by shaking with the solid. This solution has to be used freshly made, and deteriorates in a few hours). The stain taken up by the film was removed by shaking the slide for 5–10 min in 10 mM-acetate buffer at pH 4. After rinsing and drying, the stained autoradiograph was mounted in Euparal.

Transverse sections were occasionally stained by the same procedure.

Microscopic examination was done either with direct, or with dark-field illumination. Photographs were made on Kodak P. 200 plates.

The grain counting was sometimes done from the photographs, but most of the counts were made through the microscope, with the aid of a square-pattern grid in the focal plane of the eyepiece.

For high-power work an apochromatic oil-immersion objective was used (N.A. = 1·32).

The grain yield per β-particle: the autoradiographic conversion factor

It is necessary to make use of a conversion factor to obtain a value for the activity (in μc/ml.) from the grain count in an autoradiograph. It was previously assumed (Hill, 1959) that each tritium β-particle entering the emulsion produced 1·0 grains, but more recent data suggest that the yield is rather lower than this. Barnard & Marbrook (1961) say the value should be 0·85 grains per incident β-particle for the Kodak A.R. 10 emulsion. A still lower figure of 0·69 was obtained (unpublished observation) by the use of thin sections of tritium-labelled Araldite (Hill, 1962*b*). The value used here is the mean of the more recent estimates, 0·77 grains per incident β-particle. In addition a correction has to be made for self-absorption in the section, and this is calculated with the aid of data relating absorption and screen thickness (Hill, 1959). The values so obtained are shown in Table 3.

TABLE 3. Conversion factors for obtaining a measure of the radioactivity from an autoradiographic grain count

Section thickness (μ)	Grains/100 μ^2.day for an activity of 1 μc/ml.	Section thickness (μ)	Grains/100 μ^2.day for an activity of 1 μc/ml.
1·5	0·043	0·5	0·031
1·0	0·042	0·45	0·029
0·9	0·041	0·4	0·027
0·8	0·039	0·3	0·022
0·7	0·037	0·2	0·017
0·6	0·035		

Shrinkage of the tissue occurs during preparation, so the concentration of activity in the living muscle is less than the value obtained from the sections. Longitudinal shrinkage is not significant when the muscle is held extended during fixation and dehydration, but

Carlsen, Knappeis & Buchthal (1961) have shown that there is a transverse linear shrinkage of 30 % during preparation by a procedure essentially similar to that used here (Expts. *A B*, and *C*). This means that the final volume of the muscle is only 50 % of the value *in vivo*, and it follows that all values for activity from grain counting have to be divided by 2 to obtain the concentrations in the living muscle. The results given below and in Table 4 are corrected in this way.

An attempt was made to estimate the shrinkage of the muscle of Expt. *D*, which was freeze-dried. This was done by comparison of the fibre sizes with those in the muscles fixed with osmium tetroxide. A reliable result could not be obtained, because there was too much variation in the fibre size. A rough estimate suggested that the shrinkage was perhaps rather greater in the freeze-dried muscle than in the others. In the absence of accurate data it was thought best to assume that there was a volume shrinkage of 50 % in the freeze-dried, as in the other muscles.

RESULTS

Autoradiographs of the radioactive albumin in the inter-fibre spaces

Observations at low magnification. Autoradiographs of cross-sections of a muscle are shown in Pls. 1 and 2. The inter-spaces are seen clearly marked by dense deposits of silver. The radioactive material which must have been adhering to the surface of the muscle on withdrawal from the albumin solution appears to have been largely washed away; this is not surprising, because the muscle was moved around vigorously in the fixative immediately after immersion.

A similar view, by dark-field illumination, is seen in Pl. 3, fig. 1.

Occasionally, fibres are seen to have become highly permeable to the albumin. These fibres, which were probably damaged or dead, acquired a concentration almost equal to that in the inter-spaces. One such fibre is seen in Pl. 2, and a high-power view of another is shown in Pl. 3, fig. 2.

The variation in the width of the inter-spaces. Though it is fairly clear from a low-power view (Pl. 2) that the inter-spaces are not of the same width in different parts of the muscle, this feature is not well shown under such conditions. The exposure required in an autoradiograph suitable for low-power photography is so long that 'over-hitting' of the emulsion occurs in the active regions, the rate of grain production then falls off, and the contrast between 'rich' and 'poor' areas becomes progressively reduced. As a consequence, the variability in inter-fibre spacing is not very conspicuous.

At higher power an autoradiograph made with a shorter exposure time shows the effect more clearly (Pl. 4, fig. 1).

No attempt has been made to measure the widths of the larger inter-spaces (it is worth noting that these often appear (Pl. 2) to be linked together, to form long channels which may traverse the entire muscle), or to estimate the total volume of the inter-spaces in the muscle. Such quantitative considerations have been confined to the very narrow inter-spaces. The assumption is made that the activity of the material in the

inter-spaces was equal, *in vivo*, to that of the external soaking solution; the width of the space at that time may then be calculated from grain counts. It turns out that there are numerous regions where the distance between fibres must, before fixation, have been less than 0·1 μ (1000 Å). For instance, in Pl. 4, fig. 2, the 19 μ length of inter-space between the arrows has yielded only 30–40 grains, and it was calculated that *in vivo* it must have been only 300–400 Å wide. The space, as seen, is 0·6 μ wide, and must be presumed to be an artifact caused by a parting of the fibres during fixation and dehydration.

The apparent narrowness, *in vivo*, of a considerable proportion of the inter-spaces cannot be attributed to a redistribution of material during fixation. This is proved by examining autoradiographs made with the freeze-dried muscle; they show similar regions of low grain count.

'Phasing' of striations. It was noticed that in regions where fibres showed close contact with one another the striations had a tendency to be lined up with one another, that is to say the A bands of one fibre lay opposite the A bands of the neighbouring fibre, and I opposite I; an example is seen in Pl. 4, fig. 2. This point is discussed later.

The volume of the space accessible to albumin within the fibre

Autoradiographs viewed at low power: the variation between fibres. The volume of the internal space accessible to albumin was found to be variable. This is dealt with quantitatively in the following section. Observation by low-power dark-field illumination serves to show, in a non-quantitative way, how the activity of the fibres varies from one part of the muscle to another. The muscle of Pl. 3, fig. 1, was exposed to the radioactive solution for only 1 hr, and it is doubtful (p. 277) whether the fibres in the middle of the muscle had reached diffusion equilibrium by this time; it will be noticed that the fibres possessing the lowest activity are near the middle of the muscle. However, the inference that this is a diffusion effect may not be correct, for there are some 'rich' fibres deep in the muscle and, furthermore, a number of 'poor' fibres were present near the middle of the muscle in Expt. *B*, in which the muscle was exposed for five times as long. A few extremely dense areas (showing almost pure white) are probably accounted for by dead or damaged fibres which have developed an abnormal permeability.

The muscle of Expt. *B* showed a wider range of variation than that seen in Pl. 3, fig. 1, and this was perhaps due to radiation damage.

Though there was, on the whole, a rather 'random' distribution of 'rich' and 'poor' fibres in the deeper parts of the muscles, the fibres near the surface showed a more or less constant degree of activity. This was intermediate in amount, but not much greater than the minimum. These

fibres, alone, were well preserved by fixation, and the location-analysis had to be confined to them.

TABLE 4. Intracellular activity following exposure to tritiated albumin

Expt.	Autoradiograph exposure (days)	Mean activity (μc/ml.)	Extreme values (μc/ml.)	
			High	Low
A	1·9	53 (0·17)	115 (0·37)	30 (0·10)
	5·9	40 (0·13)	110 (0·36)	20 (0·06)
	9·9	—	—	28 (0·09)
	1·9 (α)	—	—	46 (0·15)
B	0·86	86 (0·28)	134 (0·43)	50 (0·16)
	4·9	72 (0·23)	238 (0·77)	42 (0·13)
	8·8	—	—	49 (0·16)
C	4·8 (β)	4·9 (0·12)	7·8 (0·18)	2·9 (0·07)
	21·0	5·6 (0·13)	6·7 (0·16)	4·0 (0·09)
D	4·8 (β)	5·0 (0·12)	7·8 (0·18)	2·0 (0·05)
	21·0	6·1 (0·14)	7·4 (0·17)	3·7 (0·09)

The figures in brackets give the percentage of the muscle volume which is accessible to albumin.

(α) This figure gives the activity found from longitudinal sections used for the location analysis. All the others were taken from transverse sections through the entire muscle.

(β) At this time of exposure the grain counts were low, and the 'high' and 'low' values are the means of counts made over several fibres, so that each observation is based on a total of not less than 60 grains.

Grain counts: the volume of the internal space as a percentage of the fibre volume. The method for deriving a volume from a grain count has already been described. The results are summarized in Table 4. They mostly refer to counts made with transverse sections, and in these about twenty to thirty fibres were included in the count. The fibres were selected so as to constitute a representative sample, with about the correct proportions of fibres with the different degrees of activity. The values given for 'mean activity' were based on counts totalling at least 200 grains, and the standard deviation ($\sqrt{n}$) was less than 7 %; but in some instances 'extreme values' were based on counts as low as 60 grains (S.D. 13 %), and in these much of the inconsistency could be accounted for by random variation in the radioactive decay rate.

The conclusions are as follows. First, the mean value for the volume of the internal space accessible to albumin is in the range 0·12–0·28 % of the volume of the fibre. Secondly, the amount of variation, as between individual fibres, is not the same in the different experiments. It is least in Expts. *C* and *D*, where the highest values were only about twice the lowest. It is considerably more in Expts. *A* and *B*, where as much as sixfold variation is seen; it seems likely that this came about because the radiation produced an abnormally high permeability in some of the fibres. (Only a comparatively small number of fibres were affected, so the 'mean activity' is not greatly altered by their counts having been included in the mean.) Thirdly, there is reasonable consistency in the values recorded in the different experiments for the 'low activity' fibres. It appears that the 'low' values for Expt. *B* are significantly higher than those in the other experiments, but it is interesting to observe that the 'low' values for Expts. *C* and *D*, after an exposure of 22 hr, are not markedly higher than those in Expt. *A*, where the muscle was exposed to the radioactive solution for only 1 hr. This means that the rate of diffusion was not a limiting factor even at 1 hr. It would also suggest that only these 'low activity' fibres were in normal condition, at least in the muscles of Expts. *A* and *B*; it might, therefore, be more nearly correct if the volume of the internal space were given, say, as 0·10–0·15 % of the volume of the fibre.

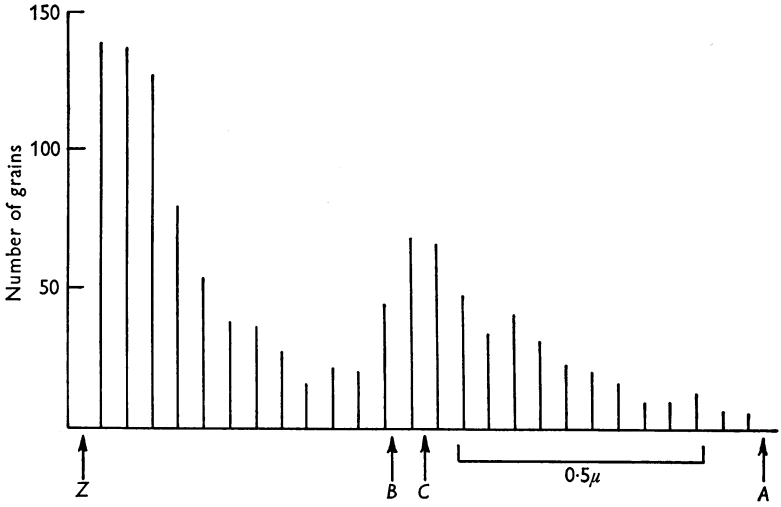
It was satisfactory to find that the values obtained with the freeze-dried muscle were about the same as the others. This shows that the latter are not in error as the result of there having been some re-distribution between the inside and outside of a fibre during fixation with osmium tetroxide.

The location of the albumin-accessible space

The location analysis was done in the usual way (Hill, 1959, 1962*a*, 1964) with longitudinal sections cut from the extreme surface of the muscle used in Expt. *A*. The activity (Table 4) was about 46 μc/ml. (0·15 % of fibre volume), and an exposure of 6 days was allowed. The grain density was rather low, and twenty-nine photographs were required for the analysis; one of these is reproduced in Pl. 4, fig. 2.

The grain distribution is shown in Text-fig. 1. The pattern is at least largely attributable (Hill, 1962*a*, *b*) to two narrow transverse sources, one lying along the Z line (in the centre of the I band), and the other at a site in the A band near the A–I boundary. The sarcomere length in the part of the muscle used for the analysis was 2·63–2·76 μ, mean 2·65 μ. The length of the A filaments in the embedded muscle is taken to be 1·45 μ (Page & Huxley, 1963), and the position of the A–I boundary is shown in Text-fig. 1. The second source lies about 0·1 μ from this boundary.

The method of presentation in Text-fig. 1 is such that the distribution attributable to the source at the Z line is recorded only on one side of the peak. If the distribution were re-drawn so that it lay on both sides of the Z line (as of course it actually does) it would then have a peak only half as high as that shown. It would be almost equal in height with the peak near the A–I boundary. This means that the amount of radioactive material present at the Z lines is approximately equal to the amount near the A–I boundaries.



Text-fig. 1. Longitudinal distribution of grains in an autoradiograph. Section thickness, 0·45 μ; sarcomere length, 2·65 μ. A total of 1126 grains is represented. These are located at twenty-six regions between the centre of the I band (arrow *Z*) and the centre of the A band (arrow *A*). The vertical lines indicate the number of grains at each position. The peak of one frequency distribution is at *Z*; the other is at *C*. The position of the boundary between the A and I bands is given by arrow *B*.

It can be shown that the steepness of the rise to the peak at the A–I boundary, from the trough between the two peaks (Text-fig. 1), is greater than might be thought possible on theoretical grounds. Similar anomalies have been observed on other occasions (Hill, 1962*a*). It is possible, therefore, that the peak at the A–I boundary has for some reason become unduly accentuated. If so, the amount of radioactivity at the A–I boundary is appreciably less than the amount at the Z line. However, a more elaborate analysis does not seem justified, and for the purpose of the following discussion the most straightforward assumption is accepted, namely, that the activity is equally divided between the Z line and the A–I boundary locations.

There are two of the active sites near the A–I boundary for each one at the Z line, so that quantity of radioactive material at each A–I position

is 50 % of that at each Z line. The volume of the albumin-accessible space varies from 0·12 to 0·28 % of the volume of the fibre. Taking a mean value of 0·20 % it follows from the results of the location analysis that the space at the Z line occupies 0·10 %, and that at each of the other positions at the A–I boundary is 0·05 % of the volume of one complete sarcomere.

The freeze-dried muscle could not be used for a location analysis because the structure was not well enough preserved.

The possible use of the electron microscope for autoradiography. The resolving power with autoradiography can be appreciably increased by employing the thin-layer emulsion method, with viewing under the electron microscope. The technique has its drawbacks (Hill, 1964), and it was not tried here because it was doubtful whether much would have been gained. The method might perhaps have been employed for a *transverse* analysis. This was done in the experiments with adenine nucleotide (Hill, 1964), where it was shown by this means that the nucleotide lay in the inter-fibrillar spaces rather than in the body of the fibrils. However, a similar problem cannot be said to exist here, because it can hardly be doubted that the albumin is confined to the reticular system in the inter-fibrillar spaces.

DISCUSSION

It has been shown that albumin can diffuse into the interior of the muscle fibre of the toad. One half of the space which is accessible is located in the middle of the I band. This is also the position of the T-system of intermediary vesicles. As further evidence to help in deciding whether the one is identical with the other, a comparison of their volumes must be made. It is therefore necessary to make an estimate of the volume of the T-system.

The volume of the T-system. The tubules of this system form a complete network in the inter-fibrillar spaces. The tubules are 'shared' between adjacent fibrils and, in effect, each fibril is associated with one half of the ring of tubule which surrounds it. Values for the internal dimensions of the tubules have kindly been supplied by Dr Sally Page (unpublished observations). They are based on measurements made from electron micrographs of frog's muscle which had been fixed in glutaraldehyde. Fewer data are available for toad's muscle, but the indications are that there is no marked difference between the two kinds of muscle. In frog's muscle the cross-section of an intermediary vesicle is roughly elliptical in form, with an internal diameter on the short axis of 250 Å, and, on the long one, 900–1300 Å. Taking a mean value of 1100 Å for the latter dimension, and assuming a perfect ellipse for the purpose of calculation, it works out that the volume of one-half of a ring of tubule is equal to $2{\cdot}03 \times 10^9$ Å^3.

The diameter of the myofibrils in the toad's sartorius is about 0·6 μ, at a sarcomere length of 2·5 μ (Hill, 1964). The volume of a fibril over a length of one sarcomere is $0{\cdot}71 \times 10^{12}$ Å^3. The total volume of the space in the fibre associated with one sarcomere is greater than this, because allowance has to be made for the inter-fibrillar spaces and for the mitochondria. It is estimated that about 30 % of the whole is accounted for by these additional components. This means that $1{\cdot}0 \times 10^{12}$ Å^3 represents the volume of fibre which is associated with one sarcomere length of myofibril.

Thus, according to this calculation, the volume of the T-system is 0·20 % of the volume of the muscle fibre.

The space accessible to albumin at the Z line averages 0·10 % of the fibre volume. This is sufficiently close to the estimated value for the T-system for it to be concluded that these spaces are probably one and the same.

The identity of the space at the A–I boundary. In amphibian muscle the physiological evidence (Huxley & Taylor, 1958; Huxley, 1964) and that available from electron micrographs (see, for instance, the review by Porter, 1961) point to the existence of a transverse system at the Z-line region, but not at the A–I boundary. It was therefore surprising to find a comparatively large space at the latter position.

In other muscles the situation may be different. In the lizard (Robertson, 1956; Huxley & Straub, 1958; Huxley, 1959) and in the crab (Huxley, 1956) the physiological and morphological evidence suggests that a T-system is located near the A–I boundary. Veratti (1961), in a paper first published in 1902, made a wide survey of different muscles; he showed the existence of transverse reticula at the Z line (but not at A–I) in amphibian muscle, and at the A–I boundary (but not at Z) in mammalian, bird and reptile muscle. In certain crustacean muscles, and in some insect larvae, he showed clearly that transverse reticula may be present at *both* of these sites. Veratti was very strongly of the opinion that every type of muscle really possesses both systems; he was convinced that only the erratic nature of his staining methods prevented this from being apparent in his preparations. The results from the present experiments suggest that there may have been some truth in Veratti's rather intuitive conviction, though the situation remains largely obscure.

It is worth drawing attention to the fact that the location of the space near the A–I boundary is precisely that at which, under certain conditions, adenine nucleotide may become concentrated, and where a clear-cut transverse band of basophilic material may be demonstrated (Hill, 1960). It should, however, be pointed out that, whereas the albumin is likely to lie in the inter-fibrillar spaces, the nucleotide (under the conditions

referred to) is more likely to have been bound to some component of the fibril. The spatial correspondence may therefore be fortuitous and of no particular interest.

How does the space at the A–I boundary become filled? The simplest view, though there is no morphological evidence for it, is that the A–I space has its own transverse continuity, with entry holes at the fibre surface. The alternative is to suppose that this space is a part of the complex longitudinal reticulum which is present throughout the inter-fibrillar spaces, and that it becomes filled through longitudinal connexions with the T-system at the Z line. Such an idea is not easily made consistent with the results obtained by autoradiography, but it need not be ruled out. If the volume per unit length of the supposed connecting-tubules were comparable with that of the T-system it is fairly obvious that the autoradiographic distribution would have the form actually found only if these connexions were very few in number, less than one per fibril. On the other hand, the longitudinal connexions might be a good deal narrower than the channels of the T-system. If they were circular in cross-section, and had an internal diameter of 160 Å, their capacity, per unit length, would be only one-tenth that of the T-system, and their contribution to the autoradiographic grain-yield might be negligible.

Is the space at the A–I boundary an artifact due to radiation damage? There was considerable variation, as between fibres, in the volume of the albumin space found by the present method. It was suggested that some of this variability might be attributed to radiation damage. The fibres used for location analysis had an albumin space of 0·15 %, which is much lower than the space recorded in fibres which were definitely suspected of having been damaged. But some fibres had an even smaller space, around 0·10 %, and it might be suggested that these, alone, were in normal condition. Unfortunately, such fibres were not close enough to the surface of the muscle to have been well preserved by the fixative, and they could not be used for the location analysis.

The question has to remain open.

Other 'special regions' of the muscle fibre. Harris (1963) has shown that at least 10 % of the muscle volume is freely accessible to certain ions, such as those of methyl sulphate, which are incapable of penetrating the muscle as a whole. Fatt (1964) has suggested, from a study of the electrical properties of frog's sartorius muscle, that a 'channel-system' exists, which (if it is filled with material possessing a conductance equal to that of the myoplasm) appears to occupy 2 % of the muscle volume.

The volumes of the spaces referred to by both Harris and Fatt are of a different order of magnitude from that under consideration here, and it is probable that other, larger spaces must be involved.

Endo (1964) has given a striking demonstration showing that certain fluorescent dye molecules can rapidly pass into, and out of, the central region of the I band of a frog's muscle fibre. Quantitative data are not so far published to indicate what proportion of the muscle volume is accessible to the fluorescent material, and it remains to be seen whether it is of the same order of magnitude as that which is occupied by the T-system, or is accessible to albumin.

Hodgkin & Horowicz (1960) have observed that in an isolated muscle fibre the rate of change of membrane potential produced by a sudden fall in potassium concentration is slower than the change produced by a rise, and they suggested that this could be explained by assuming that potassium ions may be retained for a short time in a special region of the fibre whose volume was estimated as being 0·2–0·5 % of the volume of the fibre. This region is not much larger than the T-system, or than the space accessible to albumin, and one is tempted to suggest that they are one and the same.

Further investigations. It would be interesting to investigate the size and location of the space accessible to large molecules, such as albumin, in mammalian, avian, reptilian or crustacean muscles, where the arrangement of the transverse tubular system is thought to be fundamentally different from that found in amphibian muscle. Similar experiments with cardiac muscle might also prove worth doing. It would not be surprising to find that the volume of the space accessible to albumin in mammalian cardiac muscle is much larger than it is in amphibian skeletal muscle. The transverse tubular system has not proved so difficult to identify in cardiac muscle, and its volume as seen in electron micrographs of the heart of sheep (Simpson & Oertelis, 1962) and of human and rabbit cardiac muscle (Nelson & Benson, 1963) appears to be considerably larger than it is in most skeletal muscles.

Other muscles have very conspicuous and relatively capacious reticular systems. Exceptionally well-developed systems have been demonstrated in two very fast muscles, one in a fish (Fawcett & Revel, 1961) and the other in a bat (Revel, 1962). At the other end of the scale, the reticulum is poorly developed in the slow, tonus fibres of the frog (Peachey & Huxley, 1962); and Peachey (1961) has shown that in the muscle of *Amphioxus* almost no reticulum can be seen. Muscles such as these might be considered for investigation by the method described in this paper.

Another suggestion arises from some observations made on muscles which had been fixed in acetic acid and ethanol (Hill, 1964). Vesicles were seen at a position (in the I band) where none is found in material fixed in the conventional manner. One is therefore tempted to suppose it possible that some other 'unconventional' method of fixation, or of staining, might

once more show an alternative morphological pattern, and perhaps reveal the 'missing' tubular system which the present experiments indicate may exist near the A–I boundary.

'Phasing' of striations. The method used in these experiments provided some additional information concerning the spacing which must have existed between the fibres of a muscle while it was still living. It was found that many of the fibres were in remarkably close contact with one another (the spacing being less than 1000 Å) and there appeared to be a tendency for the striations of these closely packed fibres to be 'in phase' with one another. Such 'phasing' of the striations of adjacent fibres has been noticed in the past (Buchthal & Knappeis, 1940), in living muscle; but, since it was previously assumed that the inter-space volume was uniformly distributed between the fibres, it had been difficult to conceive how any mechanism, postulated as responsible for the 'phasing', could have acted over the required distance, since this would have had to be as much as 2 μ. (An inter-space volume of 10 %, in a bundle of square-section rods of side 40 μ would, if evenly distributed, require a spacing of 2 μ.) However, if the fibres which show the 'phasing' are as close together as is now suggested, it is less difficult to accept the notion of some sort of interaction between fibres.

The 'phasing' is perhaps found only in a young animal whose muscles are still developing, with the effect being confined to regions where fibres have recently divided. Or it might be due to a non-specific affinity of similar structures for one another, due to surface or to long-range colloidal forces (Barer, 1948).

One other possible reason which is relevant to the main subject of this paper can be mentioned for the existence of the 'phasing'. It is thought that the T-system of tubules in the muscle communicates with the exterior through the sarcolemma at the Z-line region. If fibres are 'in phase', and in very close contact, this system of communication might, in effect, extend between adjacent fibres; this could conceivably be of some functional or metabolic importance for the muscle.

The matter needs further investigation; this could at least establish the frequency with which adjacent fibres show the 'phasing'. It is possible that this frequency is not greater than could be accounted for by chance, and the matter should be put on to a quantitative basis.

SUMMARY

1. Sartorius muscles of the toad (*Bufo bufo*) were soaked in a solution containing tritium-labelled albumin. The muscles were then fixed with osmium tetroxide, or by freeze-drying. Autoradiographs of the embedded

muscles showed that a small amount of albumin had penetrated into the fibres, and the space accessible to these molecules was estimated as 0·12–0·28 % of the fibre volume.

2. The location analysis showed that in any one sarcomere about half the albumin space (0·10% of the fibre volume) lies at the position of the Z line. The remainder consists of a pair of spaces (each being 0·05 % of the fibre volume) lying at the ends of the A band, close to the A–I boundary.

3. The T-system of tubules (made up of the central elements of the triads) lies at the Z line, and its volume, calculated from electron micrograph data, is about the same as that of the albumin-accessible space at this position. It seems likely, therefore, that the albumin entered the T-system. The morphological identity of the space near the A–I boundary is unknown.

4. The method has provided some information concerning the spacing which must exist between the fibres in the living muscle, as distinct from that seen in the fixed preparation. A point of interest is that this spacing is sometimes much less than that to be expected if the inter-space volume were uniformly distributed. It was noticed that fibres which had been close together in the living state often had their A and I bands lined up with one another.

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Note added in proof. Since this work was completed two independent investigations by Huxley (1964) and by Page (1964) have been reported which show that the iron-containing protein ferritin is able to enter the T-system of tubules in frog's muscle from the extracellular space. A paper by Endo (1964) has provided further details of his study on the entry of a fluorescent dye into the living muscle fibre of the frog: it appears that about 1% of the fibre volume is accessible to this dye, and it therefore seems likely that regions other than the T-system must be penetrated by it.

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EXPLANATION OF PLATES

PLATE 1

Autoradiograph of a cross-section of a sartorius (Expt. *B*). Section thickness, 0·6 μ. Exposure, 6 days. The fibre inter-spaces, filled with radioactive albumin, are marked by dense deposits of silver. Scale bar, 100 μ.

PLATE 2

Autoradiograph of a cross-section of a sartorius (Expt. *A*). The section was cut at less than 90° to the long axis and the fibre outlines are therefore elliptical. Thickness, 0·6 μ. Exposure, 7 days. The silver deposits marking the fibre inter-spaces are seen to be not uniformly dense, and this is due to unequal spacing between adjacent fibres. There is a tendency for the wider inter-spaces to be linked together to form long wide channels, which may traverse the entire width of the muscle. Scale bar, 50 μ.

PLATE 3

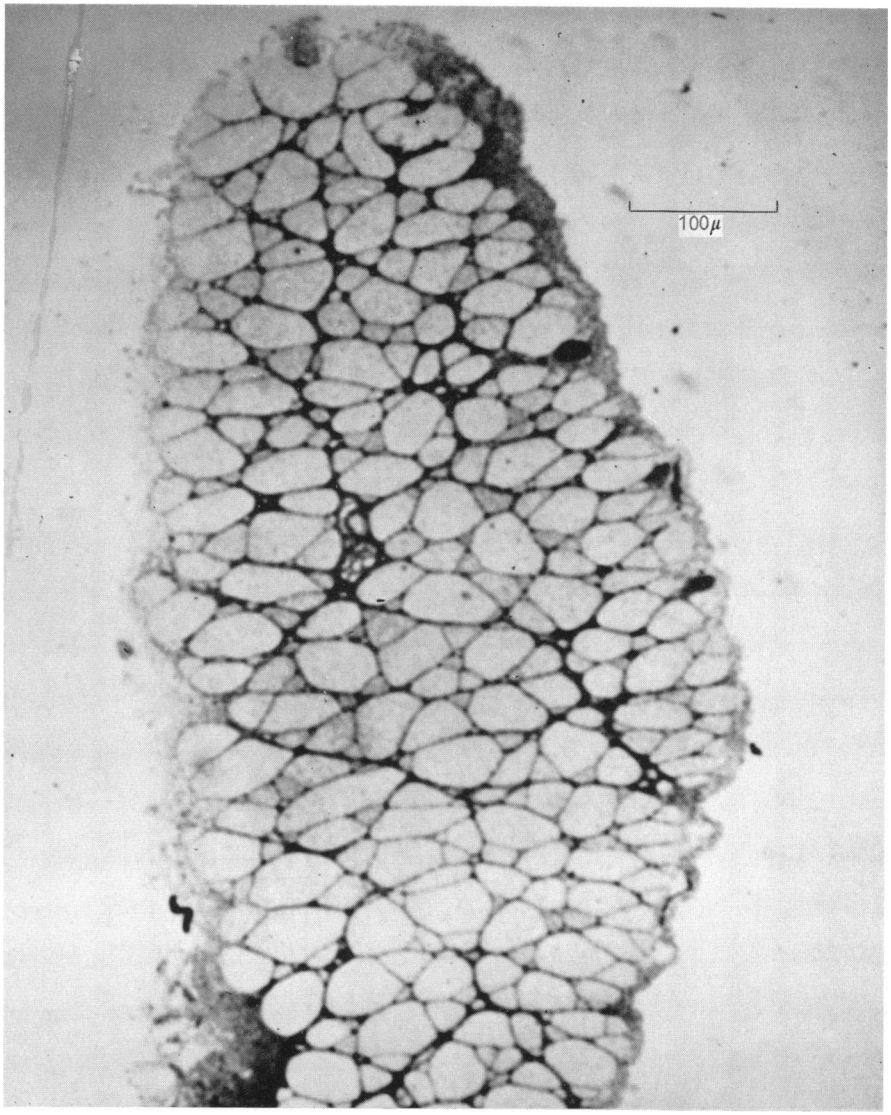
Fig. 1. Autoradiograph of a cross-section of a sartorius, cut at less than 90° to the long axis (Expt. *A*). Dark-field illumination; the silver deposits show light. Thickness, 0·6 μ. Exposure, 7 days. The radioactivity of the interior of the fibres is seen to be variable. A few extremely dense areas (showing almost pure white) lie over dead fibres, which have become abnormally permeable to the radioactive albumin. Scale bar, 50 μ.

Fig. 2. Autoradiograph of a cross-section of a part of a muscle which shows a damaged fibre (Expt. *C*). Thickness, 0·6 μ. Exposure, 62 days. This fibre, with the dense silver deposit, must have been abnormally permeable to the albumin. It is surrounded by normal fibres. Scale bar, 10 μ.

PLATE 4

Fig. 1. Autoradiograph of a cross-section of a muscle, showing the variability in the width of the inter-spaces between neighbouring fibres. Thickness, 0·6 μ. Exposure, 13 days. The inter-space marked with an arrow possesses very little radioactivity, and calculation shows that, *in vivo*, the spacing of the fibres must have been less than 1000 Å. Note the dense pockets in the angles between the fibres. This section was stained with basic fuchsin, to show the inter-spaces. Scale bar, 10 μ.

Fig. 2. Autoradiograph of a longitudinal section of a muscle. This was one of the twenty-nine photographs used for the location analysis (Expt. *A*). Thickness, 0·45 μ. Exposure, 5·9 days. The section was stained with basic fuchsin; the A band and Z line are dark. There is an example here of an inter-space which possesses low radioactivity, and it was calculated (see text) that at the region between the arrows the space, *in vivo*, was only 300–400 Å across. The present space (0·6 μ) is an artifact. At regions of close contact the fibres often have their bands 'in phase' with one another, as seen here. Scale bar, 10 μ.



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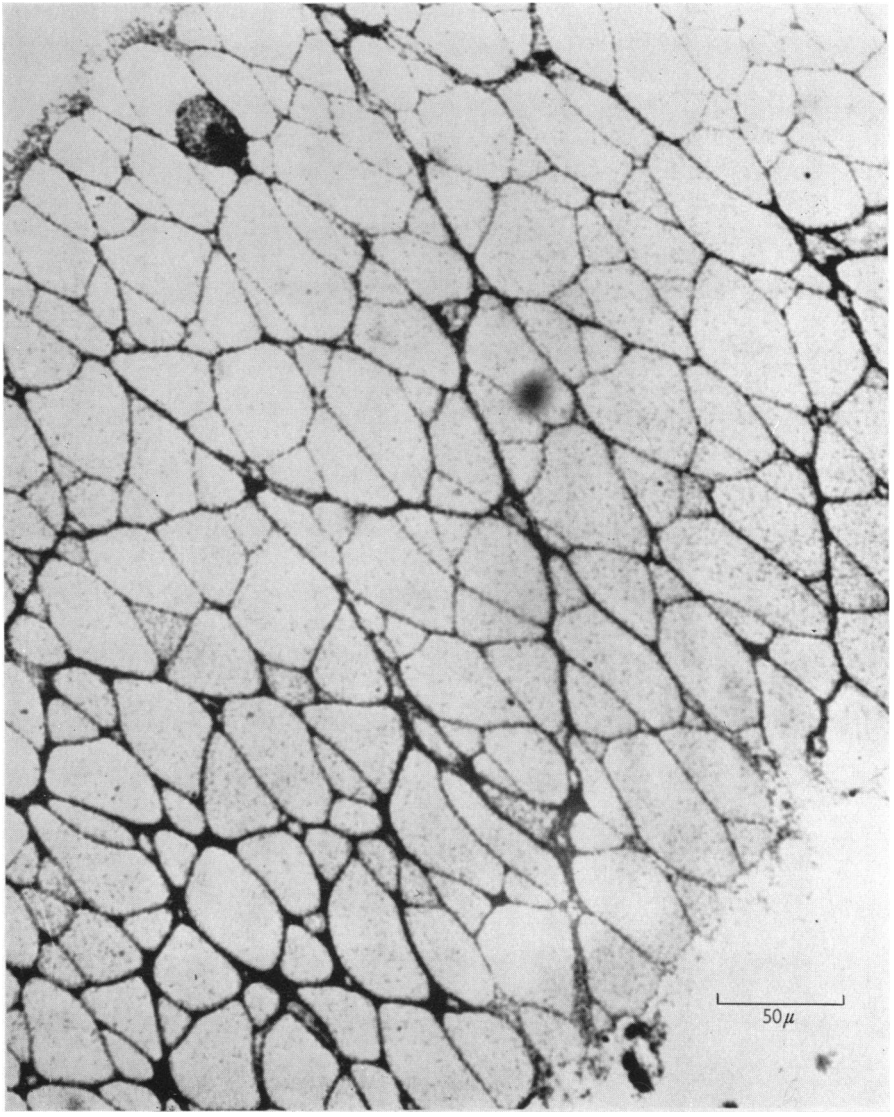

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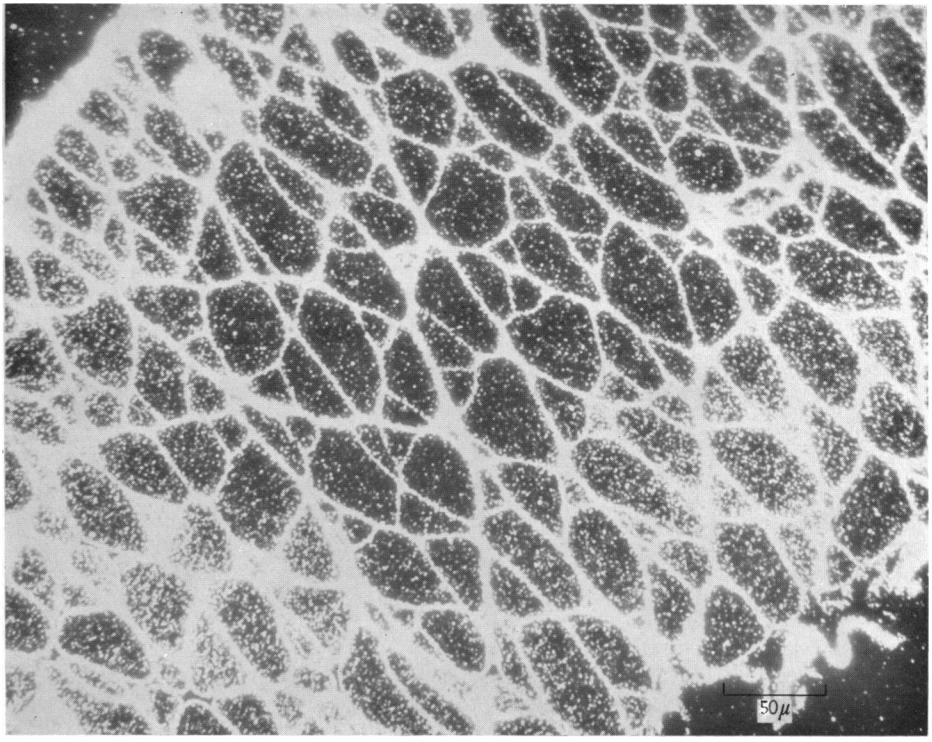

Fig. 1

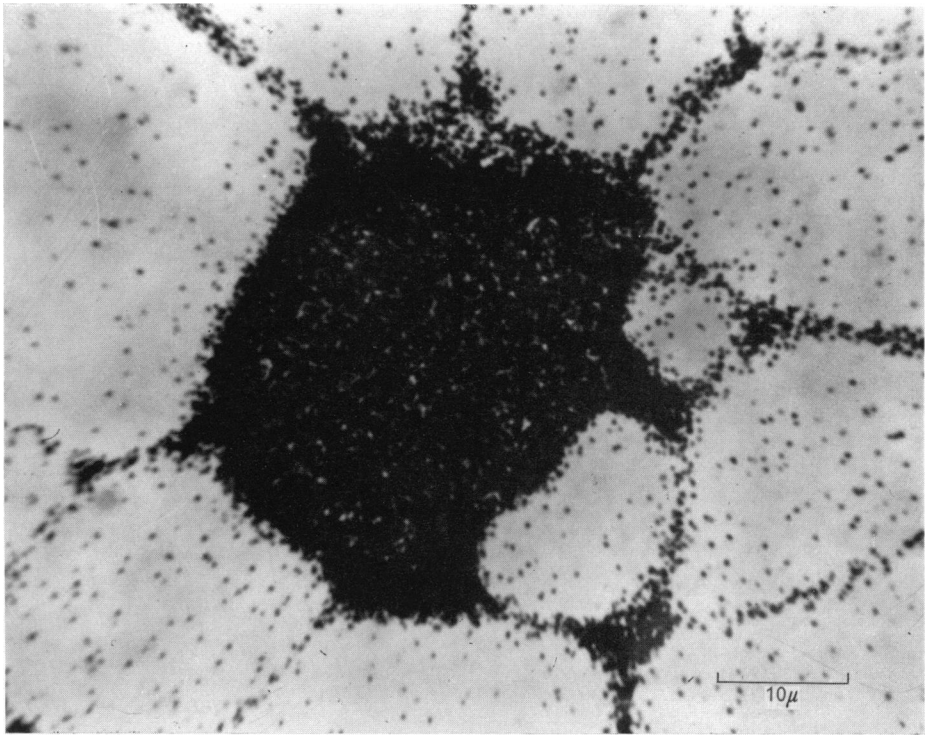

Fig. 2

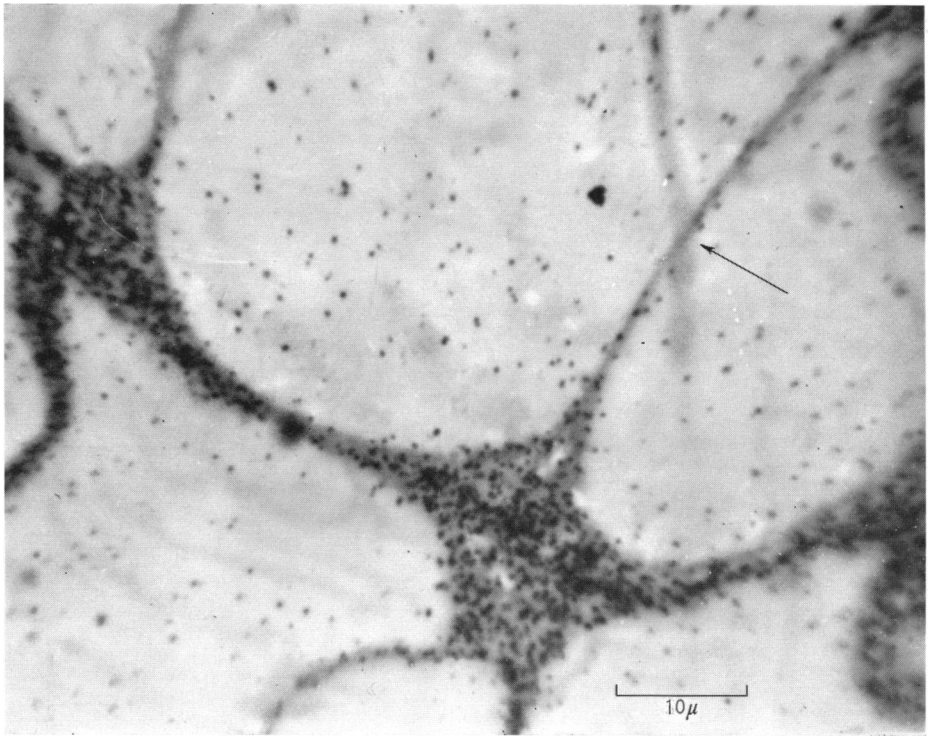

Fig. 1

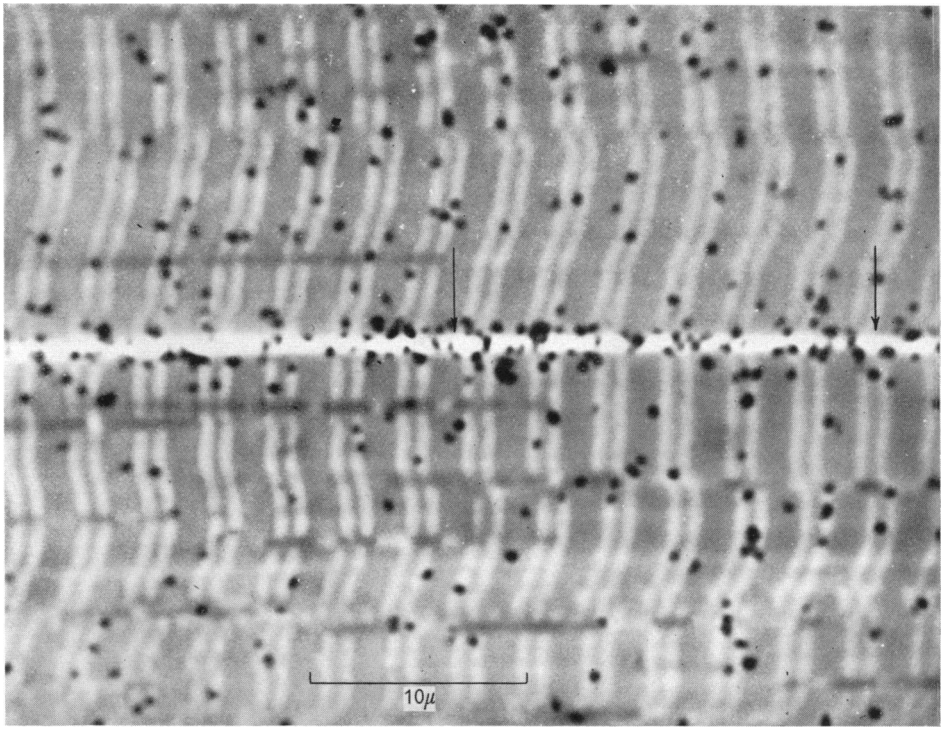

Fig. 2