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THE OSMOTIC PROPERTIES OF STRIATED MUSCLE FIBRES IN HYPERTONIC SOLUTIONS

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It has been known for many years that, when muscles are placed in hypertonic solutions, their loss in weight is generally less than would be expected if the muscle were a simple osmotic sac. Even in the strongest solutions muscles lose only about 20% of their initial weight (see Loeb, 1897; Cooke, 1898). Overton (1902) showed that even when allowance was made for dry matter and for an extracellular space of about 20% (corrections not adequately appreciated by his predecessors), there was still a discrepancy between the calculated and the measured changes in the weight of the muscle. He accounted for this by proposing that about a fifth of the water was bound within the cell in the form of 'Quellungswasser', which could not be displaced except by very large gradients of vapour pressure (see his pp. 139–142, 155). Overton's conclusion was disputed by Hill (1930), who showed that almost all the fibre water could dissolve solutes in a normal manner. The assumption that all the water in muscle fibres participates in osmotic effects was later shown to lead to a satisfactory explanation of the weight changes in mildly hypotonic and hypertonic solutions (Boyle & Conway, 1941). It seemed to us that the discrepancy in strongly hypertonic solutions might be explained by Overton's own qualitative observation, that in such solutions the extracellular space appeared to be increased.

In this paper we have confirmed and extended this observation of Overton's by actual measurement of the extracellular (sucrose) space in solutions with various osmotic strengths. This permits, by subtraction, a direct calculation of the amount of fibre water as a function of osmotic strength. It then turns out that in solutions up to four or five times as concentrated as normal Ringer's solution the muscle fibres behave as simple semipermeable bags containing a fixed amount of solute: there is no need to invoke binding of any appreciable part of the fibre water.

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METHODS

All the experiments were performed on sartorius or sternocutaneus muscles of frogs (*Rana temporaria*). The weight of the sartorii varied from 41·4 to 136·3 mg (mean = 71·7 mg, S.D. = 16·3 mg, n = 75); muscles of this size strike a reasonable compromise between speed of diffusion and accuracy of weighing and chemical estimation.

Solutions. Our normal Ringer's solution contained (mM): NaCl 115·5, KCl 2·0, $CaCl_2$ 1·8, Na phosphate buffer, pH 7·0, 2. Solid sucrose was added in order to prepare hypertonic solutions. Roughly speaking, 7·5 g sucrose added to 100 ml. of Ringer's solution doubles the osmotic strength. However, in these experiments we have been concerned with such concentrated solutions that they depart substantially from ideal behaviour. To allow for this we have expressed all concentrations as effective milliosmoles/kg water (θ);

$$\theta = n.\phi.m; \tag{1}$$

where m = molal concentration; ϕ = osmotic coefficient; n = number of particles given on complete dissociation of one molecule. This procedure puts all the solutions on a common basis, because it is the value of θ that determines the activity of water (a) in each solution, and thus decides in which direction water will tend to move. For

$$0{\cdot}018.\theta = -\ln a \simeq -\ln\left[\frac{\text{(vapour pressure of water in solution)}}{\text{(vapour pressure of pure water)}}\right].$$

The appropriate values for ϕ have been taken from Robinson, Smith & Smith (1942) and Conway (1952); unfortunately, information is available only for each chemical substance considered separately. For mixed solutions it is accordingly necessary to assume that each constituent acts independently. This is probably quite accurate, because the situation is dominated by sodium chloride and sucrose, which are unlikely to interact.

Thus, in the case of our Ringer's solution, which has a density at 20° C of 1·00415 g/ml. (Wilkie, 1953), 1 l. contains 997 g of water. NaCl (ϕ = 0·93) contributes 215 m-osmole/kg water; KCl (ϕ = 0·99) contributes 4 m-osmole/kg water and the remaining constituents contribute 5–11 m-osmole/kg water, depending on the unknown degree to which they interact. We have assumed a value of 230 m-osmolal for the whole solution. It is often convenient to express the strength of solutions as a multiple of normal Ringer's solution, i.e. as a multiple of 230 m-osmolal.

The measurements on sucrose solutions by Robinson *et al.* (1942) show how the osmotic coefficient, ϕ, varies with molality, m. Their results can be described quite accurately by the simple empirical equation:

$$\phi = 0{\cdot}094m + 1. \tag{2}$$

When x g of solid sucrose (mol. wt. = 342) is added to 100 ml. of Ringer's solution, the resulting molality of sucrose is

$$m = \frac{x}{34{\cdot}2 \times 0{\cdot}997}. \tag{3}$$

Combining this with equations (1) and (2), and adding 230 m-osmole/kg for the salts, we arrive at the final working equation for the effective osmotic strength of the whole solution,

$$\theta = \frac{1000x}{34{\cdot}2 \times 0{\cdot}997}\left[\frac{0{\cdot}094x}{34{\cdot}2 \times 0{\cdot}997} + 1\right] + 230 \text{ m-osmole/kg water.} \tag{4}$$

The more concentrated solutions have a density appreciably greater than 1 g/ml. We need to know the value of this density in order to make calculations about the weight of the various constituents of the muscle, to which the extracellular fluid makes a substantial contribution. Fortunately sucrose forms simple additive solutions in Ringer's solution, so their density can be calculated from the equation:

$$D = \frac{x + 100{\cdot}415}{x/1{\cdot}588 + 100} \text{ g/ml.} \tag{5}$$

(1·588 g/ml. is the density of solid sucrose). This equation was checked by direct measurement and found to be accurate.

Weighing. One important source of variation in investigations of this kind is the technique employed for removing surplus solution from the muscle before weighing it. Blotting may damage the outer fibres; accordingly, we have followed the advice of Dr R. H. Adrian and have merely drained the muscle by pulling it slowly up the side of a glass beaker. When this technique is employed, successive weighings agree well (coefficient of variation, S.D./mean = 1–1½%) and the muscle survives well. For convenience in handling, the muscle is suspended from the tibial end by a loop of nylon monofilament (weight 1·6 mg/m). Obviously a drained muscle contains more extracellular fluid than a blotted one. In order to discover how much more, a series of ten muscles were weighed after our standard draining procedure, and again after blotting on dry filter paper. The muscle was stroked on to the paper, then moved to a dry place and stroked again, until no damp mark could be seen. This is intentionally a severe blotting procedure, which may damage the muscle, but it would be expected to remove as much fluid as possible and thus to set a lower limit for the extracellular fluid. Other published results, e.g. those obtained by blotting on damp filter paper, must lie somewhere between our 'blotted' and our 'drained' values. The mean value of (drained weight)/(blotted weight) was 1·26 (S.D. = 0·079). The values of this ratio in each case did not seem to be related to the drained weights of the muscles, which varied from 63·3 to 124·0 mg (mean = 80·5; S.D. = 18·9).

Sucrose was estimated by the anthrone method (Sattler & Zerban, 1948) as modified for small quantities of tissue by Norman, Randall & Bohr (1959). Care was taken to allow for the non-linearity of calibration that was sometimes encountered.

RESULTS

Direct observation

As Overton (1902) reported, there does appear to be more space than usual between the fibres of a muscle soaked in a hypertonic solution. However, the effect is striking only in rather strong solutions (e.g. $> 2 \times R$ that is > 460 m-osmolal), where the whole appearance of the muscle is altered and the fibres themselves look transparent and refractile. We could not satisfy ourselves purely from direct observation that the absolute, and not merely the relative, size of the extracellular space had increased.

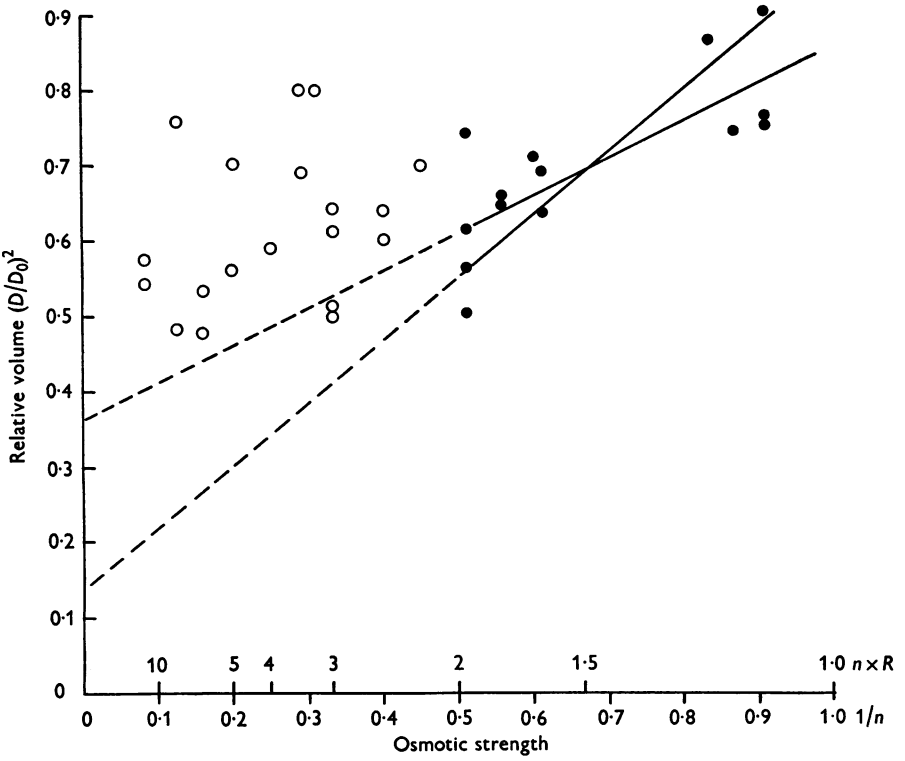
In any case, our primary interest is not in the extracellular space as such but rather in the variation in fibre water. In preliminary experiments this was estimated roughly by measuring the diameters of muscle fibres when they were immersed in solutions of different osmotic strength. The sternocutaneus muscle was used because its fibres are spread out in a thin sheet. It was pinned out in a small chamber under only enough tension to keep it flat, illuminated obliquely from beneath and examined under a binocular microscope ($\times 70$) equipped with an eyepiece micrometer. In each experiment the diameters of about fifteen fibres were measured, first in normal Ringer's solution, then in the test solution. The mean diameters of the two groups, D_0 and D, were then calculated.

Method of calculation. If the fibres retain the same cross-sectional shape

as they shrink, and do not change in length, their relative volume in the hypertonic solution is given by

$$V/V_0 = (D/D_0)^2,$$

where V and V_0 are the volumes in the hypertonic solution and in Ringer's solution respectively. When the relative volume $(D/D_0)^2$ is plotted against the reciprocal of the relative osmotic strength ($1/n \times R$ in our terminology),

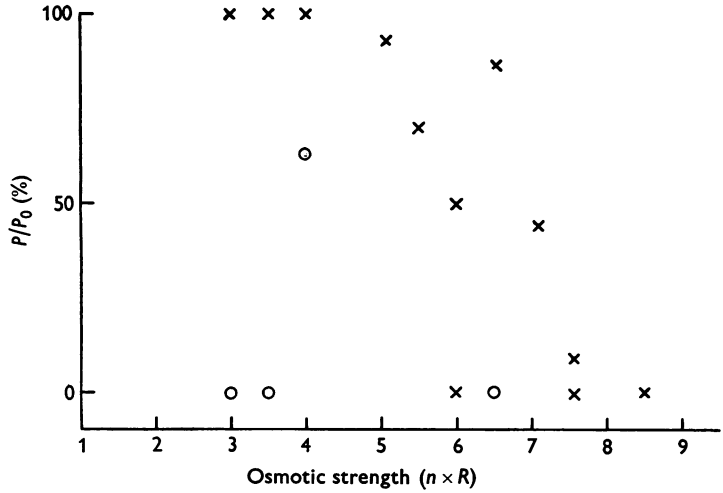


Text-fig. 1. The variation of fibre volume with osmotic strength. Abscissae. Osmotic strength, $n \times R$; i.e. $n \times 230$ m-osmolal, reciprocal scale; or $1/n$, linear scale. ● $1 \times R$–$2 \times R$; the regression lines for these points are indicated. ○ above $2 \times R$; no significant correlation between the variables.

the points would fall on a straight line if the fibre were a perfect osmometer. The intercept on the Y-axis measures the fraction of the fibre volume that does not participate in the osmotic process.

The actual experimental results (Text-fig. 1) show a good deal of scatter. However, up to $2 \times R$ (filled circles), relative volume and osmotic strength are strongly correlated (correlation coefficient, $r = 0{\cdot}77$; $P = 0{\cdot}001$), and the slope of the regression lines is reasonable. Their intercept on the vertical axis indicates (very inaccurately) an inactive volume of 25–30 %. If the regression lines are constrained to pass through the origin 1·0, 1·0

they are steepened somewhat and intercept on the y axis at 0·09 and 0·16. Beyond $2 \times R$ (open circles) there is no correlation between the two variables ($r = 0{\cdot}28$; $P > 0{\cdot}1$). The fibres thus appear to behave as osmometers only up to $2 \times R$, but not beyond. It seemed possible that stronger solutions might damage the fibres, perhaps making them permeable to sucrose. Overton certainly thought that even mildly hypertonic solutions were harmful, and both Hill (1930) and Fenn (1936) assumed that a fraction of the muscle fibres died during experiments where they were exposed to a raised osmotic pressure. It thus seemed necessary to discover what were the limits of osmolality within which muscles would survive.



Text-fig. 2. The survival of frogs' sartorii after soaking for 2 hr in hypertonic solution at 0° C: the muscles were allowed to recover in Ringer's solution. Ordinates, tetanic tension after recovery as a percentage of the original tension. Abscissae, osmotic strength, $n \times R$. ×: these muscles were freshly dissected. ○: these muscles had been used in some other experiment, but until they were placed in the hypertonic solution they showed no signs of deterioration; evidently slight fatigue predisposes to osmotic injury.

Survival in hypertonic solutions

The isometric tetanic tension developed by the muscle was used as a measure of the number of surviving fibres. All the experiments were performed on sartorii at 0° C in oxygenated solutions. After a preliminary soaking for at least 30 min in Ringer's solution, the tension developed in a 1 sec tetanus was measured by a mechano-electronic transducer (RCA 5734). The muscle was then soaked in the test solution for 2 hr. At the end of this period it was replaced in normal Ringer's solution, and was stimulated at intervals in order to test its recovery. The time required for maximum recovery depends on the strength of the test solution. Up to

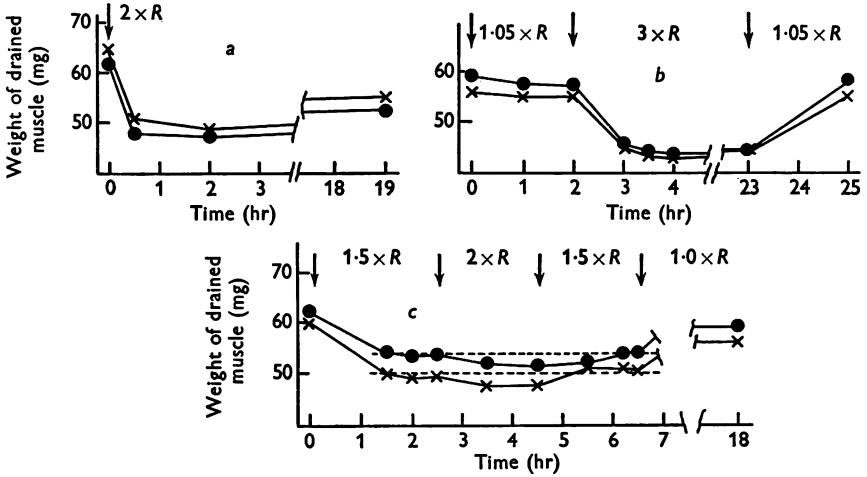
$3{\cdot}5 \times R$, 2 hr were sufficient, but in the stronger solutions 24 hr were required. The results of these tests are shown in Text-fig. 2 (crosses) where it is seen that after $4 \times R$ recovery is complete, and after $7–8 \times R$ some recovery takes place.

While adding and after removing the stronger solutions, the mechanical apparatus was rearranged as an isotonic lever with small load (approximately 0·2 g wt.) in order to detect whether or not contracture developed. Above about $6 \times R$ there was slight and usually transient shortening when the hypertonic solution was applied, but no mechanical change was seen on replacing normal Ringer's solution.

The circles in Text-fig. 2 show the result of tests in which the muscle had already been used once for some other purpose, e.g. a mechanical experiment or another osmotic experiment. Muscles were only used in this way if the first procedure had left tetanic tension completely unchanged. Nevertheless, such muscles clearly are more liable to be damaged by hypertonic solutions than those being used for the first time. Perhaps some effect similar to this is responsible for the reports of damage mentioned above.

The time required for equilibration in hypertonic solutions

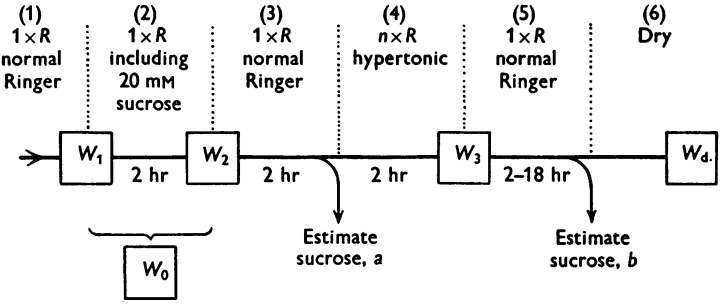
Another essential preliminary is to discover how long must be allowed for a muscle to come to osmotic equilibrium. Serial weighings of muscles



Text-fig. 3. The time course of the change in weight in hypertonic solutions. Abscissae, time; note the interruptions in the record. The two lines show the changes in the paired sartorii. *a*, muscles in $1 \times R$ exposed to $2 \times R$ and left to soak for 19 hr. *b*, muscles in $1{\cdot}05 \times R$ exposed to $3 \times R$, left to soak in it for 21 hr, then replaced in $1{\cdot}05 \times R$. *c*, muscles originally in $1 \times R$ exposed to the solutions shown; note that roughly the same weight (interrupted horizontal line) is reached in $1{\cdot}5 \times R$ both from $1 \times R$ and from $2 \times R$.

soaking in hypertonic solutions show that the weight change has two fairly distinct phases (Text-fig. 3). The first phase is complete within 2 hr or less. Much slower weight changes may occur over the succeeding 24 hr, but they are small, irregular and almost certainly the result of a different process from the one that we are studying. The slow changes in Text-fig. 3*a* and *b* are both increases, but in fact decreases are equally common. Further evidence that the weight after 2 hr is truly a function of osmotic strength is given in Text-fig. 3*c*, which shows how approximately the same weight in $1{\cdot}5 \times R$ is approached both from $1 \times R$ and from $2 \times R$.

Unfortunately, change in weight of the whole muscle is no guide to what is happening in solutions stronger than about $2{\cdot}5 \times R$, but there seems no reason, either on theoretical grounds or from a comparison of the curves in Text-fig. 3, to suppose that the time constant of change becomes longer in the stronger solutions. We have therefore adopted 2 hr as the standard period for coming to osmotic equilibrium for muscles of the size that we used. Fenn (1936) shows how equilibration time varies with muscle weight.



Text-fig. 4. Diagram to illustrate the procedure for estimating fibre water. For details see text.

Determination of fibre water

As mentioned in the introduction, our main measurements of fibre water were made by first determining the extracellular water (sucrose space), then subtracting this from the total water in the muscle. In each of fifty-nine muscles the fibre water was determined at 4–8° C, first in a solution of normal osmolality, then in a hypertonic solution. The six stages of this procedure are illustrated in Text-fig. 4.

(1) Starting with the muscle in normal Ringer's solution, it was drained and weighed ($= W_1$).

(2) It was then soaked for 2 hr in a solution with the same effective osmolality as Ringer's solution (230 m-osmolal) and the same composition, except that an appropriate amount of sodium chloride had been replaced

by sucrose, whose concentration was 20 mM. At the end of this soaking period the muscle was drained, weighed ($= W_2$) and

(3) placed in 5 ml. of normal Ringer's solution. W_2 tended to be less than W_1 by about 3 %: the mean was taken as the starting weight of the muscle, W_0. After 2 hr the 5 ml. of solution contained some sucrose that had soaked out of the muscle. The solution was made up to 10 ml. and the sucrose concentration in two duplicate 2 ml. portions was estimated by the anthrone method. Combining this estimate with the known concentration of sucrose in solution (2), it is possible to calculate how many microlitres of solution (2) were included with the muscle when it was weighed for the second time, i.e. how many microlitres of extracellular fluid were present. Calling this quantity a μl., the contribution W_{e0} that this makes to the weight W_2 is a μg, since the density of the solution (1·004 g/ml.) is not significantly different from 1·0. The muscle (W_2) consists of extracellular fluid (W_{e0}), fibre water (W_{f0}) and dry matter (W_d), so

$$W_{f0} = W_2 - W_d - a. \tag{6}$$

(4) In the mean time the muscle had been placed for 2 hr in the hypertonic solution ($n \times R$) under test. At the end of this period it was drained and weighed ($= W_3$).

(5) It was then placed in 5 ml. of normal Ringer's solution for from 2 to 18 hr, in order to soak out the sucrose within it. This was estimated exactly as in (3) and the extracellular fluid b μl. calculated as before. Then where

$$W_f = W_3 - W_d - b \times D, \tag{7}$$

D g/ml. is the density of the $n \times R$ solution calculated from equation (5). Note that it does not make any difference to the calculation of fibre water what technique has been used for blotting or draining the muscle so long as all the sucrose in solution that was weighed in W_2 and W_3 was estimated chemically.

(6) Finally, the muscle was dried over silica gel, and weighed again ($= W_d$).

Accuracy of sucrose determinations: a statistical test based on the difference between duplicate estimations

The sucrose concentration chosen for solution (2), 20 mM, is a compromise between the wish that the solution should be as similar as possible to normal Ringer's solution, and the need that there should be enough sucrose to estimate.

Each sample of eluting solution was divided into two duplicates before chemical treatment, so as to permit two independent determinations of optical density, k and k'. We have devised the following test in order

to use the difference $(k-k')$ to estimate the precision of the mean $(k+k')/2$: suppose each measurement of k to consist of a true part, t, and an error, e. Then

$$k = t+e$$

and

$$k' = t+e'$$

so $(k-k') = e-e'$ and $(k+k')/2 = t+(e+e')/2$, because t is the same for both duplicates, though it is different for different samples. When $(k-k')$ is plotted against $(k+k')/2$ for all the samples it is evident that the difference of errors is independent of the mean and is distributed about zero with a standard deviation s. From sampling theory it can be shown that the standard deviation of $(e-e')$ must be about $s \times \sqrt{2}$, while the standard deviation of $(e+e')/2$ is about $s/\sqrt{2}$. The standard error applicable to each mean is thus about one half the S.D. of all the observed differences $(k-k')$. In our experiments the coefficients of variation (standard error/mean) were about 5 %—from 2 to 8 % in normal muscles and from 2 to 12 % in hypertonic ones.

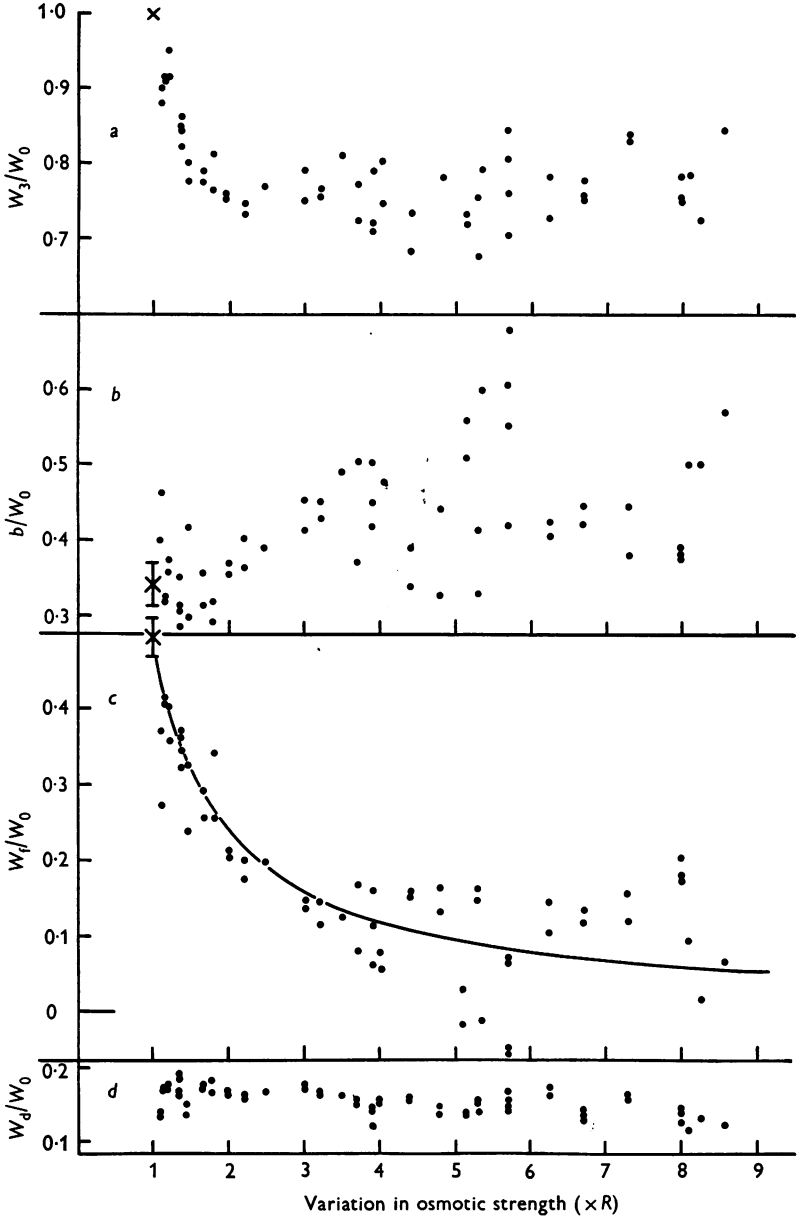
Experimental results

The results of these experiments are shown in Text-fig. 5. In order to put muscles of different weight on to the same basis, the quantities for each muscle are expressed as a fraction of its initial weight, W_0.

Text-fig. 5*a* shows the way in which the weight of the whole muscle varies with osmotic strength. Beyond about $2 \times R$ the weight does not diminish significantly: this fact was established a long time ago, as mentioned in the introduction. However, part of the failure to lose weight is in a sense an artifact, for in strong solutions the loss of weight of the fibres is offset by the increased density of the solution filling the interspaces. If this effect was allowed for, it would lower the points at the extreme right-hand end by 0·07–0·08.

Text-fig. 5*b* shows how the extracellular space alters with osmotic strength. Even if the volume of the extracellular fluid remained constant, its weight would increase in hypertonic solutions because of their greater density. In order to present a truer picture of the variation in volume of the extracellular space, we have plotted the weight (b/W_0) that the extracellular fluid would have had if its density had remained 1 g/ml. It can be seen that the volume of extracellular space is roughly constant at 0·35 up to about $2 \times R$, then it increases to nearly 0·5 in $4 \times R$. Beyond that point the results become so scattered that it is hard to generalize, and it is not altogether clear that the increase in extracellular fluid is maintained. The very large values that are sometimes encountered may result from death of some of the fibres, which permits sucrose to permeate inside them.

Text-fig. 5*c* shows the variation in fibre water with osmotic strength.



Text-fig. 5. The variation with osmotic strength (abscissae) of: *a*, drained muscle weight, W_3; *b*, extracellular volume, *b*; *c*, weight of fibre water, W_f; *d*, dry weight, W_d. All these are expressed as a fraction of the original drained weight, W_0. The extracellular volume *b* is expressed as the equivalent weight of water (or Ringer's solution). The mean values of *b* ($n = 22$) and W_f ($n = 20$) at $1 \times R$ are based on muscles that had been exposed to only mildly hypertonic solutions. Confidence limits at $\pm 2 \times$ S.E.

The curve shows what would be expected theoretically if the fibres behaved as simple osmotic bags containing a fixed amount of an ideal solute. Up to about $4 \times R$ the experimental points lie close to this line, but slightly to the left of it. This shows straight away that no large amount of water can be 'bound' in the sense that it is not available to dissolve the solutes within the fibres in a normal manner: for in this case the experimental points would lie above the line. The effect actually observed is that at a given value of the ordinate, i.e. of the internal concentration, the effective osmolality of the cell contents is less than its ideal value. The effect is not large enough to merit a very extensive discussion. It may well be that the osmotic coefficient, ϕ, of the cell contents diminishes as the concentration within the cell rises. The type of behaviour is certainly observed in solutions of salts (especially those with polyvalent ions): but it is impossible to be more precise, for even though the osmotically active constituents of muscle are known (Boyle & Conway, 1941, p. 15) their combined osmotic coefficient cannot be estimated theoretically.

Beyond about $4 \times R$ the experimental points become irregular. This is partly a consequence of the fact that in this region the fibre water is determined as a small difference between relatively larger quantities (hence the negative values between $5 \times R$ and $6 \times R$); but also because in some cases a large proportion of the fibres may have become permeable to sucrose. Notwithstanding these difficulties, it appears that a substantial group of points lie above the line; and it may be that this is the commencement of a process that is known to occur in dehydrated muscles—that their water is 'bound' in the sense that it can only be removed by a relatively large gradient of vapour pressure.

Text-fig. 5*d* shows the dry weight as a fraction of the drained weight. This fraction is almost constant, but close examination of the points shows that they do tend to fall towards the right-hand end. This impression is confirmed when the first 26 points at the left-hand end are compared with the last 10 points at the right-hand end. The means and standard errors of the two groups are, respectively, $0{\cdot}167 \pm 0{\cdot}003$ and $0{\cdot}139 \pm 0{\cdot}0046$, so the difference between the two means is highly significant; it shows that solid matter is lost from the muscle as a result of soaking it in a strongly hypertonic solution. The loss of phosphate and of nucleotides from dehydrated muscle has been investigated previously (Dydyńska, 1961), and we have also found more recently that about 20% of the muscle's potassium is lost during a 2 hr soaking in $4 \times R$, perhaps as a consequence of the very large difference in potassium concentration across the cell membrane. However, the loss of dry weight is greater than can be accounted for by all these substances together: the other substances lost have not yet been identified.

The loss of solid material into solutions 4 and 5 (see Text-fig. 4) only becomes appreciable above about $5 \times R$, where we have independent evidence that fibres are damaged. When loss occurs it introduces an error into our calculations, for in equation (6) the W_d employed should actually be larger than the one finally measured. For this reason the mean values at $1 \times R$ plotted in Text-fig. 5 are based on 26 experiments in which only moderately hypertonic solutions had been used and this error does not arise. The loss of phosphates and of nucleotides mainly occurs when the muscle is returned to normal Ringer's solution (solution 5) and probably most of the other substances are also lost at this stage. If so, our estimate of fibre water in hypertonic solution, from equation (7), is slightly too high.

DISCUSSION

Despite Overton's (1902) opinion to the contrary, it seems now to be widely accepted that when resting muscle fibres are placed in physiological solutions whose effective osmotic strength is not too far from normal (say $0{\cdot}7 \times R$ to $2 \times R$, 160–460 m-osmolal) water is gained or lost according to simple osmotic laws (see Hill, 1930; Fenn, 1936; Chao & Chen, 1937; Boyle & Conway, 1941; Sato, 1954). Ernst (private communication) also finds that small variations of vapour pressure alter the water content of a muscle in the same way as that of a simple solution. The chief result in this paper is to show by a new method that the same simple law governs water movement up to much higher concentrations, approximately $4 \times R$ or nearly 1000 m-osmolal. In such a solution 75% of the fibre water is withdrawn; the consequent high concentration of intrafibrillar solutes inhibits contraction (see Howarth, 1958) and causes some leakage of potassium, but no permanent harm is done. Incidentally, this concentration is nowhere near what can be borne by some specialized marine creatures (Bayliss, 1960, p. 73).

Beyond $4 \times R$ it appears that damage is done to the frog's cell membrane, for the fibres no longer behave like osmotic sacs and they also do not recover their contractility completely. This accords very well with the earlier demonstration (Dydyńska, 1961) that when muscles are dehydrated by silica gel, the withdrawal of 75% or more of the water is followed by irreversible changes and the leakage of phosphate and nucleotides.

The increase in extracellular fluid above $2 \times R$ probably occurs by a process akin to plasmolysis, the fibres being held together in the muscle in such a way that the whole structure has some rigidity and each fibre can shrink towards its own central axis. Tasker, Simon, Johnstone, Shankly & Shaw (1959) have shown that the opposite process occurs in

hypotonic Ringer's solution, where the extracellular space is reduced. These authors, who compared three different methods of measuring the extracellular space, conclude that the measurement is subject to very wide scatter. Their value for the sucrose space, 26·5 ± 8·3 % (mean ± S.D., $n = 72$), is lower than ours, 34·0 ± 7·2 % ($n = 22$), because it appears that their muscles were blotted while ours were not: the scatter is about the same in both cases. Both sets of values are much higher than those given by Boyle, Conway, Kane & O'Reilly (1941). Tasker *et al.* (1959) stress, quite correctly, the danger of using an assumed 'mean' value for the extracellular volume in calculations about muscle. To this we would add that the extracellular volume is a quantity as ill-defined as the water capacity of a sponge: it can be made more precise only by describing *exactly* the draining or blotting procedure applied to the muscle in question. It should be emphasized that our value for the normal fibre water 49·7 % (S.D. = 6·6 %, $n = 20$) applies only to muscles drained according to the procedure that we describe.

Free and bound water in muscle

Our results support Hill's (1930) conclusion that practically all the water in muscle must be 'free', in the sense that it is available to dissolve solutes which then affect the vapour pressure (or activity) of the water in the same way that they would do *in vitro*. As Hill pointed out, other definitions of 'freedom' may lead to other conclusions, but this need not involve any real contradiction. For example, it is well known (see e.g. Brooks, 1934; Ernst, 1958) that in order to remove the last traces of water from a muscle it must be exposed to a very low vapour pressure. In this sense, the last trace of water is 'bound'. What is not so widely realized is that a perfect solution, if it continued to obey Raoult's law up to the very high concentrations involved, would show similar behaviour. Moreover, even if the muscle does behave as a perfect osmotic sac, it does not follow that the content of solutes within it must always remain constant. If the content alters, so must the fibre volume. Such an alteration is probably the explanation of Ernst's results on the effects of lactic acid and of stretching.

Fibre volume and fibre water

There is a discrepancy between the results shown in Text-fig. 1 and those in Text-fig. 5*c*. The microscopic observations indicate that fibre volume does not diminish above $2 \times R$, while the chemical estimations show that fibre water continues to diminish up to $4 \times R$. There is no reason to doubt either result, for although our method of estimating fibre diameter was admittedly crude, it gave the same answer as that found by Sato (1954) using a much better technique. Unfortunately we did not know of Sato's

work until it was pointed out to us recently by Professor A. F. Huxley. In any case, Sato's experiments did not extend above $2 \times R$, so they do not bear on the discrepancy under discussion.

One possible explanation might be that from $2 \times R$ to $4 \times R$ part of the sucrose space is actually within the muscle fibres: it was in order to investigate this possibility that the electron microscopical study described in the Appendix was undertaken. The same suggestion has been made by Johnson & Simonds (1962), who showed that in rabbit heart the sucrose space was considerably larger than the extracellular space observed histologically. It is shown in the Appendix that in hypertonic solutions elements of the endoplasmic reticulum do indeed become dilated with fluid, and that at some points these dilated tubules appear to open on the surface of the fibre. However, the total volume of the dilated regions does not appear to be sufficient to account for the discrepancy: to do so they would have to occupy one third of the volume of the fibre in $4 \times R$. Nor can it be assumed that they are filled with sucrose solution. Various other possible explanations are put forward in the Appendix and further work will be needed to establish whether or not the paradoxical appearance under the microscope is connected with the macroscopic results given in this paper.

APPENDIX: AN ELECTRON MICROSCOPIC STUDY OF MUSCLE IN HYPERTONIC SOLUTIONS

BY H. E. HUXLEY, SALLY PAGE AND D. R. WILKIE

METHODS

Frogs' sartorii were mounted at body length on glass frames and treated at 2–4° C with $3{\cdot}5 \times R$ solution, some for 2 hr as described above, some for 24 hr. This osmotic strength was chosen so as to produce the maximum effect without risk of irreversible damage. A control was provided by keeping the paired muscle for similar periods in normal Ringer's solution. The muscles were then fixed for 1 hr at 4° C in a 1 % solution of OsO_4 buffered at pH 7·3–7·5 with veronal acetate (Palade, 1952), either (i) without added sucrose (i.e. about $0{\cdot}76 \times R$), or (ii) with sucrose added to make the fixative $1{\cdot}0 \times R$, or (iii) with sucrose added to increase the osmolality to $3{\cdot}5 \times R$. The tissue was dehydrated in an alcohol series, stained overnight in a 1 % solution of PTA in absolute alcohol, and embedded in araldite (50 ml. araldite, 50 ml. hardener 964 B and 1·5 ml. accelerator 964 C). Sections were cut with a glass knife on a Porter–Blum microtome and viewed in a Siemens Elmiskop 1 at 80 kV with an objective aperture of 50 μ.

RESULTS

Plate 1, fig. 1 and Pl. 2, fig. 1 show a normal control muscle in transverse and longitudinal section: Plate 1, fig. 2 and Pl. 2, fig. 2 show comparable sections of muscles that have been exposed for 2 hr to $3{\cdot}5 \times R$. In the transverse sections the fibrils of the normal muscle are clearly defined by the sarcoplasm and the elements of the sarcoplasmic reticulum that sur-

round them. After exposure to hypertonic solution the fibres appear shrunken and the interfibrillar spaces at the level of the A-band have become so much reduced that individual fibrils can no longer be distinguished. The spacing between thick filaments has become reduced from $354 \pm 2{\cdot}4$ Å (mean ± S.E., $n = 12$) to $302 \pm 4{\cdot}8$ Å ($n = 16$). To the left of Pl. 1, fig. 2, in the I-band, dilated vesicles can be seen.

The shrunken appearance of the fibres is also evident in the longitudinal section (Pl. 2, fig. 2). The sarcolemma can be seen clinging to the outer fibrils; often it outlines clusters of pinocytotic vesicles that project from the surface of the fibre. However, the most striking feature of the longitudinal section is again the presence of swollen vesicles within the I-band. These can here be identified as elements of the triads and they contrast markedly with the shrunken appearance of the sarcoplasmic tubules in the A-band. Most frequently it is one, or sometimes both, of the outer elements that is enlarged; however, it is not uncommon to find that the central element alone is enlarged. Frequently it is impossible to decide with certainty which element of the triad is swollen.

The occurrence of dilated vesicles is somewhat patchy throughout the muscle; it does not seem to vary systematically, for example, from the outside to the inside of the muscle or of its fibres. In regions where the triads are not clearly swollen they have a normal appearance; we have never been able to recognize one that was shrunken. The dilated vesicles, like the triads of normal muscle, are sometimes empty, sometimes filled with finely granular material.

As is seen in Pl. 2, fig. 2, the sarcoplasmic tubule from the A-band often runs close to and alongside the outer element of the triad. In no case was its lumen seen to be continuous with that of any element of the triad. This puzzling feature contrasts with the situation in normal muscle, where these tubules often appear to be continuous with the outer elements of the triads.

The effect of soaking for different times in hypertonic Ringer's solution

Most of the experiments were done on muscles that had been soaked in the hypertonic Ringer's solution for 2 hr. However, other experiments were done in which the muscle was soaked for 10 min and 24 hr respectively. In all cases the shrunken appearance of the fibrils of the sarcoplasm and of the tubules in the A-band was similar. The appearance of the dilated vesicles was similar in the 10 min and the 2 hr experiments, but in the 24 hr experiment we seldom saw dilated outer elements. Almost all the vesicles that could be identified after 24 hr were dilated inner elements, but there were many spaces in the muscle at the level of the I-band that seemed not to be bounded by membranes. It may be that these are dilated outer elements of the triads whose membranes have not been preserved.

In many places the dilated inner elements form channels running across the fibre at the level of the Z line. Exactly what happens when these channels approach the surface of the fibre cannot be settled without serial sections. Two types of termination at the surface membrane have been observed. In one (Pl. 3, fig. 3) the channel ends blindly, being separated by a membrane from the extracellular space. In the other (Pl. 3, figs. 1 and 2) the channel opens through the surface membrane and its lumen is continuous with the extracellular space. It is not clear whether this second type of termination is formed from the first by the collapse of the bounding membrane.

Effects of the tonicity of the fixative. In the first experiments, where the muscles were fixed in $0{\cdot}76 \times R$ OsO_4, there was the possibility that the dilatation of the vesicles might have arisen from exposure to the hypotonic fixative. This was ruled out by the experiments in which $1 \times R$ and $3{\cdot}5 \times R$ fixative was used: the tonicity of the fixatives made no difference to the appearance either of normal muscles or of muscles from hypertonic solution.

Recovery. Complete physiological recovery would have been expected in the muscles soaked for 2 hr in $3{\cdot}5 \times R$; morphological recovery was also found to be complete. No tests were made for physiological or morphological recovery after 24 hr exposure.

DISCUSSION

It seems paradoxical that dilated regions should be found in fibres that are otherwise very markedly shrunk. There are several possible explanations:

(1) It might be possible for extracellular solutions to enter the vesicles through specially permeable regions or actual pores where the triads lie near to the surface membrane. If so, a slight fall in hydrostatic pressure within the fibre, resulting from its rigidity and shrinkage, would cause the vesicles to become dilated.

(2) Active secretion of solute into the vesicles could cause water to follow passively.

(3) Fluid within tubules in the A-band might be passively squeezed into the I-band by the shrinkage of the sarcoplasm and the subsequent packing together of the myosin filaments. However, as mentioned above, we do not see continuity between the vesicles and the tubules in shrunken muscle, unlike normal muscle.

None of these explanations is altogether adequate and the situation remains puzzling.

SUMMARY

1. The changes of weight of frogs' muscles in hypertonic solutions have been studied. The strength of solutions is expressed as a multiple of normal Ringer's solution: thus $n \times R = n \times 230$ m-osmolal.

2. In solutions made hypertonic with sucrose, fibres are injured only above $4 \times R$.

3. Measurements of fibre diameter indicate that the fibre behaves as an osmometer up to $2 \times R$.

4. Measurements of the sucrose space show that this remains approximately constant up to $2 \times R$, then increases markedly up to $4 \times R$. Above this point the measurements are scattered, probably because the fibres are injured.

5. The fibre water alters up to $4 \times R$ as though it were contained in a simple osmometer.

6. The discrepancy between (3) and (5) could arise if part of the sucrose space were within the fibres.

7. A statistical test is described for assessing the accuracy of duplicated estimations.

8. *Appendix.* Electron micrographs of fibres in hypertonic solution show that elements of the endoplasmic reticulum near to the Z line are dilated, though the tubules in the A-band are shrunk and the protein filaments there are packed together more closely than usual. After prolonged exposure to hypertonic solution, dilated tubules can be seen opening on the surface of the fibre.

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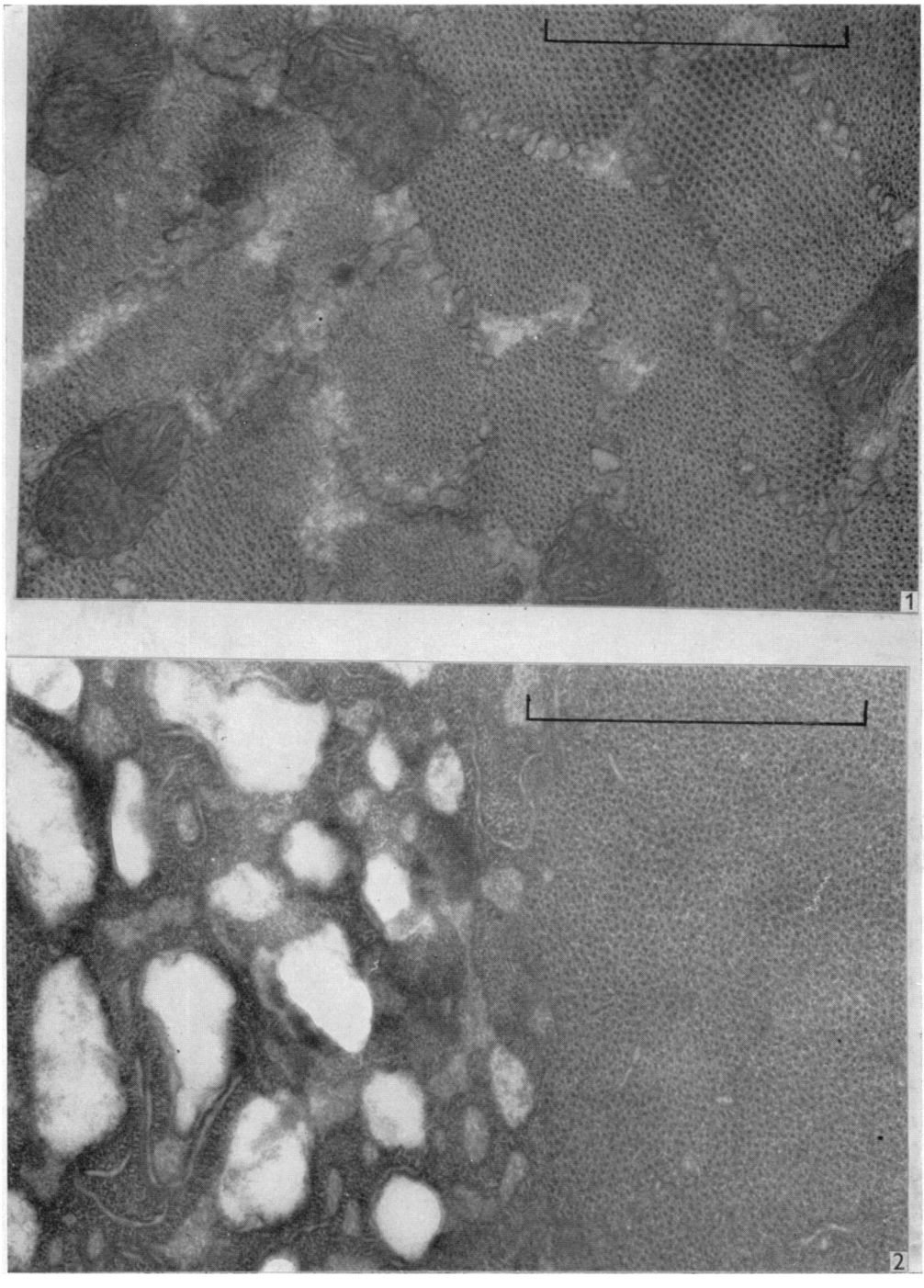

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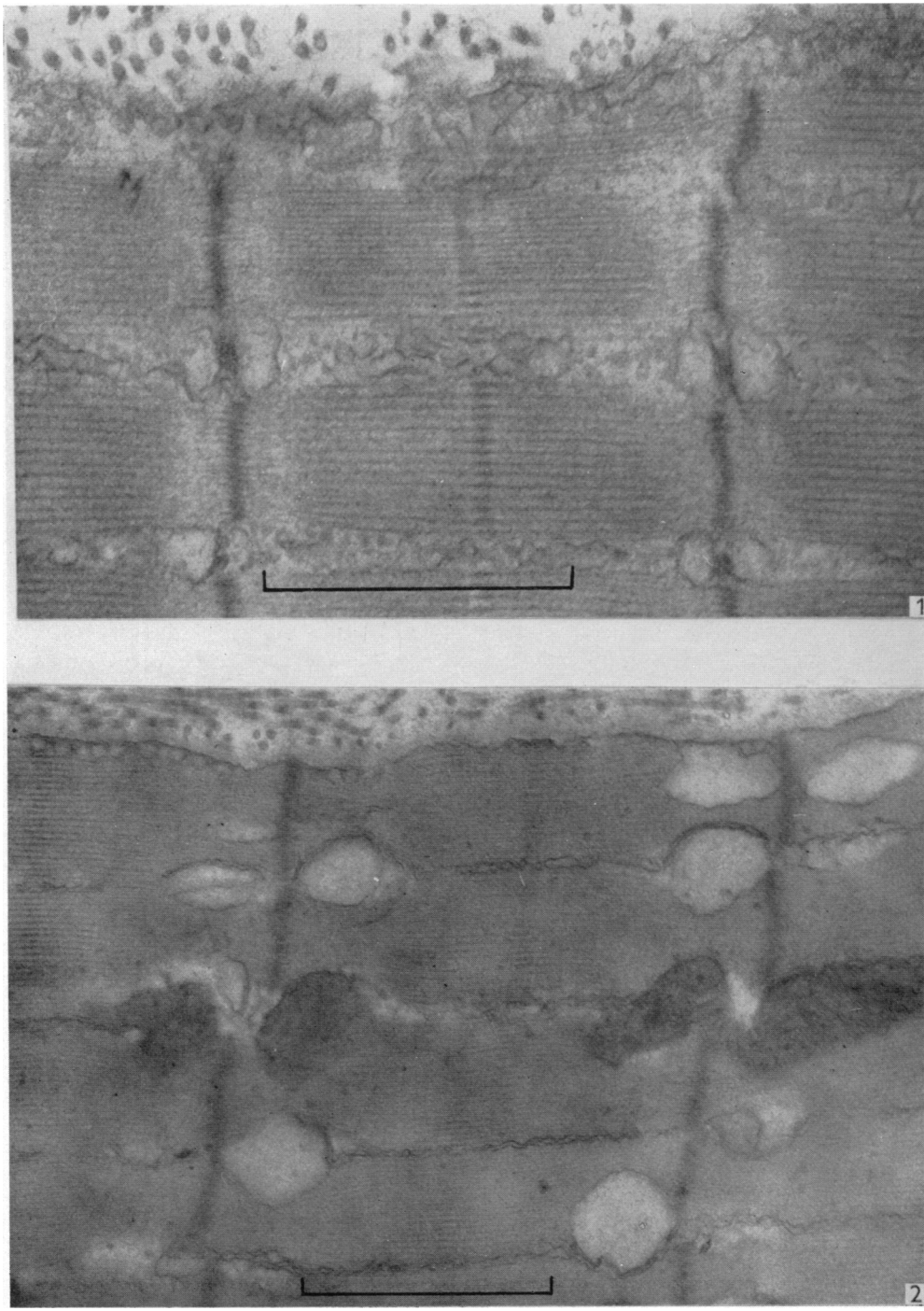
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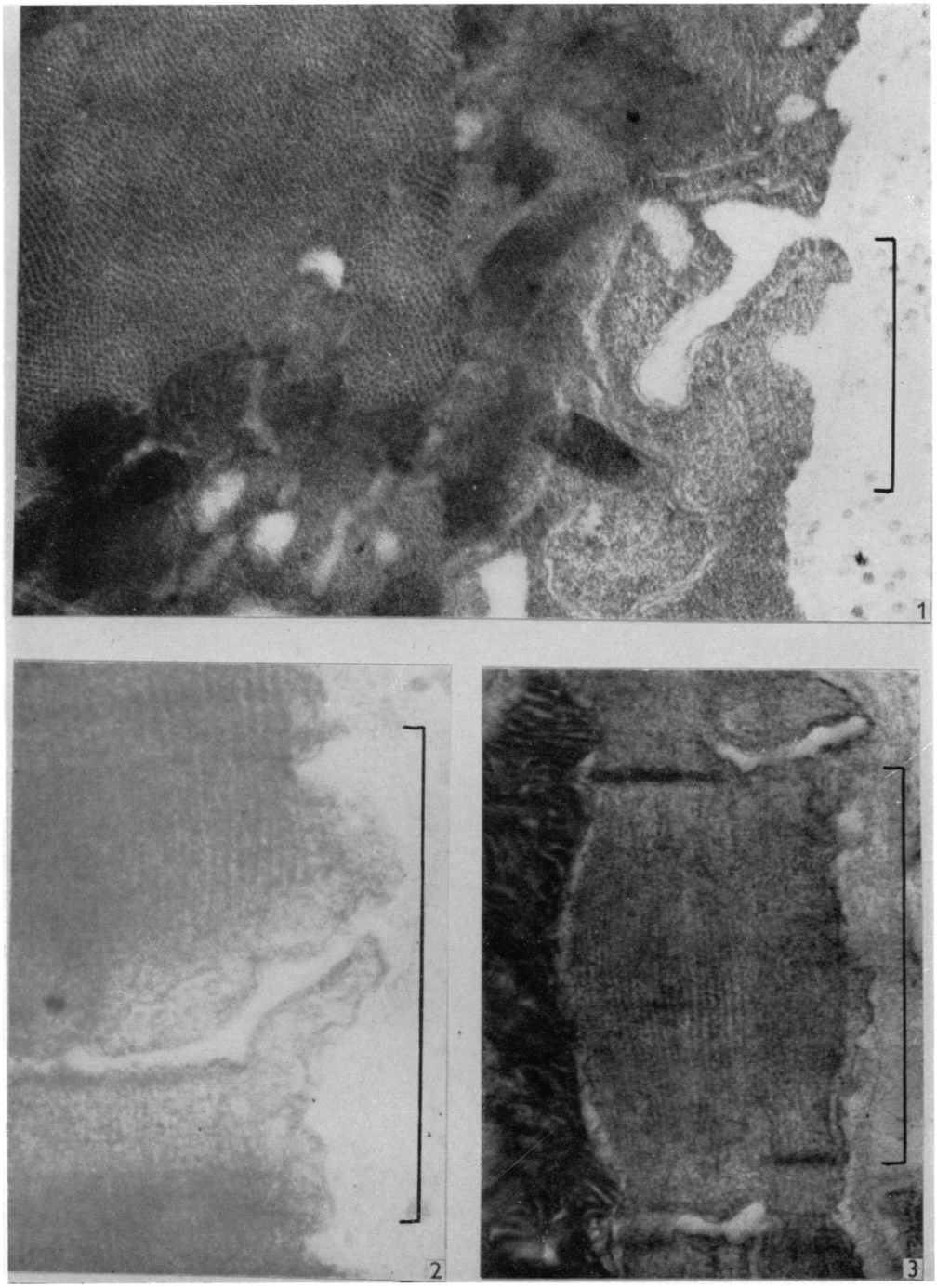
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EXPLANATION OF PLATES

PLATE 1

Scale bars 1 μ. Fig. 1. Transverse section of normal muscle fixed in $0{\cdot}76 \times R$ OsO_4. Fig. 2. Transverse section of muscle treated with $3{\cdot}5 \times R$ solution for 2 hr, and fixed in $0{\cdot}76 \times R$ OsO_4.

PLATE 2

Scale bars 1 μ. Fig. 1. Longitudinal section of normal muscle fixed in $0{\cdot}76 \times R$ OsO_4. Fig. 2. Longitudinal section of muscle treated with $3{\cdot}5 \times R$ solution for 2 hr, and fixed in $0{\cdot}76 \times R$ OsO_4.

PLATE 3

Scale bars 0·5 μ. Fig. 1. Transverse section of muscle treated with $3{\cdot}5 \times R$ solution for 24 hr, and fixed in $3{\cdot}5 \times R$ OsO_4. Figs. 2 and 3. Longitudinal section of muscle treated and fixed as in Fig. 1.