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DIFFUSION OF INORGANIC PHOSPHATE INTO AND OUT OF THE SKELETAL MUSCLES AND BONES OF THE FROG.

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If a skeletal muscle from a frog is immersed in a Ringer's solution containing urea, the urea distributes itself between the muscle and the solution in such a manner as to indicate that the whole of the water of the muscle is available to dissolve urea [Eggleton, 1930]. Recently it has been found that the same statement is true for the amino acid histidine [Eggleton and Eggleton, 1933], but the behaviour of the dipeptide carnosine in similar experiments indicated that only part of the water of the muscle was available to carnosine diffusing in from surrounding saline. This fraction was of the order of 30 p.c. of the total water of the muscle.

It has been shown by Stella [1928] that inorganic phosphate can be exchanged between muscles and surrounding saline solutions, and this communication presents the results of an attempt to determine what proportion of the water in the muscle is concerned in this phosphate exchange. The investigation is more difficult than was the case with urea and histidine, for in this case the muscle already contains a quantiy of the particular solute under investigation, and further, this quantity does not remain even approximately constant in an excised muscle unless special precautions are taken.

To reduce this last source of error to a minimum the experiments described in this paper have been performed at a temperature of 2° C. and, with certain stated exceptions, in an atmosphere of oxygen. The first experiments were designed to determine what, under these conditions, is the apparent concentration of phosphate (c) in the muscle when measured by the exchange of phosphate between such muscles and Ringer's solutions containing various concentrations of phosphate.

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THE VALUE OF c IN RESTING OXYGENATED MUSCLES.

Two workers have already made estimates of this value. Stella [1928], working with intact hindlimbs of the frog (English and Dutch: *R. temp.* and *R. esc.*) at a low temperature, concluded that a concentration of 8 mg. P per 100 g. water in a surrounding saline prevented loss or gain of phosphate by these preparations. Semeanoff [1931], working with isolated sartorii (Hungarian *R. esc.*) at room temperature, found a value of approximately 20 mg. P per 100 g. water. The experiments recorded in Table I were performed in essentially the same manner as those of Stella and Semeanoff.

The evaluation of the amount of phosphate exchanged requires a measurement of the initial and final volume of Ringer's fluid as well as of the initial and final concentration of phosphate in it. Stella ignored the possibility of water exchange; Semeanoff states that she observed none. In the present experiments water exchange was always taken into account, and proved to be an important consideration where the concentration of phosphate in the Ringer's fluid altered only slightly. The calculation took the form:

Phosphate exchanged
= (Initial volume of Ringer's fluid × initial concentration of phosphate)
− (Final volume of Ringer's fluid × final concentration of phosphate).

In the majority of experiments described in this communication the Ringer's solutions were buffered at an initial pH of 8·0 (in distinction to that of 7·1–7·2 used by Semeanoff and Stella), so that they might be more comparable in this respect with phosphate-free solutions (buffered with bicarbonate). In a certain number, however (Table I, Group B 1, β; Table III, Group A 2; Table VIII, Group A 2), the Ringer's solutions were buffered at pH 7·1, but no difference was observed in the properties of muscle under investigation. It was noted, however, that in the conditions here used (approximately equal quantities of muscle and Ringer's fluid, in a CO_2-free atmosphere) the pH of the Ringer's fluid was ultimately about 7·4 for resting muscles whether the initial pH was 7·1 or 8·0. The quantity of saline was therefore sufficiently small for its reaction to be controlled by the muscle. In the case of fatigued muscles an initial pH of 7·1 in the saline was changed to 6·8.

The results quoted in Table I indicate that the immediate past history of the frog may affect the result obtained. The muscles of frogs (Hungarian *R. esc.*) which had been kept at 2° C. for only a few hours before use appeared to be in equilibrium with a phosphate concentration of about

20 mg. P per 100 g. water. If the frogs had been for 2–3 days at this temperature, the equilibrium concentration appeared to be between 10 and 15 mg. P per 100 g. water. Stella's frogs were kept "for several days" at 2° C., and his lower equilibrium value of 8 mg. per 100 g. water may be due to this difference. Semeanoff's frogs were not cooled, and her experiments were carried out at room temperature.

TABLE I. The exchange of inorganic phosphate (I.P.) between resting frog muscles and Ringer's solutions containing I.P., at 2° C.

Group	Muscle preparation	Duration of exp. hours	I.P. gained by muscle mg.	I.P. concentration in Ringer's fluid finally, mg. per 100 g. water	Equilibrium value (*c*) mg. I.P. per 100 g. water
A	Isolated muscles	3	−0·0535	7·5	
		3	−0·0147	11·4	
		22	−0·046	17·1	
		22	−0·006	20·8	About 20
		3	0·0045	19·7	
		22	0·009	30·9	
		3	0·022	29·9	
B 1 α	Isolated muscles	3	−0·0063	13·25	
		3	−0·0225	14·1	
		3	0·006	12·7	
		2½	0·0006	13·2	
β	„	3	−0·0023	11·4	10–15
		3	0·005	11·1	
		3	−0·0027	11·6	
B 2	Thigh preparation	19½	−0·0478	9·84	
		19	0·01	12·7	
		18	0·13	14·83	

The frogs used for Group A had been kept at 2° C. for a few hours only; those used for Group B had been cooled for 2–3 days.

THE PROPORTION OF THE MUSCLE CONCERNED IN THE PHOSPHATE EXCHANGE.

Having established that a Ringer's solution containing phosphate in a concentration of 8 mg. per 100 g. water is not altered by exposure to resting muscle, Stella raised the very pertinent point that "these values" might "refer to an equilibrium of phosphate between the Ringer's solution on the one hand, and the liquid, blood or lymph, on the other hand, which is present in the interspaces between the muscle fibres, and that from such experiments we get no idea of what is happening inside the muscle fibres themselves." He performed certain experiments designed to measure the proportion of the muscle concerned in the phosphate exchange. His values lay between 50 and 80 p.c. of the weight of the muscle tissue on his hindlimb preparations, and he concluded that the whole of the muscle was probably concerned in the exchange.

The experiments, the results of which are given in Table II, were designed to the same end, though they differ in two respects from Stella's. In the first place, isolated muscles were used, and in the second place the amount of phosphate gained by the muscle was measured by analysis of the muscle and not of the Ringer's fluid. The technique and interpretation were as follows.

One of a pair of muscles was killed without previous immersion, while the other was placed in a large volume (10 g. per g. of muscle) of oxygenated Ringer's fluid, containing phosphate, for a given time, and then killed. Proteins were removed by the use of trichloro-acetic acid, and the total acid-soluble phosphorus determined in each muscle [Martland and Robison, 1926]. Any increase in the total acid-soluble phosphate content was taken to be a measure of the amount of inorganic phosphate diffused into the muscle. Direct determination of the inorganic phosphate was considered less likely to yield accurate results, owing to the relatively large amount and ready lability of phosphagen present in such resting muscles. The inorganic phosphate concentration in the Ringer's fluid finally was also determined.

As in the case of Stella's experiments the interpretation of the results required some assumption as to the concentration of inorganic phosphate in that part of the muscle concerned in the phosphate exchange. On the basis of the experiments given in Table I, this was taken to be 12½ mg. per 100 g. water. The amount of water in the muscle concerned in the exchange was then obtained by dividing the amount of phosphate diffused by the difference between the final phosphate concentration in the Ringer's fluid and the assumed

TABLE II. Diffusion of inorganic phosphate (I.P.) into resting frog skeletal muscle (measured by direct analysis of the muscles).

Duration of exp. hours	T.P. content of muscle			I.P. conc. in Ringer's fluid finally	Proportion of muscle water (α) into which I.P. has diffused
	Initial	Final	Increase	mg. per 100 g.	p.c.
	mg. per 100 g. muscle H_2O				
½	157½	183½	26	126	23
1	156	188	32	126	28
2	157½	191½	34	126	30
2	162	184	22	120	20
2	158	187	29	120	27
3	171	187½	16½	126	15
4	157½	186½	29	126	25
4	164	178	14	120	13
4	179½	209½	30	120	28
5½	174½	207½	33	126	30
8	164	192½	28½	120	26
8	179½	199	19½	120	18
23	172	199½	27½	126	24
23	171½	206½	35	126	31
23	180½	202	21½	126	19
				Mean	24
				Probable error of mean	±2

For derivation of α see text.
T.P. = total acid-soluble phosphate.

value of the initial concentration in the muscle. The result is expressed in Table II as a fraction of the total water of the muscle, which is taken to be 80 p.c. of the weight of the muscle [Hill and Kupalov, 1930].

The value of this fraction, which will be called α, may be expected to increase as the period of immersion is lengthened, until the muscle is at equilibrium with the Ringer's fluid. The results in Table II show that equilibrium is attained very rapidly by the small muscles (sartorii, semitendinosi and graciles minores) used in these experiments. There is a rather large random variation, but there is no evidence of a tendency for the value of α to increase when the period of immersion is increased from 1 hour to 23 hours. Only 24 p.c. of the water of the muscle appears to be concerned in the exchange of phosphate between muscle and saline[1]. It may be remarked in passing that this value depends only slightly on the value assumed for c. If c be taken as 8 mg. per 100 g. water, α becomes 23 p.c., and if it be 20 mg. per 100 g., α has the value of $25\frac{1}{2}$ p.c. But no reasonable estimate of the value of c would give values of α as high as those obtained by Stella.

It is natural to identify this fraction of the muscle water with the fluid occupying the spaces between the muscle cells, and to identify the remaining fraction, which appears to be completely enclosed by semi-permeable membranes, with the cells themselves. For the sake of brevity these fractions will be called "interspaces" and "cells" respectively, although we have no direct experimental evidence as to whether these fractions are identical with the anatomical interspaces and fibres: for histological appearances cannot be relied upon, since shrinkage of the cells during preparation of the tissue is inevitable. A system of close packed cylinders of uniform radius enclose interspaces amounting to 9 or 22 p.c. of the total bulk of the system according as the axes of the cylinders are arranged in triangles or squares. It is not likely that the muscle fibres are literally close packed, and moreover the blood vessels and possibly also the connective tissue must be added to the interspaces, but on the other hand, any deformation or variation in size of the fibres would lead to a smaller proportion of interspaces. It seems impossible therefore, on the evidence at present available, to decide whether the fraction of the muscle water concerned in the phosphate diffusion corresponds only to the intercellular spaces, or includes certain parts of each cell.

[1] A corroboration of this finding is found in Semeanoff's work on sartorii. Her published data are sufficiently detailed to permit a calculation of the value of α. The values vary from 26 to 56 p.c., with an average value of 45 p.c. This value, though higher than that found in the present work, shows clearly that the whole of the muscle water is not concerned in the exchange of phosphate.

This failure to confirm Stella can be explained satisfactorily, but it is convenient to defer the explanation to a later stage. Meanwhile this rather unexpected result was confirmed by another type of experiment, in which the value of α was determined without previous assumption of the value of c. Pairs of muscles were again used, but in this case one was placed into an approximately equal amount of Ringer's fluid containing a high concentration of phosphate, and the other into the same volume of fluid of low phosphate concentration. Determination of the changes in phosphate concentration in the two Ringer's solutions (and of any slight change in volume due to water exchange between muscle and saline) yielded values for the amount of phosphate diffused into the first muscle and away from the second muscle. If sufficient time was allowed for equilibrium to be attained between the Ringer's fluid and the muscle, the measurements made enabled one to calculate the initial phosphate concentration in the "interspaces" of the muscle as well as the value of α. The only assumptions made in such a calculation were: (1) that when no further exchange of phosphate occurs between muscle and Ringer's fluid, the concentration in any part of the former into which phosphate has been able to diffuse is identical with that of the latter; and (2) that water forms 80 p.c. of the muscle weight. It then follows that

$$\frac{\text{Phosphate initially present in "interspaces"} + \text{Amount of phosphate diffused in from Ringer's fluid}}{\text{Amount of water in "interspaces"}}$$

= Phosphate concentration in Ringer's fluid when equilibrium has been attained.

If α = that fraction of the muscle water contained in the "interspaces,"

c = initial concentration of phosphate in the "interspaces" (in mg. per 100 g. water),

w = weight of muscle initially (in g.),

P = final concentration of phosphate in Ringer's fluid (in mg. per g. water),

x = amount of phosphate diffused into muscle from Ringer's fluid (in mg.),

then
$$\frac{4/5 \times w \times \alpha \times c + x}{4/5 \times w \times \alpha} = P. \qquad \text{.....(1)}$$

When this equation is applied to each muscle of a pair (one in Ringer's fluid of a high phosphate content, one in low) the two unknowns α and c can readily be calculated. In the experiments with living muscles, the "high-phosphate" Ringer's fluid contained 30–60 mg. P per 100 g. and

the "low-phosphate" 5–15 mg. per 100 g.; with muscles in rigor, the values were 150–280 and 0–45 respectively. Phosphate was added in the form of an isotonic solution in order not to affect the total osmotic pressure.

The results of several experiments on resting muscles are given in Table III. A considerable degree of error is to be expected, since the calculations are based on small differences, and the number of measurements made in any one experiment is large (twenty).

TABLE III. The proportion of muscle water occupied by diffusible inorganic phosphate in resting skeletal muscle of the frog.

Group	Duration of preliminary soaking hours	Duration of exp. hours	Proportion of muscle water occupied (α) p.c.
A 1	0	1–1½	28, 22, 32
A 2	0	3	28, 12, 22
B	½	2	25, 15
C	2	21	12, 15, 52
D	1	23	21, 20, 15, 26, 26
			Mean 23 ±3*
E	44	6	42, 28, 26, 36, 24
F	95	24	30
G	120	19	24
H	1½	65½	33, 32, 27, 28, 42
			Mean 31 ±2*

For derivation of α see text.

* Probable error of mean.

The proportion of the muscle water occupied by diffusible phosphate in the normal muscle appears to lie between 20 and 30 p.c., and exchange between muscle and Ringer's fluid is restricted to this part of the muscle whether the experiment occupies 1 (in the case of thin muscles such as the sartorius) or 24 hours. The prolonged preliminary immersion in phosphate-free Ringer's fluid of the muscles in groups E–G was undertaken for another purpose, but the equilibrium experiments subsequently performed on them indicate that such prolonged soaking has little effect on the value of α. There is some evidence of a slight increase, but the results are not sufficiently consistent to warrant a definite conclusion on the matter. The only values of c which can be compared with those given in Table I are from group A; these were 17, 12, 17, 12, 9 and 13 mg. P per 100 g. water in α respectively. In the other groups, in which the muscles were immersed in phosphate-free Ringer's fluid for varying periods of time before the equilibrium experiment was performed, the value of c was naturally lower (practically zero in groups E–G).

This type of experiment therefore confirms those given earlier in leading to the conclusion that phosphate from a surrounding Ringer's fluid will diffuse freely into only 20–30 p.c. of the muscle water in the case of resting muscles, and that the phosphate concentration in this fraction is of the order of 15 mg. per 100 g. water.

Muscle-bone preparation.

It is convenient at this point to consider why Stella found, with thigh preparations, the value of the fraction α to be between 50 and 80 p.c., whilst preparations of isolated muscles give values in the neighbourhood of 25 p.c. Experiments were performed with thigh preparations of different sizes, the technique being similar to Stella's. His thigh preparations weighed about 3 g. each. The smallest preparation used in the present work weighed 5 g. It gave a value of α of 60 p.c. (Table IV). Larger

Table IV. Diffusion of inorganic phosphate (I.P.) into the resting thigh preparation of the frog.

Exp.	Duration of exp. hours	Wt. of muscle g.	Wt. of Ringer's fluid g.	I.P. conc. in Ringer's fluid finally mg. per 100 g.	I.P. diffused into prep. mg.	α p.c.
1	24½	25·8	30	74·4	3·0	24
2	24	16·3	18·5	61·0	1·99	31
3	19	12·7	17·0	81·8	2·07	30
4	18	10·4	18·5	62·5	1·55	37
5	18	7·4	17·0	41·6	0·73	41
6	23½	6·5	17·2	76·3	1·7	51
7	24	5·5	18·0	84·0	1·55	53
8	24	5·0	20·0	47·6	0·828	60

The fraction of the muscle water occupied by diffusible phosphate (α) is calculated on the basis of an assumed initial concentration in this fraction of 12½ mg. per 100 g. water (Table I). For details see text. In Exps. 1–6 Hungarian frogs (*R. esc.*) were used, and in 7 and 8 Dutch frogs (*R. esc.*).

preparations yielded lower values of α. Indeed it became obvious that the apparent value of α varied inversely with the size of the preparation, and suggested at first sight that insufficient time had been allowed for phosphate equilibrium to be established between these larger preparations and the surrounding saline. The actual rate of diffusion of phosphate into preparations of different sizes was therefore determined. Two such cases are depicted in Fig. 1, curve I showing the rate of diffusion into a preparation containing 25 g. of muscle, and curve II into one containing 5 g. of muscle. In both a steady rate of phosphate entrance is reached between 12 and 18 hours, the magnitude of which (per g. of tissue) varies inversely with the size of the preparation. Further light is thrown on the matter by investigation of the behaviour of a bone preparation (a

thigh preparation with all the muscle removed) exposed to such Ringer's fluids. It is seen (curve III, Fig. 1) that phosphate diffuses into such a preparation continuously over the periods in question. The results quoted in Table V show equally clearly that bone is capable of absorbing large

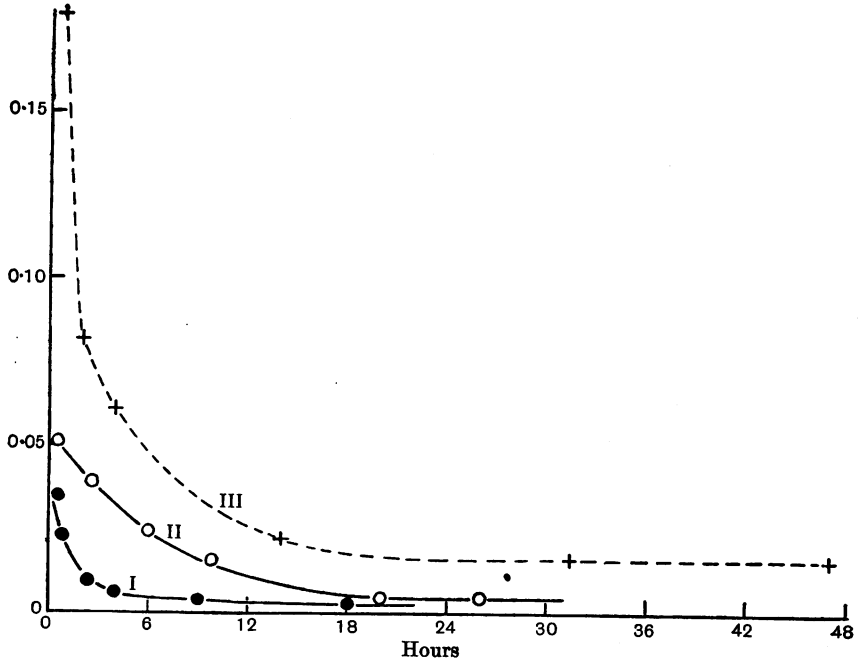

Fig. 1. Rate of diffusion of inorganic phosphate into muscle-bone and bone preparations. Curve I, muscle-bone preparation containing 25 g. muscle. Curve II, muscle-bone preparation containing 5 g. muscle. Curve III, bone preparation. The concentrations of phosphate in the Ringer's fluid surrounding the preparation at the end of the experiment were 74, 84 and 68 mg. P per 100 g. respectively. Ordinates: rate of diffusion in mg. P per g. tissue per hour.

TABLE V. Diffusion of inorganic phosphate (I.P.) into and out of bone.

Exp.	Duration of exp. hours	Wt. of bone g.	I.P. conc. in Ringer's fluid finally mg. P per 100 g.	Change in I.P. content of bone mg.
A	24	3·0	41·9	+0·585
B	23	2·9	41·4	+0·94
C	47	2·3	67·7	+3·15
D	23	2·6	1·6	−0·18
E	20	1·0	4·5	−0·42

In Exps. A–D the thigh preparation freed from muscle was used (two preparations in Exp. C); in Exp. E intact femurs (4). Hungarian frogs (*R. esc.*) were used in Exps. A, B and D; Dutch frogs (*R. esc.*) in Exps. C and E.

amounts of phosphate from surrounding Ringer's solutions. Attempts to express the behaviour of the Ringer-bone system in terms of simple diffusion lead to impossibly high values for the water content of the bone —200 p.c. by weight in one experiment. This is a strong indication that the phosphate, once it has diffused into the bone, is stored in an osmotically negligible form.

Much work has been done on the deposition of insoluble phosphate in slices of rat bone. Robison, Macleod and Rosenheim [1930] conclude that two mechanisms may be concerned in this deposition in experiments *in vitro*: (*a*) the phosphatase mechanism, dependent on the presence of a phosphoric ester in the medium surrounding the bone, and (*b*) the "inorganic" mechanism, which favours deposition in bone of phosphate from supersaturated solutions: the relative importance of the two mechanisms depending upon a number of factors. The subject has been recently reviewed by Kay [1932], but it is impossible to compare directly the present results with those discussed by him, since the latter concerned only rat bone in slices while the former were obtained on intact frog bones. Since no phosphoric ester was present in the Ringer's solutions used, the "inorganic" mechanism must be entirely responsible for phosphate deposition in these experiments, and the controlling factors undoubtedly include (1) the concentration of inorganic phosphate in the surrounding Ringer's fluid (40–100 mg. P per 100 g.), (2) the concentration of calcium ions (10 mg. $CaCl_2$ per 100 g.), (3) the *p*H (usually 8·0), and (4) the absence of protein, other than traces diffused from the preparation.

The variation in the apparent value of α in the muscle-bone preparations would seem therefore to be due to the varying extent to which the bone present is participating in the phosphate exchange. In the smaller preparations, the proportion of bone to muscle is greater than in the larger; in weight the ratio varies from over 0·3 in the small to less than 0·2 in the large preparations, while the ratio of bone surface to the bulk of muscle will vary in the same direction and to a greater extent. The rate of diffusion of phosphate will also tend to be lessened in the larger preparations by the mass of tissue to be traversed between Ringer's fluid and bone, since the diffusion coefficient of phosphate in muscle is less than one-twentieth of that in Ringer's fluid [Stella, 1928].

It is impossible to assess exactly the true value of α in the muscles of these preparations, but it seems likely that it is not materially different from that found in isolated muscles, since the larger the preparation (that is, the greater the proportion of muscle to bone) the more nearly does the value of α approximate to that of isolated muscle.

Rigor muscles.

In the equilibrium experiments already quoted, there was no significant movement of water between muscle and Ringer's fluid, but when the behaviour of dead muscles was investigated, such movement was

considerable and could not be ignored. The question then arose as to whether all parts of the muscle were sharing equally in this water exchange, or whether any one fraction was playing the major part. If water exchange concerned only that part into which phosphate could not readily diffuse, viz. $(1-\alpha)$, then the equation already used for the calculation of α and c in resting muscles was still valid. But if water and phosphate were diffusing into and from the same part of the muscle, equation (1) would no longer apply; it would have to become

$$\frac{4/5 \times w \times \alpha \times c + x}{4/5 \times w \times \alpha + y} = P, \qquad \ldots\ldots(2)$$

where y = the amount of water entering the "interspaces" of the muscle.

A series of equilibrium experiments was therefore performed, on both resting and dead muscles, in order to determine which equation yielded the more consistent results; movement of water was magnified in the experiments on resting muscles by the use of hypo- and hypertonic Ringer's fluids. The results given in Table VI show that in the case of living, but not of dead, muscles the use of equation (1) is fully justified.

TABLE VI. Diffusion of inorganic phosphate into frog sketal muscle from hyper- and hypotonic Ringer's fluid.

Exp.	State of muscle	Ringer's fluid	α_1 p.c.	α_2 p.c.
A	Resting	1·75 normal	29	49
B	,,	,,	30	48
C	,,	0·5 normal	29	6
D	,,	,,	18	−1
E	Rigor. Frog kept 6 hours at 42½° C.	3·0 normal	99	95
F	,,	,,	110	96
G	,,	,,	131	104
H	,,	,,	119	105
I	,,	,,	132	107

α is the proportion of the muscle water occupied by diffusible phosphate. α_1 is calculated on the hypothesis that water exchange concerns only the remaining part $(1-\alpha)$ of the muscle water; in the calculation of α_2 it is assumed that this fraction itself (or a part of it) is concerned in the exchange of water. For the method of calculating see equations (1) and (2).

In regard to these results on dead muscles, two points of interest emerge: (*a*) phosphate can now diffuse into the whole of the water of the muscle; and (*b*) into whatever part of the muscle water is drawn by osmotic forces, phosphate can also diffuse[1]. The application of equation (1) to such muscles yields an impossibly high value for α.

These results were corroborated by others of a more direct type. Either the muscles (dissected after the frog had passed into rigor) were

[1] The part of the muscle which swells or shrinks is probably still the "cells," that is the fraction $1-\alpha$ of living muscles, but this is now included in that part of the muscle containing diffusible phosphate.

soaked several hours in a phosphate-free Ringer's fluid—analysis of some of them at this stage showing a phosphate content equal to that in the fluid outside—and then transferred to a Ringer's fluid of high phosphate content; or they were placed without preliminary soaking into a Ringer's fluid containing either more or less phosphate than themselves. In all cases the phosphate content of the muscle and of the Ringer's fluid was determined at the end of the experiment, and also the degree of water exchange between the two. The results of several such experiments are given in Table VII. In most cases the muscles gained water during the experiment, but in two (C 1 and C 2) movement in the opposite direction was induced by addition of cane sugar to the Ringer's fluid. The results of this set of experiments (C) corroborate those given by the less direct method (Table VI). In Exps. A and B, however, the whole water of the muscle does not seem to be available for dissolving phosphate diffusing in from the surrounding saline. But if the water initially present in the muscle is alone assumed to be available, then impossibly high values are obtained for α (110–120 p.c.). It would appear that in these muscles the breakdown of barriers to phosphate diffusion is nearly, though not yet quite, complete.

TABLE VII. Diffusion of inorganic phosphate (I.P.) into the skeletal muscles of the frog in rigor (measured by direct analysis of muscles).

Exp.	Type of exp.	Duration of exp. hours	Initial water content of muscle g.	Water gain by muscle g.	I.P. content of muscle finally mg.	I.P. conc. in muscle water finally mg. per 100 g.	I.P. conc. in Ringer's fluid finally mg. per 100 g.	α p.c.
A 1	Initial content of	1	0·101	0·031	0·1253	95	128	74
2	I.P. in muscle re-	2	0·132	0·02	0·1723	113½	125	91
3	duced to 5 mg.	2	0·133	0·045	0·187	105	125	84
4	per 100 g. water	4	0·183	0·067	0·276	110½	125	89
B 1	Initial content of	18½	0·57	0·132	0·763	109	120	91
2	I.P. reduced to	18½	0·572	0·112	0·76	111½	120	93
3	about 10 mg. per	18½	0·91	0·271	1·302	110½	120	92
4	100 g. water	18½	0·717	0·146	0·924	107	120	89
C 1	Initial content of	17	0·576	−0·023	0·709	128	127½	100
2	I.P. about 85 mg.	17	0·581	−0·014	0·3745	66	62½	105
3	per 100 g. water	17	0·469	0·012	0·642	133½	130	102
4		17	0·52	0·009	0·342	64½	62½	103

α is the ratio of the two preceding columns.

In Exps. C 1 and 2 hypertonic Ringer's fluid was obtained by addition of 15 p.c. cane sugar. This appreciably affected the specific gravity of the fluid, for which due allowance was made in calculating the phosphate concentration per 100 g. of water.

In fully advanced rigor therefore, the evidence from both direct and indirect types of experiment indicates that the whole of the muscle water,

including the water which passes into the muscle from the surrounding saline during the course of the experiment, is available to dissolve phosphate. This does not necessarily entail a mechanical rupture of all the membranes in the muscle, since the dead muscle swells in salt solution and the swelling can be prevented by cane-sugar solution. These may be still membranes relatively impermeable to cane sugar and to some constituents, including probably the protein of the muscle "cells," but it may be that the ability of cane sugar to prevent swelling is due to its slow diffusion, relative to water.

MUSCLES OTHER THAN IN THE RESTING STATE OR IN HEAT RIGOR.

Values for α and c were also determined in muscles other than those resting or in rigor. The results (Table VIII) were calculated by the use of equation (1); little movement of water between muscle and Ringer's fluid occurred in any of them, however, so that the results would not be appreciably altered had equation (2) been applied. No changes comparable with those observed in rigor are to be observed in the value of α.

TABLE VIII. The effect of various treatments upon the concentration of diffusible inorganic phosphate (I.P.) in frog skeletal muscle and the proportion of muscle water occupied by it.

Group	Condition of muscles	Duration of exp. hours	Proportion of muscle water occupied (α) p.c.	Conc. (c) of I.P. in α mg. per 100 g. water
A 1	Fatigued *in situ* before	3–5	15, 11, 20, 46, 24	20, 18, 24, 17, 20
A 2	dissection	3	22, 26, 31	19, 24, 25
B	,, Greater fatigue	2½–5	12, 17, 33, 44, 19	21, 31, 35, 27, 32
C	Anaerobiosis	17	26, 24, 33, 28	21, 14, 38, 24
D	H_2 + coal gas 23 hours at 16° C.	2½–5	36, 32, 28, 49	47, 23, 28, 27
E	H_2 + coal gas during exp.	17	11, 30, 34, 41, 59	66, 45, 42, 48, 40
F	Resting: initial immersion in Ringer's fluid containing 45½ mg. I.P. per 100 g.	16	21, 30	45, 48

In groups A, B and C the hydrogen used was prepared from zinc: in groups D and E the hydrogen used (from a cylinder) smelt strongly of coal gas. In all experiments the value of α was obtained by use of equation (1).

There is a slight tendency for the "interspaces" to be restricted in fatigue (Exps. A and B), and this would entail a relative increase in the remaining part of the muscle. One would expect such a result if the chemical breakdown products of fatigue were unable to diffuse out freely

and rapidly from the muscle "cells," but the present results are not sufficiently consistent to warrant a definite conclusion to this effect. Anaerobiosis in its early stages (17 hours at 2–3° C.) had no appreciable effect (Exp. C). In Exps. D and E hydrogen was used which was subsequently found to be contaminated with coal gas; the muscles were found to be inexcitable at the end of the experiment, and the value of α had increased.

The interest of the experiments in group F lies particularly in the value of c. The pairs of muscles used had been kept for 23 hours in an oxygenated Ringer's fluid containing 45½ mg. P per 100 g. They were then wiped and reweighed, and the value of c determined for each pair by means of an equilibrium experiment. The values obtained (45 and 48 mg. P per 100 g. water respectively) show clearly that the "interspaces" (containing 21 and 30 p.c. of the muscle water respectively) had come completely into equilibrium with the surrounding Ringer's fluid, and add to one's confidence in the physical meaning of the value of c obtained in other experiments.

The value of c is also of special interest in the experiments (groups A and B) on fatigued muscles. The muscles were stimulated *in situ*, series of tetani being produced by use of an induction coil, the treatment being continued for several minutes. In group B greater fatigue was induced than in group A. Direct analysis of the inorganic phosphate content of these muscles (group B) at the end of the equilibrium experiment yielded a value of 51 mg. P per 100 g. muscle water, which, taken in conjunction with the equilibrium value of 29 mg. per 100 g. in 25 p.c. of the muscle water, indicates a value of 58 mg. per 100 g. in the remaining 75 p.c. of the water. Due care was taken to avoid stimulation of the muscles afresh while they were being killed (by the use of ice-cold trichloracetic acid), and the protein-free filtrate was immediately neutralized with baryta to prevent breakdown of phosphagen. It is unlikely therefore that this value of 51 mg. P per 100 g. muscle water is fictitiously high.

A similar, though smaller, difference between the values found for the phosphate content of skeletal muscle by direct analysis and by equilibrium experiment is found in the case of resting muscles. The lowest figures yielded by direct analysis vary from 12½ to 25 mg. per 100 g. muscle water [Eggleton and Eggleton, 1929], whereas equilibrium values vary from 10 to 15 mg. per 100 g. water. It seems probable therefore that one-quarter of the muscle water contains phosphate which can diffuse away from the muscle into Ringer's solution, and that the remaining three-quarters of the muscle water contains phosphate in about twice the

concentration, restrained by semipermeable membranes from diffusing out so long as the muscle is alive, and at rest.

The value of 51 mg. per 100 g. water (only 41 mg. per 100 g. when expressed as a proportion of the muscle and not of its water content) is, however, lower than those previously found by direct analysis of the muscle. In the present series of experiments ample time for anaerobic resynthesis of phosphagen was given, whereas in the earlier recorded work [Eggleton and Eggleton, 1927] the muscles were killed immediately after stimulation had ceased, and this difference in treatment is the most likely cause of the difference in results.

Exchange of phosphate between "cells" and "interspaces" in the muscle.

The foregoing experiments lead to the conclusion that in respect of the exchange of phosphate between itself and Ringer's fluid the behaviour of a living muscle is identical with that of a quantity of water equal to a fraction (α) of the total water of the muscle, containing phosphate in a concentration c. In so far as description in these terms is successful there can be no exchange of phosphate between the two fractions of the water of the muscle: the fact that the numerical value of α is found to be the same within the limits of experimental error for experiments of 2 hours' duration as for those of 24 hours implies that any exchange of phosphate between "cells" and "interspaces" must be subject to far greater hindrance than exchange between the "interspaces" and the Ringer's fluid outside. Yet in the experiments on fatigued muscles and muscles maintained under anaerobic conditions, the value of c is found to be higher than that in resting muscles. There are several possible explanations of this result.

(1) An increase in c would result from a decrease in the size of the "interspaces" due to withdrawal of water into the remainder of the muscle by osmotic forces. A minute decrease in α is sometimes observed as a result of fatigue, but the decrease of 50 p.c. which would be required on this hypothesis to account for the doubled value of c is never found.

(2) An increase in c might result from the breakdown of some phosphoric ester present in the "interspaces." But it is generally agreed that all inorganic phosphate formed within physiological limits in muscle is derived from phosphagen. (The hexosephosphate and the adenylpyrophosphate, which account for practically all the organic phosphate other than phosphagen, give rise to no inorganic phosphate within physiological

limits of fatigue or oxygen lack.) But the inability of phosphagen to diffuse from muscle seems to indicate that it is not contained in the same fraction as the diffusible inorganic phosphate (*i.e.* the "interspaces").

(3) It seems more likely that in certain circumstances and within certain limits phosphate can diffuse from the "cells" to the "interspaces." Such a diffusion was observed in isolated oxygenated muscles when the surrounding Ringer's fluid was maintained practically phosphate-free by frequent renewal, but the rate of diffusion was so minute as to be inappreciable within 24 hours, and throws no obvious light on the diffusion observed in muscles fatigued or suffering from oxygen lack. It may be, as Embden suggested in 1921, that membranes become more permeable when the muscle is in the active state; it is also conceivable that the membranes concerned maintain, whenever possible, a constant ratio of phosphate on the two sides, and that diffusion outwards into the "interspaces" occurs to some extent whenever the concentration of phosphate inside the "cells" is increased or that in the "interspaces" lowered. Whatever be the underlying mechanism of the action of such semipermeable membranes, the evidence available indicates that this property of the membranes serves greatly to hinder diffusion outwards of inorganic phosphate produced within the muscle "cell."

To this property of the muscle "cell" boundary may perhaps be ascribed the observation that the increase in concentration of inorganic phosphate in the blood of an exercising animal is relatively small in comparison with the increase in the muscles themselves. Havard and Reay [1926] found only a small rise in the concentration of inorganic phosphate in the blood of athletes, excercised to the point of exhaustion; we have no direct knowledge of the concentration of this ion in the muscles themselves under such conditions, but in view of the large increase in the concentration of lactate in the blood it seems probable that the muscles were inadequately oxygenated. Direct determination of the phosphate concentration in blood and muscle of artificially exercised anæsthetized cats confirms this interpretation. The technique employed in such experiments has been described elsewhere [Eggleton and Evans, 1930]. These workers found (unpublished experiments) that stimulation sufficiently severe to cause an increase of 140 mg. lactic acid per 100 g. blood, led to an increase in its inorganic phosphate concentration of only 6 mg. P per 100 g., although the muscles analysed at the same time showed increases of 40–80 mg. P per 100 g. tissue. In lesser degrees of fatigue the same relationship between the increase in concentration of phosphate in muscle and blood was still observed. In such experiments

the major part of the musculature of the body was caused to contract, so that the volume of circulating blood was considerably less than that of active muscle, and equilibrium between the two should have been rapidly established. It might be argued that in such intact animals, phosphate was diffusing out from the muscles into the blood stream and being as rapidly removed by other tissues such as the kidney and bone. But no perceptible decrease in the total acid-soluble phosphate content of such muscles was apparent after fatigue, and one is driven to the conclusion that the muscle cell membranes in the body permit very little inorganic phosphate to pass through, despite a high concentration of this ion within the cell.

Summary.

1. When an excised frog muscle is immersed in Ringer's fluid until equilibrium is reached in respect of phosphate exchange (2–5 hours at 2–3° C.) analysis of either muscle or Ringer's fluid indicates that only 20–30 p.c. of the water of the muscle has been involved in the diffusion system. It is therefore suggested that the muscle cells are bounded by membranes which are impermeable in these circumstances to phosphate.

2. The different result previously obtained on the thigh preparation by Stella is due to the fact that the bone present can take up or give out considerable quantities of inorganic phosphate, thereby masking the capacity of the muscle tissue itself.

3. In heat rigor, the whole of the water of the muscle becomes ultimately freely available to phosphate diffusing in from a surrounding Ringer's fluid; the phosphate contained initially in such a muscle will also diffuse out freely and rapidly.

4. In other conditions of the muscle (fatigue, etc.) little change is observed in the relative size of the "interspaces." When the muscle is poisoned with coal gas a definite increase is noted.

5. Under the conditions in which these experiments were performed the phosphate concentration with which resting muscles (isolated muscles or thigh preparations) are in equilibrium lies between 10 and 15 mg. P per 100 g., while the phosphate concentration with which fatigued muscles are in equilibrium is about 30 mg. P per 100 g. Such fatigued muscles on direct analysis yield a value of 50 mg. P per 100 g. muscle water. These results are discussed in relation to those obtained by Stella and Semeanoff.

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