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A PHYSIOLOGICAL STUDY OF PHOSPHAGEN IN PLAIN MUSCLE.

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Prior to 1927 the only chemical constituent of plain muscle examined with reference to activity of the tissue was lactic acid [Lovatt Evans, 1925]. Stimulation of frog and tortoise stomach and cat bladder resulted in an increase of from 10 to 70 mg. lactic acid per 100 g. tissue (10–600 p.c. of the amount initially present); the recovery process was not studied. The existence of phosphagen in plain muscle has been known for several years; the presence of creatine phosphoric acid in the stomach of the rabbit and frog and in the uterus of the rabbit and guinea-pig was demonstrated by Eggleton and Eggleton [1929] and in the stomach muscle of birds by Zanghi [1929], while argininephosphoric acid, discovered by Meyerhof and Lohmann [1927] in the striated muscle of invertebrates, was found also in invertebrate plain muscle by Meyerhof [1928] and by Needham *et al.* [1932]. The latter authors did not study the physiological relationships of the phosphagen, but Meyerhof performed a few experiments which indicated that breakdown of phosphagen occurred during activity of such plain muscle. No attempts to demonstrate resynthesis of phosphagen during recovery from fatigue in plain muscle, either vertebrate or invertebrate, have yet been reported. The work here recorded was undertaken in order to fill this gap.

The retractor muscle of the foot of *Mytilus edulis* was chosen as the experimental material in preference to a vertebrate muscle owing to the completeness with which it can be isolated free from non-muscular tissue (such as nerve cells and mucous membrane). This muscle survives well under cool, aerobic conditions; it exhibits no spontaneous contractions and is uniform in its response to electrical stimulation.

The whole muscle, consisting of both anterior and both posterior horns, connected through the central portion (the protractor muscle and the foot containing the nerve ganglion being cut away), weighed from 0·25 to 0·5 g. and was therefore of a size suitable for chemical analysis. By means of a thread at either end it was attached below to a fixed point and above to a suitably weighted lever, so that contraction occurred isotonically. The stimulus was applied through two nickel mesh electrodes at the upper and lower ends of the muscle chamber, the stimulus reaching the muscle through the sea water in which it was bathed. In all cases where a series of contractions were required, alternating current (50 cycle) at 16 volts was used as the stimulus. The amount of current passing between the electrodes was of the order of 60 milliamps, of which only a small fraction passed through the muscle itself. When a tetanus was being evoked, a continuous stimulus was applied and the voltage varied to give a maximal response for the longest possible time. In some experiments of this type direct current was employed in place of alternating, but no gross difference was observed in the effects of the two types of stimulus. The sea water was kept stirred by a current of air or of nitrogen and the whole muscle chamber immersed in a water bath at 8–12° C. At the conclusion of an experiment the muscle was removed as rapidly as possible (not more than 1 min.) and killed by grinding in ice-cold 4 p.c. trichloroacetic acid. The protein-free filtrate was rapidly neutralized with finely ground baryta till pink to phenolphthalein and the phosphagen and inorganic phosphate content determined as described in a later section.

The dissection of the retractor muscle is tedious owing to its close adherence to surrounding tissues and multiple insertion into the shell. The amount of handling involved in freeing it completely seems to be the most likely cause of the low level of phosphagen observed in the freshly dissected muscle (Table I A). When such a muscle is left to rest for two or three hours in aerated sea water the phosphagen is rebuilt (Table I B). This phosphagen is, in all probability, argininephosphoric acid (see later) and is therefore referred to as such in the tables. The average level of phosphagen in the freshly dissected muscle is only 11·5 mg. per 100 g. of tissue (expressed in terms of P); in the muscles aerated for a few hours it has risen to an average value of 24 mg. per 100 g. The total phosphate, which expression is used throughout to signify phosphagen + inorganic orthophosphate, varies widely in different muscles, but the fraction of it in the form of phosphagen appears to be fairly constant (column 3, Table I) and the difference between the two groups can be more readily seen by a comparison of this fraction in the two. In the freshly dissected

TABLE I. The argininephosphoric acid content of the retractor muscle of *Mytilus* in the resting state.

Temperature ° C.	Duration of aeration hours	(1) Argininephosphoric acid mg. P per 100 g.	(2) Inorganic phosphate mg. P per 100 g.	(3) Ratio (1)/(1)+(2)
		A.		
20	Freshly dissected	8·7	21	0·3
17	,,	10·5	22·8	0·32
17	,,	16·9	17·2	0·49
17	,,	9·8	24·2	0·29
		B.		
10–12	2¼	16·4	5·5	0·75
15	3½	24·1	6·3	0·79
15	3½	19·6	5·5	0·78
9	3	18·5	4·9	0·79
7–10	4	21·9	5·3	0·8
8½	6	30·1	7·2	0·81
10–12	2¼	33·5	7·0	0·81
9	6	24·1	5·4	0·82
12	4	22·4	3·9	0·85
8½	6	25·2	3·9	0·86
8½	6	32·8	4·5	0·88
8½	4½	19·1	2·3	0·89

muscle this fraction is only 0·3–0·5; after a few hours in aerated sea water it has risen to 0·8; the muscles are at the same time in much better condition with regard to their mechanical response to stimulation than those freshly dissected. In all experiments therefore the muscles were allowed to rest in cool aerated sea water (8–12° C.) for a few hours before use.

Some inorganic phosphate must undoubtedly be lost to the surrounding medium from the cut ends of the muscle during this process; it is impossible to determine accurately the extent of this loss, for individual muscles vary too widely in their total phosphate content. But the loss is small in comparison with this individual variation and there is no indication from the figures in Table I that it is continuous; the total phosphate content of muscles left 6 hours in sea water is not lower on the average than that of muscles left only 2¼ hours.

The figures in Table II A show the effect on the phosphagen content of the muscle produced by a series of contractions. A stimulus lasting 15 sec. of the type described above was given once a minute in this first series of experiments. The intermittent mechanical response, after the initial staircase effect, decreased gradually in size until, at the times noted, the muscle failed to contract despite continued stimulation. Analysis showed that in such fatigued muscles the ratio of phosphagen to total phosphate was only 0·3–0·5 (Table II A).

A second series of resting muscles was stimulated continuously until the sustained mechanical response (of tetanus form) fell to zero, in spite of an increase in the strength of stimulation. The actual times of length of response recorded in Table IIB cannot be accurately compared since the strength of stimulus and consequent magnitude of mechanical response was not identical in all cases. In these muscles tetanized to exhaustion the ratio of phosphagen to total phosphate varied from 0·46–0·67.

TABLE II. The effect of fatigue on the argininephosphoric acid content of the retractor muscle of *Mytilus*.

Duration of stimulation min.	Gas used for stirring	(1) Argininephosphoric acid mg. P per 100 g.	(2) Inorganic phosphate mg. P per 100 g.	Ratio (1)/(1)+(2)
A. 15 sec. stimulus (16 v. A.C.) once per minute.				
120	Air	9·4	8·2	0·53
180	None	12·8	12·1	0·52
60	N_2	17·5	18·2	0·49
120	None	12·4	13·2	0·48
60	N_2	13·0	15·7	0·45
75	None	10·2	14·6	0·41
210	Air	6·3	14·7	0·3
B. Continuous stimulation (variable voltage, A.C. or D.C.).				
12	N_2	15·6	7·2	0·67
4	None	16·2	8·3	0·66
10	N_2	15·1	7·9	0·65
5	None	12·5	8·2	0·6
23	Air	16·2	12·6	0·57
12	N_2	11·1	9·1	0·55
10	N_2	8·6	7·4	0·54
12	N_2	12·8	14·7	0·46

The results, though not as striking as those obtained with skeletal muscle, in which the ratio of phosphagen to total phosphate can fall from a resting value of 0·8 to a value of 0·1 in extreme fatigue, prove beyond doubt that when fatigue is induced in a previously resting plain muscle, phosphagen disappears and inorganic phosphate appears. The figures in Table II reveal a further similarity between the processes occurring in the two types of muscle; in this, as in skeletal muscle, appreciable quantities of phosphagen are present even when the muscle is so fatigued that it fails to respond mechanically to further stimulation. It is possible that lactic acid accumulation is responsible for this failure, but there may be other, at present unknown, physical or chemical changes occurring which prevent further contraction. In this muscle, as in skeletal and cardiac muscle, complete absence of phosphagen has only been observed when the muscle is dead, *i.e.* incapable of recovery.

The question arises, is the breakdown of phosphagen which we have found to be associated with fatigue of the muscle irreversible and merely indicative of approaching death of the tissue, or is the chemical change to be interpreted as a genuine physiological accompaniment of the mechanical change? The latter alternative is favoured by the observations, summarized in Table III, which show that completely exhausted muscles, allowed to rest under aerobic conditions for 2–3 hours, recover not only their full mechanical response but also their phosphagen content. The figures in Table III A were obtained from muscles which had been fatigued in the same way as those recorded in Table II A; that is to say, they were initially in a resting state with a phosphagen : total phosphate ratio of about 0·8 which fell to about 0·45 as a result of fatigue. One hour's subsequent aeration restored this ratio to 0·65, while two or more hours of such treatment fully restored it to the initial resting value of 0·75–0·84. That this increase in the ratio of phosphagen to total phosphate

TABLE III. The argininephosphoric acid content of the retractor muscle of *Mytilus* after recovery (aerobic and anaerobic) from fatigue.

Duration of recovery hours	(1) Argininephosphoric acid mg. P per 100 g.	(2) Inorganic phosphate mg. P per 100 g.	Ratio (1)/(1)+(2)
	A. Aerobic.		
1	12·8	7·0	0·65
2	30·7	9·9	0·75
2	18·1	4·5	0·8
3	24·5	5·9	0·8
3	26·4	5·0	0·84
4	22·2	5·9	0·79
	B. Anaerobic.		
1	18·3	15·0	0·55
2	19·6	14·6	0·57
2	12·5	14·9	0·45
2	14·8	14·2	0·51

is not due merely to diffusion away of the inorganic phosphate formed during activity is seen by comparison of the actual amounts of phosphagen present in the two series. In the fatigued muscles (Table II A) the phosphagen content had been reduced to 11·5 mg. P per 100 g. on the average; in such muscles allowed to recover completely under aerobic conditions the phosphagen content had risen to 24 mg. P per 100 g.

Rest alone, in the absence of oxygen, failed to promote resynthesis of phosphagen in any comparable degree; in view of the fact that the nitrogen used was not purified and therefore almost certainly contained traces of oxygen, the slight increase in the phosphagen content of the muscles

under these conditions (a ratio of 0·45–0·57 as compared with one of 0·3–0·54 in fatigued muscles) may well have been due to this impurity. It is not impossible, however, that a small amount of anaerobic resynthesis occurs as in skeletal muscle. This partial resynthesis of phosphagen was accompanied by a partial recovery (about 30 p.c.) in the mechanical power of the muscle.

Identity of phosphagen present in *Mytilus* muscle.

Recently, Baldwin [1933] has suggested the possibility of the existence of a phosphagen other than argininephosphoric acid in invertebrate muscle, on the basis of the different rate of acid hydrolysis encountered in some species (*Octopus vulgaris, Eledone moschata*). Arnold and Luck [1933] have also postulated the presence of an argininephosphoric acid compound different from phosphagen, in some invertebrates (*Lumbricus*, etc.), since acid hydrolysis at room temperature resulted in the liberation of inorganic phosphate whereas boiling the solution did not. It was considered advisable therefore to ascertain the behaviour of the phosphagen present in the retractor muscle of *Mytilus* towards acid hydrolysis.

In view of the inconstancy of the results obtained on acid hydrolysis of crude muscle extracts [Meyerhof, 1928], the phosphagen was first separated in the form of its barium salt (alcohol precipitation after separation of water-insoluble barium salts). This was then hydrolysed in $N/10$ or $N/100$ HCl at 28° C. for varying periods of time (up to 70 p.c. hydrolysis). Its hydrolysis constant, calculated from the equation for a monomolecular reaction, was 8×10^{-3} in $N/10$ and $8{\cdot}5 \times 10^{-3}$ in $N/100$ acid. This hydrolysis constant is similar to that found by Meyerhof and Lohmann [1928] for a pure preparation of argininephosphoric acid; its maximum rate of hydrolysis occurred in $N/100$ HCl, the constant being $8{\cdot}5 \times 10^{-3}$*. The effect of molybdate on the rate of hydrolysis was also similar in the two cases; a thirtyfold reduction was observed, the constant in $N/100$ HCl + 0·3 p.c. molybdate being only $2{\cdot}7 \times 10^{-4}$. The hydrolysis rate of phosphagen in crude muscle extracts was greater in $N/10$ HCl ($k = 7 \times 10^{-3}$) than in $N/100$ HCl, a result also in agreement with that obtained by Meyerhof on muscle extracts from the invertebrates *Holothuria, Pecten* and *Sipunculus* ($k = 5{\cdot}4$–$7{\cdot}7 \times 10^{-3}$ in $N/10$ HCl).

The phosphagen described by Baldwin [1933] as a possible new member of the series possessed distinctly different properties. Its

* Calculated from the measurements given by these authors.

minimum hydrolysis constant occurred in $N/10$ HCl (5×10^{-3}) and the hydrolysis rate was reduced only fourfold by the presence of molybdate.

Although the phosphagen present in the retractor muscle of *Mytilus* has not been isolated, it seems highly probable, from the results given above, that it is argininephosphoric acid.

Method of estimation of argininephosphoric acid.

The method originally proposed by Meyerhof and Lohmann [1928] for the estimation of argininephosphoric acid in invertebrate muscle was shortly afterward modified by Lohmann [1928], and since that time has remained the standard method for determination of this phosphagen. In the original method a protein-free acid extract of muscle was hydrolysed at 37° C. for 15 hours and the orthophosphate liberated taken as a measure of the argininephosphoric acid initially present. Lohmann subsequently showed that at 37° C. pyrophosphate is also hydrolysed to some extent in the strength of acid used, and recommended incubation at 28° C. in $N/20$ acid for 15 hours. Under these conditions he found that added pyrophosphate was untouched and assumed that the adenosinetriphosphoric acid (adenylpyrophosphate) present in the muscle would behave in the same way.

This method is unsatisfactory from two points of view: a 15-hour incubation time (extended to 20–24 hours by most workers for the sake of convenience) is tedious and unsuited to such extensive series of determinations as are necessary in any quantitative study of the behaviour of argininephosphoric acid during physiological activity of the muscle; and secondly, there is no convincing proof that the phosphate liberated during such hydrolysis comes only from argininephosphoric acid. The exact position of the pyrophosphate radicle in adenosinetriphosphoric acid is unknown; and, since a muscle extract fails to exhibit certain properties characteristic of pyrophosphate solutions (for example, Davenport and Sacks [1929] showed that a muscle gave no distinctive blue colour with the Folin phosphate reagents, whereas pyrophosphate added in small amount produced the reaction), the conditions of its hydrolysis may well differ from those of simple pyrophosphate. If this were so, then the values obtained for argininephosphoric acid would be too high and apparent variations in its content in the muscle with changes in physiological condition would differ from the real changes to some unknown extent.

These difficulties are overcome if the argininephosphoric acid is separated, before hydrolysis, from the ortho- and pyrophosphates. The

separation is carried out by means of their barium salts exactly as in the case of creatinephosphoric acid [Eggleton and Eggleton, 1929] and the soluble barium argininephosphate, freed now from the "pyrophosphate," can be hydrolysed in a few minutes by boiling in acid solution. An additional difficulty present in the case of *Mytilus* muscle is incidentally overcome by the use of this technique, for the muscle contains some substance which forms an opalescent solution in trichloroacetic acid, and this prohibits the direct estimation of phosphate by colorimetric methods. The substance in question is, however, carried down in the barium phosphate precipitate, and in the estimation of the orthophosphate content of this fraction is again carried down by the barium sulphate precipitate and so removed before the colorimetric reading is made.

The method appears to be as accurate in the case of argininephosphoric acid as it was shown to be in that of creatinephosphoric acid. The barium salt of argininephosphoric acid is completely water soluble. In two experiments the residual barium phosphate precipitate was washed several times with barium trichloroacetate; the first washing extracted 2–3 p.c. of the total barium argininephosphate originally present, the second washing yielded a further 1 p.c. and subsequent washings continued to extract only traces of some ester hydrolysable by acid as in the original method.

Hydrolysis in $N/10$ HCl of the argininephosphoric acid in the soluble barium fraction is complete in 3 min. at 100° C. This was shown to be so in several experiments, for more prolonged boiling (up to 10 min.) failed to cause any further increase in the figure. In three experiments the orthophosphate liberated by 3 min. boiling was compared with that produced by 24 hours' hydrolysis at 28° C. and found to be identical.

The completeness of the separation of the barium salts was for a while in doubt, since the insoluble barium fraction, however frequently washed, yielded small amounts of extra inorganic phosphate after hydrolysis in $N/20$ trichloroacetic acid at 28° C. The same result was obtained, however, when frog muscle, which contains no argininephosphoric acid, was used, and it appears that the "pyrophosphate" fraction of either vertebrate (frog) or invertebrate (lobster, *Mytilus*) muscles is hydrolysed to a small extent during 15 hours in $N/20$ trichloroacetic acid at 28° C. The constant of hydrolysis under these conditions is approximately $4{\cdot}5 \times 10^{-5}$. Comparison with sodium pyrophosphate showed that this also is hydrolysed to a slight extent under the same conditions, its rate of hydrolysis being approximately half that of the muscle "pyrophosphate."

SUMMARY.

1. In the resting aerated retractor muscle of *Mytilus edulis* the ratio of phosphagen to the sum of phosphagen and inorganic phosphate is 0·8.

2. Fatigue induced by a series of contractions reduces this value to 0·45 and by a tetanus to 0·6.

3. Rest under aerobic conditions restores the phosphagen content of such fatigued muscles to its original resting level, full mechanical recovery occurring at the same time. Under anaerobic conditions the ratio remains practically unchanged after the same period of recovery and the power of mechanical response is restored to only a slight extent.

4. It is concluded that phosphagen bears the same close relation to activity in this plain muscle as it has previously been shown to do in skeletal and cardiac muscle.

5. The behaviour of the phosphagen present in *Mytilus* muscle towards acid hydrolysis resembles closely that of argininephosphoric acid.

6. A method for the rapid estimation of argininephosphoric acid is described, based upon its separation from pyro- and orthophosphate before hydrolysis.

7. Both sodium pyrophosphate and muscle "pyrophosphate" are hydrolysed to a slight extent in $N/20$ acid at 28° C., the rate of hydrolysis being roughly twice as fast in the latter case as in the former.

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