FURTHER OBSERVATIONS ON PHOSPHAGEN.

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The collection of experimental results presented in this paper is too inconsecutive to be used as the basis of any theoretical discussion of phosphagen, but the results are published in the hope that they will be of practical use to other workers in the field of muscle chemistry.

Phosphagen is the name given to a substance which is present in considerable quantities in resting voluntary muscle, and is of the nature of an ester of phosphoric acid, remarkable for the ease with which the phosphoric acid is hydrolysed off in dilute acid solution. From the skeletal muscles of rabbits we have isolated a material which shows this typical lability and which is a compound of creatine[1] and phosphoric acid(1). But it is not yet certain whether this substance is identical with, or is a breakdown product of, phosphagen.

The extreme lability of phosphagen in acid solution makes it imperative that its extraction by the following method from the muscle should be completed in the minimum of time. To precipitate proteins in the least bulky and most easily filtrable form, 4 p.c. CCl_3COOH has been found best (40 c.c. per grm. of muscle), filtration being carried out within 12 minutes of the grinding of the muscle. It is not at present possible to discover by direct estimation whether the phosphagen extraction is complete in this time: presumably it is, since the sum of the phosphagen and inorganic phosphate extracted is not increased by longer extraction (up to 24 hours). Neither is there any appreciable increase in the amounts extracted of creatine, lactic acid, or acid-soluble organic phosphorus (Table I).

Even with an extraction as short as 10 minutes there nevertheless occurs some breakdown of the extracted phosphagen in the acid medium. If phosphagen be dissolved in 4 p.c. CCl_3COOH at a dilution comparable to a muscle extract, at room temperature half of it is hydrolysed in

[1] Confirming Fiske and Subbarow (11).

TABLE I.

Time of extraction in minutes ...	10	60	180	1180
Phosphagen P and Inorganic P ...	89	88	87	88
Organic P (other than phosphagen)	69	67	68	72
Lactic acid	139	133	136	139
Total creatine	500	490	510	510

Five grm. of muscle were ground up in 200 c.c. of 4 p.c. CCl_3COOH and divided into four portions which were filtered at the stated times. Organic P estimated in duplicate, and lactic acid in triplicate. Results are expressed in mg. per 100 grm. of muscle.

200 minutes. In 10 minutes about 5 p.c. is hydrolysed. It is therefore necessary to apply a correction for this loss, and to correct the value for inorganic phosphate in the opposite sense.

As to the technique of estimating both phosphagen and inorganic phosphate we have found Briggs' method, suitably modified, most useful. The details of the technique we have published previously(2).

With regard to the plotting of the "colour ratios" it is necessary to take readings only in three groups at, say, 3, 6, and 8 minutes from the beginning of the reduction, and a reading at 50 minutes. The reciprocals of the readings lie in a straight line for the first 8 minutes of the reduction, and extrapolation back to zero time is therefore simple and accurate. We have been able to test this method on artificial mixtures of phosphagen and inorganic phosphate, and have obtained perfect results in all cases save where the proportion of phosphagen to phosphate is very low. Thus the method would tend to make a badly fatigued muscle appear even more fatigued (Table II).

TABLE II.

Experiment		I	II	III	IV	V
Phosphagen P	Theoretical	16	32	49	65	81
	Found	12	31	49	66	81
Inorganic P	Theoretical	71	53	36	18	0
	Found	76	55	35	17	0
Total	Theoretical	87	85	85	83	81
	Found	88	86	84	83	81

Results expressed in mg. P per 5 litres of solution (actually 5 c.c. were used for each analysis).

It may be mentioned that Meyerhof has elaborated an alternative technique in which the inorganic phosphate is precipitated by an alkaline magnesia mixture and estimated separately. The result subtracted from the total as estimated in the ordinary Briggs' method gives the phosphagen content of the muscle.

The estimation of free creatine. In 1911 a method was published by Walpole(3) for the estimation of creatine (as distinct from creatinine) in urine. The method is based on the fact that creatine condenses with

diacetyl in alkaline solution to give a pink coloured product. Several other substances containing the guanidine group (4), including arginine, display the same property. We have found that the creatine combined in phosphagen is not estimated by this method, and have made use of this fact in studying the fate of the phosphagen creatine of the muscle when the phosphagen has been caused to disappear. In its present form the method does not give accurate quantitative results, and the experiments cited in Table III must be taken only as a very rough indication.

TABLE III. Free creatine in frog's gastrocnemius.

Experiment	I	II	III	IV	V
Resting	190	170	210	160	200
Fatigued	430	—	—	370	—
In rigor	—	350	—	—	460
Incubated in $NaHCO_3$ ...	—	—	415	—	—
Ditto in presence of NaF	—	—	460	—	—

The results are expressed as mg. of creatine per 100 grm. of muscle. Creatine estimated by the method of Walpole. (In Exp. I the phosphagen P fell from 47 to 8, and in Exp. II from 50 to 0.)

Comparative study. In an earlier publication (2) we referred to the fact that plain muscle appeared to contain no phosphagen. We have followed up this clue[1] by studying the muscles of a number of animals, with some interesting results. Whilst the voluntary muscles of the vertebrates studied, even as low in the scale as *amphioxus*, all contained phosphagen, we were unable to demonstrate its presence in the muscle of any invertebrate examined. Parallel estimations of the acid-soluble creatine brought out clearly its physiological relationship. Muscles containing no phosphagen contained no acid-soluble creatine (Table IV).

Meyerhof has confirmed this discovery as regards the crab, and has further stated (5) that although phosphagen is absent, there is present a similar substance having rather less lability in acid, and (in a recent private communication) probably containing arginine in the place of creatine. In view of the work of Kutscher (6), who found that the muscles of the crayfish were rich in arginine but contained no creatine, it seems possible that this substance isolated by Meyerhof from the crab muscle may be present in the muscles of other invertebrates. It is a curious fact that the colour reaction given by free creatine with diacetyl is given also by arginine. It is possible that the guanidine residue common to these two compounds (and responsible for this colour reaction) may be the reason for their relationship in muscle physiology. In a more complete comparative study of muscles one

[1] Acting at the suggestion of Sir Walter Fletcher, whose helpful interest in this work we gratefully acknowledge.

must be prepared to meet (as Meyerhof has suggested) a series of "phosphagens" serving similar functions in different types of contractile tissue.

TABLE IV. A comparison of different muscles with respect to their content of phosphagen.

		Inorganic P	Phosphagen P	Creatine
VERTEBRATES:				
Snake	(dorsal)	65	40	—
Guinea-pig	(gastrocnemius)	58	22	—
Dog fish	(coraco-mandibular)	51	18	460
Plaice	(dorsal)	91	37	—
Cottus	(dorsal)	130	13	410
Frog	(heart)	20	5	—
	(gastrocnemius)	30	50	450
	(stomach)	20	0	—
Ray	(coraco-mandibular)	50	40	440
Tortoise	(hind limb)	64	15	—
Rabbit	(gastrocnemius)	26	62	—
	(soleus)	47	32	—
Amphioxus	(whole body)	57	33	420
INVERTEBRATES:				
Lobster	(tail)	74	0	0
Crab	(claw)	48	0	—
Aplysia	(foot)	2	0	—
Pecten	(adductor)	114	0	0
Holothuria	(longitudinal band)	12	0	—
Mytilus	(adductor)	50	0	0
Aurelia	(contractile tissue at circumference)	0·7	0	—
Earthworm	(whole body)	Trace	0	—

The results are expressed in mg. of P (or of creatine) per 100 grm. of muscle.

Anaerobiosis. The coraco-mandibular muscle of the ray (*R. clavata*) is peculiarly suited to certain types of muscle studies, and we take this opportunity of drawing attention to its advantages. It is a flat, straight-fibred muscle which can be dissected from the fish without any injury. In the adult the muscle weighs up to 20 grm., and is 8–10 cm. long. When cut up into several samples the distribution of phosphate, lactate, etc. is found to be very constant. We have used this muscle to study the effect of resting anaerobiosis on the phosphagen. Cut up into a dozen pieces and kept in an atmosphere of hydrogen the muscle lost most of its phosphagen during the first hour or two, the curve of breakdown resembling a negative exponential. During the first 6 hours the lactic acid production was practically linear, at the rate of about 70 mg. p.c. per hour. But the fact which makes these experiments of special significance is that until 80–90 p.c. of the phosphagen has disappeared the inorganic phosphate production can be ascribed entirely to phosphagen breakdown. Expressed in another way the sum of inorganic phosphate and phosphagen phosphate is constant until the muscle has passed beyond

the physiological range of anaerobiosis. Fig. 1 gives the results of one of five entirely concordant experiments of this nature. A difference is here

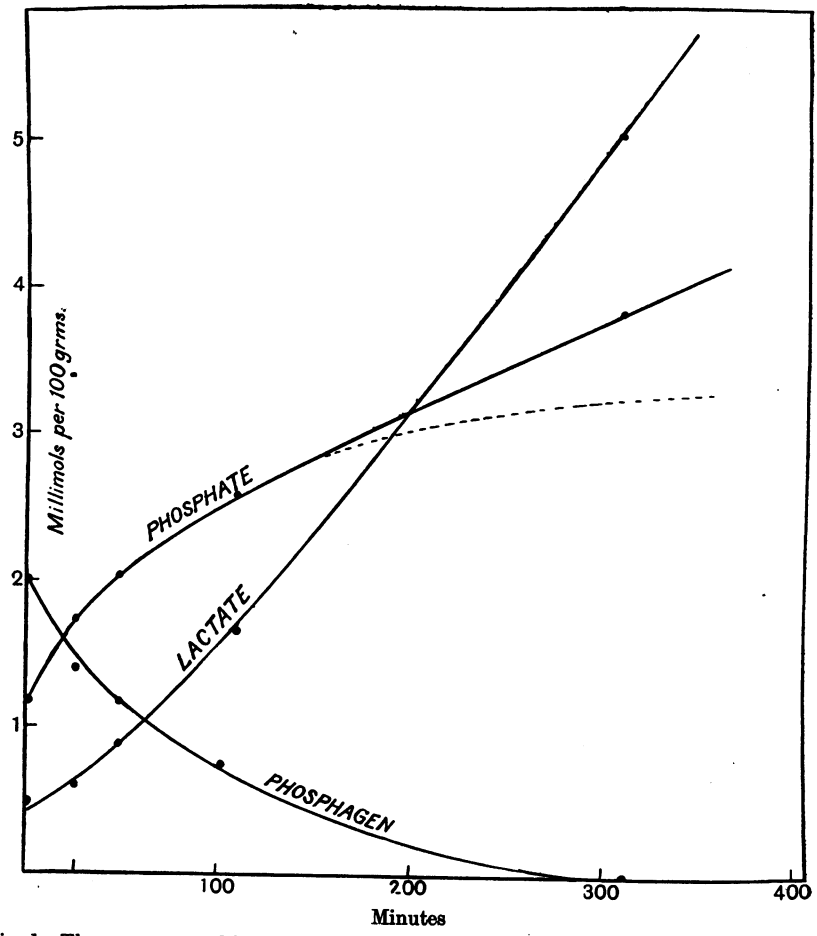

Fig. 1. The coraco-mandibular muscle of a ray (*R. clavata*) was cut into six pieces and allowed to remain in hydrogen at 16° C. One piece was taken for analysis at each of the stated times. The dotted line is the curve of phosphate production which would have been given had the phosphate been derived solely from phosphagen. At the end of 24 hours the last sample contained 700 mg. p.c. lactate, and 162 mg. p.c. phosphate (as P).

observed between the breakdown of phosphagen in resting anaerobiosis and its breakdown in muscular fatigue[1]. In the latter case (Table V) the inorganic phosphate produced accounts for only 60–80 p.c. of the

[1] The disappearance of phosphagen in activity is about 100 times as rapid as in esting anaerobiosis.

phosphagen removed, the remainder being "stabilised," and in the subsequent oxidative recovery of the muscle the fraction of the phosphagen which has been "hydrolysed" is rebuilt before the fraction which has been "stabilised." Indeed we have not so far in isolated muscles found any sign of "unstabilisation" of the "stabilised" fraction. It is to be observed also that the production of lactic acid in anaerobiosis is not directly related to the phosphagen breakdown. We have shown that this is true also in muscular activity, and Meyerhof has reached the same conclusion. When one considers also that the resynthesis of phosphagen during oxidative recovery is nearly complete when the lactic acid removal is only just begun, it is clear that one is dealing with a mechanism chemically distinct from, though perhaps physiologically related to, the production of lactic acid.

TABLE V. The fate of phosphagen phosphorus in muscular activity.

	Phosphagen P		Inorganic P		Organic P		Total		Change due to activity			
	R	*F*	*R*	*F*	*R*	*F*	*R*	*F*	Phosphagen P	Inorganic P	Organic P	Total
A	61	10	32½	74½	80	89	173½	173½	−51	+42	+ 9	0
B	50	6	36	75	84½	90	170½	171	−44	+39	+ 5½	+ ½
C	41½	15½	43½	60½	76½	85	161½	161	−26	+17	+ 8½	− ½
D	55	45	34½	32½	73½	84½	163	162	−10	− 2	+11	−1
E	70	41½	23	45	76	81½	169	168	−28½	+22	+ 5½	−1
F	45	26	37	49	72	82	154	157	−19	+12	+10	+3

These are six out of a large number of experiments in which one gastrocnemius (*F*) of a frog was caused to contract isometrically before being analysed. The second gastrocnemius was used as the resting control (*R*). The measurements of lactic acid, tension, and length of muscle have been omitted from the table. The results are expressed in mg. of P per 100 grm. of muscle. In Exps. C, D, and E the muscles were tetanised isometrically, and in the others a series of twitches at one second intervals were given.

The influence of the fluoride ion. In a previous communication to this *Journal*(9), we referred to the curious effect of fluoride on the phosphagen in a minced muscle preparation suspended in a bicarbonate buffer. Whether fluoride was present or not, all the phosphagen disappeared, but in the presence of fluoride this disappearance did not result in the liberation of free phosphate. In Table VI we have collected together a number of experiments bearing on this phenomenon.

This behaviour of phosphagen accounts in part for the so-called "synthetic" effect of the fluoride ion discovered by Embden and Lehnartz(7). The "synthesis" observed by these authors was in the main a conversion of phosphagen phosphorus (which they estimated as inorganic phosphorus) into some acid-stable phosphoric ester. When phosphagen is present some true synthesis can occur however, for added inorganic phosphate disappears to a small extent under the action of

TABLE VI. The effect of fluoride on the fate of phosphagen phosphorus in the incubation of chopped muscle in $NaHCO_3$ buffer.

		Initial condition of muscle		After incubation in buffer	
				(*a*) No NaF	(*b*) In *M*/10 NaF
		Phosphagen	Inorganic	Inorganic	Inorganic
1	Resting	45	28	91	25
2	,,	41	42	—	26
3	,,	47	33	—	20
4	,,	—	—	115	21
5	,,	50	40	—	28
6	Fatigued*	—	56	—	20
7	,, *	—	53	—	20
8	Resting	—	—	—	21
	Fatigued*	—	—	—	21

* Fatigued by 3′ isotonic tetanus.

In these experiments frog gastrocnemii were incubated for 3 hours at 38° in 2 p.c. $NaHCO_3$ buffer (after which time no phosphagen remains). The figures represent mg. of P per 100 grm. of muscle.

fluoride. Deuticke(8) has shown that muscle in rigor mortis (which incidentally contains no phosphagen) fails to give the "synthetic" effect. The same is true also of vertebrate involuntary muscle (Table VII), although the typical effect of the fluoride in inhibiting glycolysis of the organic esters is observable.

TABLE VII. The effect of flouride on the inorganic phosphate formation when chopped plain muscle (frog's stomach) is incubated in $NaHCO_3$ buffer.

	Inorganic phosphate in muscle		
	Initially	After incubation in $NaHCO_3$	After incubation in $NaHCO_3$ and *M*/9 NaF
I	15	30	25
II	20	41	26
III	20	45	28

In these experiments as in those of Table VI the chopped muscle was incubated in 2 p.c. $NaHCO_3$ solution for 2 hours, but in this case added fluoride has merely inhibited glycolysis.

A further distinction between plain and voluntary muscle is that added inorganic phosphate is not esterified by the former when the chopped muscle is incubated in the presence of fluoride.

It is safe to deduce from the experiments already quoted in this paper that the phosphate radicle of phosphagen is made use of by the muscle (when stimulated to activity, or when incubated in the presence of fluoride) to esterify some organic compound. From the constancy of the "total acid-soluble phosphorus" figures in Tables V and VIII[1] it is clear that the phosphoric ester produced is a member of the "acid-

[1] The results in Table VIII are a confirmation of those of Wechselmann (10).

TABLE VIII. The effect on the total acid-soluble phosphorus of incubation of frog's gastrocnemius in bicarbonate buffer with and without fluoride (at 38°).

	Inorg. P and phosph. P	Acid-sol. org. P other than phosph. P	Total acid-sol. P	Change in Inorg. and phosph. P	Change in Org. P	Change in Total acid-sol. P
Without fluoride:						
Unincubated	76	75	151			
Incubated	128	21	149	+52	−54	−2
Unincubated	75	77	152			
Incubated	137	13	150	+62	−64	−2
With fluoride:						
Unincubated	90	70	160			
Incubated	77	79	156	−13	+ 9	−4
Unincubated	84	70	154			
Incubated	60	94	154	−24	+24	0

Incubation for 18 hours in $NaHCO_3$ buffer both with and without fluoride produced large changes in the ratio of inorganic to organic acid-soluble P, but the sum of the two was unaltered. Results expressed in mg. of P per 100 grm. of muscle.

soluble" group, and the question remains whether or not it is of the class designated by Embden as "lactacidogen" (*i.e.* the ester or esters hydrolysed rapidly by the muscle enzymes when the minced muscle is incubated in a bicarbonate buffer). The experiments cited in Table IX

TABLE IX. The nature of the phosphoric ester formed as a result of activity.

	Inorg. and phosph. P	Acid-sol. org. P other than phosph. P	Total acid-sol. P	Change in Inorg. and phosph. P	Change in Org. P	Change in Total acid-sol. P
I. *Killed immediately:*						
Resting	88½	66	154½			
Fatigued	80½	75	155	− 8	+ 8½	+ ½
Incubated:						
Resting	104½	42½	147			
Fatigued	105½	43½	149	+ 1	+ 1	+2
II. *Killed immediately:*						
Resting	85	76	161			
Fatigued	65	93	158	−20	+17	−3
Incubated:						
Resting	116	39½	155½			
Fatigued	115½	39	154½	− ½	− ½	−1

In Exp. I one gastrocnemius was tetanised for 2 minutes isotonically under zero load, and the other gastrocnemius was a resting control. In Exp. II the stimulated gastrocnemius was tetanised isometrically for 2 minutes under 20–30 grm. initial tension. Each set of values is the mean of three independent results (*i.e.* six frogs were used for each experiment). The small apparent changes recorded in total acid-soluble P are due to small errors of manipulation (arising usually in the weighing of the muscles). They stand in marked contrast to the large rises in organic P and the compensating falls in "phosphagen and inorganic P" resulting from rapid fatigue. Results expressed in mg. of P per 100 grm. of muscle.

indicate quite definitely that this is so. Although, as a result of fatigue, the acid-soluble organic phosphorus of the muscles was increased by

about 8 mg. p.c. in one case, and 17 mg. p.c. in the other, the amount of acid-soluble organic phosphorus remaining after incubation was the same for both resting and fatigued muscles. The ester formed at the expense of phosphagen phosphorus in activity is therefore Embden's "lactacidogen."

It is in our opinion regrettable that the name "lactacidogen" has been given to a substance which in our experience is not diminished, but actually increased in amount during the contraction of an excised muscle, in which lactic acid appears; and when we identify as "lactacidogen" the substance which, during activity, is formed from phosphagen phosphorus we do not wish to be understood to express the opinion that this substance is really the source of lactic acid during muscular contraction.

The method of estimating "lactacidogen" adopted by Embden is not invalidated by the existence of phosphagen, which alike before and after incubation he has determined as inorganic phosphate, so that the difference is correct. On the other hand, all his observations on changes of inorganic phosphate during activity or in rigor are completely invalidated by the fact that a considerable fraction (in the resting muscle the major portion) of this "inorganic phosphate" is really present in an unstable organic form. In view of this latter fact it is obvious that a number of his conclusions must be revised.

Summary.

1. Muscles which are capable of rapid energy output (*e.g.* gastrocnemii of frog or rabbit) are, in their resting condition, richer in phosphagen than muscles intended for lower rates of energy expenditure.

2. The rate of disappearance of phosphagen in a muscle when resting under anaerobic conditions cannot be correlated directly with the rate of production of lactic acid. The phosphagen has diminished to an inappreciable quantity before the lactic acid production has reached a quarter of the value it finally attains.

3. The breakdown of phosphagen, whether as the result of fatigue, or rigor, or incubation of the minced muscle in bicarbonate buffer with or without the addition of fluoride, always results in the liberation of free creatine in an amount roughly corresponding to the phosphagen which has disappeared.

4. When phosphagen is present in a minced muscle which is being incubated in the presence of fluoride, some conversion of inorganic phosphate into organic can sometimes be observed. But in muscles

which contain no phosphagen we have failed so far to observe any such synthetic process.

5. The resynthesis of phosphagen which occurs when a fatigued muscle is allowed to recover is very rapid if the surrounding atmosphere is rich in oxygen (one atmosphere or more). Under these conditions the restitution of phosphagen is far more rapid than the restitution of glycogen from lactic acid. Some resynthesis has been observed by Meyerhof in anaerobic conditions, but this is easily masked by the breakdown which is always occurring in the absence of oxygen, even in a resting muscle.

6. The phosphagen destroyed by muscle when resting in anaerobic conditions, or when incubated in a bicarbonate buffer, is accounted for completely by the inorganic phosphate produced: phosphagen destroyed as the result of activity appears only in part as inorganic phosphate, the remainder being accounted for exactly by an increase in the amount of acid-soluble organic esters of phosphoric acid; phosphagen destroyed during incubation in a bicarbonate buffer in the presence of fluoride is accounted for entirely by the phosphoric esters formed. In all these cases the total acid-soluble phosphorus remains constant.

7. The phosphoric ester produced in fatigue is identical with Embden's "lactacidogen"—*i.e.* it is hydrolysed rapidly by the muscle enzymes when the chopped muscle is incubated in bicarbonate buffer.

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REFERENCES.

1. Eggleton and Eggleton. Chem. and Ind. 46. p. 485. 1927.
2. Eggleton and Eggleton. Biochem. J. 21. p. 190. 1927.
3. Walpole. This Journ. 42. p. 301. 1911.
4. Harden and Norris. Ibid. 42. p. 332. 1911.
5. Meyerhof. Naturwiss. 15. part 32. 1927.
6. Kutscher. Zeit. Biol. 64. p. 240. 1914.
7. Embden and Lehnartz. Z. Physiol. Chem. 134. p. 244. 1924.
8. Deuticke. Ibid. 149. p. 259. 1925.
9. Eggleton and Eggleton. This Journ. 63. p. 155. 1927.
10. Wechselmann. Z. Physiol. Chem. 113. p. 146. 1921.
11. Fiske and Subbarow. Science, 65. p. 401. 1927.