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A METHOD OF ESTIMATING PHOSPHAGEN AND SOME OTHER PHOSPHORUS COMPOUNDS IN MUSCLE TISSUE.

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The exact nature of phosphagen is not yet known. A protein-free extract of striated muscle contains a compound of creatine and phosphoric acid which may be identical with but is more probably derived from the phosphagen originally present in the muscle. It seems advisable to retain the term phosphagen for the substance existing in the muscle, reserving the chemical description creatinephosphoric acid for the substance isolated from acid extracts of muscle. Mr H. V. Horton, working in this laboratory, has shown that although phosphagen cannot diffuse from a living muscle, creatinephosphoric acid can diffuse readily through collodion membranes. This is true whether the creatinephosphoric acid has been extracted from the muscle by means of acid, dilute alkali, or alcohol. The inability of phosphagen to diffuse from living muscles may be due to the selective impermeability of the muscle cells to the creatinephosphoric anion, but it seems more justifiable to suppose that creatinephosphoric acid exists in the muscle in combination with some colloidal substance. Certainly the view that creatinephosphoric acid exists as such in the muscle, and that its disappearance in fatigue is a result of simple hydrolysis, raises serious difficulties. Meyerhof and Lohmann(1) have shown that creatinephosphoric acid has a large heat of hydrolysis, about 11,000 calories per gram molecule, but the total heat of contraction of a muscle has the same value relative to the lactic-acid production whether much or little phosphagen disappears simultaneously. Consideration of the myothermal data available leads us to the conclusion that the "breakdown" and "resynthesis" of phosphagen in a living muscle are reactions involving very little energy. (See A. V. Hill(2).)

The only available means of determining the amount of phosphagen in muscle is to estimate the creatinephosphoric acid in a protein-free

extract of the muscle. In the paper in which the presence of phosphagen in striated muscles was first reported (Eggleton and Eggleton(3)), we described a method for its estimation which was an elaboration of the colorimetric method of Briggs for estimating inorganic phosphate in tissues. This method has since been improved in certain respects, and as it is still the most rapid and convenient method for ordinary purposes, we take this opportunity of recording the improvements before describing the new method which is designed primarily for certain special cases.

Direct method.

Three stock reagent solutions are kept: (*a*) 5·5 *N* sulphuric acid (15 p.c. by volume), (*b*) 5 p.c. ammonium molybdate, and (*c*) 0·5 p.c. hydroquinone in 15 p.c. sodium sulphite ($Na_2SO_37H_2O$). The reagent used in an analysis is a mixture of two parts of (*a*), two of (*b*), and one of (*c*). This mixture is stable for 24 hours, but is in practice never kept more than 6 hours. 5 c.c. portions are added simultaneously to a standard phosphate solution and to the trichloroacetic acid extract of a muscle (both containing about 0·08 mg. of directly estimable phosphorus and having a volume slightly less than 10 c.c.), the volumes are rapidly adjusted to 15 c.c. and the solutions mixed. By reading the standard against the unknown in the colorimeter (the reverse of the usual procedure) the readings plotted against the time form a straight line for the first 8 minutes, and extrapolation backwards to zero time may be performed with a ruler. Theoretically two points only on this line are necessary to define its position: actually three sets of readings are made, at 2, 4, and 6 minutes, and if these do not fall on a straight line (as happens very rarely) the determination is repeated. A colorimeter one side of which is fitted with a rotating drum graduated in tenth-millimetres is more convenient than one fitted with the usual vernier scale.

The method as outlined above is accurate provided the ratio of orthophosphate to phosphagen phosphate is less than 6, and greater than 0·5, but cannot be used with confidence for well-oxygenated resting muscles in which the ratio may be 0·2 or for very fatigued muscles which may contain only a trace of phosphagen. The following device increases the range of applicability of the method to resting muscles. A quantity of the muscle filtrate expected to contain 0·1 mg. of directly estimable phosphorus is treated simultaneously with a standard containing 0·03 mg. of phosphorus (as KH_2PO_4) and the time comparison made between these two. A second standard containing 0·1 mg. of phosphorus is treated

in the course of the next few minutes, and is used for the final comparison (90 minutes).

In Table I are given the analyses (performed in collaboration with

TABLE I. Orthophosphate and phosphagen contents of excised muscles maintained in oxygen and killed in ice-cold trichloroacetic acid.

Exp.	Muscle	Duration of exposure in oxygen (hours)	Orthophosphate	Phosphagen	Ratio
1.	Sartorius	2	10	76·5	0·13
2.	Sartorius	2½	14	57	0·24
3.	Sartorius	1	16·8	58·7	0·29
4.	Sartorius	1	15·1	71·9	0·21
5.	Sartorius	2¼	15·7	65·3	0·24
6.	Sartorius	2	20	61·5	0·32
7.	Gastrocnemius	20	19·5	74·5	0·26
8.	Gastrocnemius	?	16·7	52·5	0·32
9.	Gastrocnemius	?	14·1	53·3	0·26
10.	Gastrocnemius	12	18·5	74·5	0·25

In *Exps.* 1–7 the Hungarian giant frog was used, in the last three the Dutch frog (*R. esculenta*). In *Exp.* 5 the muscle was first fatigued. Results are given in mg. of phosphorus per 100 g. of muscle.

Mr H. V. Horton) of several muscles which had been kept in oxygen for periods ranging between 1 and 20 hours. Such muscles are rich in phosphagen and frequently contain as little as 15 mg. of orthophosphate phosphorus per 100 g. of muscle. It seems not unlikely that resting muscles in the intact animal may contain even less orthophosphate. It was in the course of these experiments that Mr Horton first observed the condition of reversible inexcitability in voluntary muscle (Dulière and Horton(5)). In view of these analyses and the experiments of Sacks and Davenport(6), in which similar low values were obtained for warm-blooded animals, it seems likely that many workers on this subject are using faulty technique; for the orthophosphate values quoted for "resting" muscles are frequently higher than the phosphagen values. It seems not unlikely that the true orthophosphate content of a resting muscle in any animal is little, if any, higher than that of the blood. Stella has shown, for example, that resting frog muscles are in diffusion equilibrium with a physiological saline solution containing 8 mg. p.c. of orthophosphate phosphorus, a value only a little higher than the orthophosphate content of the blood of most vertebrates (Stella(7)). For present purposes however it is sufficient to note that in the case of really resting muscles a modified technique such as has been suggested above is essential.

For most purposes the above method is both convenient and accurate, but in certain circumstances it is valueless. When it is necessary to decide whether a tissue contains a trace of phosphagen or none whatever (a matter which is sometimes of great theoretical interest) it is essential to use some other means of estimating the phosphagen, and the following technique was designed primarily to meet this case.

NEW METHOD.

This method is based on our earlier observation (Eggleton and Eggleton(4)) that barium creatinephosphate is soluble in water: the extreme insolubility of barium phosphate should lead, and does lead, to a clean separation of the two salts. The following procedures are involved.

1. Extraction of the acid-soluble phosphorus from the muscle sample with $N/4$ trichloroacetic acid.

2. Neutralization of the extract with finely powdered solid baryta to a *p*H of approximately 9. This precipitates barium ortho- and pyrophosphates completely, but leaves the creatinephosphate in solution.

3. Separation of the precipitate from the soluble barium salts (fraction *A*) by centrifugalization.

4. Solution of the washed precipitate in HCl (fraction *B*).

5. Estimation in fraction *A* of

(*a*) directly estimable phosphorus (creatinephosphoric acid),

(*b*) total phosphorus.

Estimation in fraction *B* of

(*c*) directly estimable phosphorus (orthophosphate),

(*d*) phosphate hydrolysed to orthophosphate by seven minutes' boiling in $N/1$ HCl (pyrophosphate according to Lohmann),

(*e*) total phosphorus.

The successive stages of this technique are discussed in detail below.

DETAILED DISCUSSION OF NEW METHOD.

Stage 1. The muscle, weighing 0·5 to 1·5 g., is ground thoroughly in 5 c.c. of $N/4$ trichloroacetic acid with acid-washed sand; muscle, acid, and mortar having been cooled to 0°. Further trichloroacetic acid is added from a burette to make a total of 10 c.c. per g. of muscle used. The mixture is centrifugalized and the extract poured through a filter paper (phosphate free) into a test-tube, and a suitable volume is pipetted into a dry centrifuge tube. The procedure of centrifugalization followed by filtration is more rapid and efficient than either alone. If the muscle

be assumed to contain 85 p.c. of water the proportion of acid-soluble phosphorus extracted is represented by the ratio of the volume of extract taken to the total volume of water in the system. Thus if the muscle weighs 1·15 g. and the volume of extract taken is 10 c.c. the proportion taken is 10/(11·5 + 85 p.c. of 1·15) or 80·5 p.c. of the acid-soluble phosphorus of the muscle. That this is true for the orthophosphate, labile phosphate and total phosphate is shown by the results in Table II.

TABLE II.

Exp. 1. 2·7 g. of muscle were ground with 13 c.c. of 4 p.c. trichloroacetic acid and centrifuged. The extract was poured off and measured. The residue was extracted four times in succession with 8 c.c. of acid, each extract being measured. The five extractions took 25 minutes. The figures quoted for inorganic and labile P have been corrected for the known rate of hydrolysis of creatine-phosphoric acid in 4 p.c. trichloroacetic acid.

Volume of acid used to extract	13·0	8·0	8·0	8·0	8·05 c.c.
Volume of extract	12·6	7·65	8·0	7·9	7·9 c.c.
Proportion of acid-soluble phosphate in each extract. Theoretical*	82·4	12·6	3·6	1·0	0·3 p.c.
Do. Actual	82·3	12·5	3·4	0·7	0·2 p.c.
Proportion of inorganic phosphate	27	25	—	—	—
Proportion of labile phosphorus	36	36	—	—	—
Proportion of remainder	37	39	36	—	—

Exp. 2. As above: weight of muscle 3·78 g.

Volume of acid used to extract	15·0	10·0	10·0	10·0 c.c.
Volume of extract	12·4	12·15	10·15	10·15 c.c.
Proportion of acid-soluble phosphate in each extract. Theoretical*	67·0	24·0	5·6	1·55 p.c.
Do. Actual	67·1	24·0	5·6	1·6 p.c.
Proportion of inorganic phosphate	31	30	30	—
Proportion of labile phosphorus	26	26	25	—
Proportion of remainder	43	44	45	—

* Assuming 85 p.c. water in muscle, and instantaneous extraction. Acid-soluble phosphorus remaining in the protein precipitate at the end was extracted overnight with 10 c.c. of trichloroacetic acid.

The agreement between theoretical and the observed extraction in each successive extract is good. Moreover, the extraction must be complete in a few minutes[1], for the theoretical values quoted in the table assume instantaneous extraction.

Stage 2. The trichloroacetic acid extract in the centrifuge tube is neutralized by the addition of finely ground baryta (best sifted through a 90-mesh sieve) until neutral to phenolphthalein. A small final adjustment is occasionally necessary with $N/4$ trichloroacetic acid or saturated baryta solution. The presence of some carbonate in the powdered baryta is of no consequence. As far as this stage the operations are carried out

[1] The contrary experience of some other workers (*e.g.* Irving and Wells(8)) seems to be due to less thorough grinding of the tissue.

as rapidly as possible to minimize hydrolysis of creatinephosphoric acid, but when neutralized the solution may be left, and is indeed perhaps better left, for a short while before the next operation.

Stage 3. The mixture is centrifugalized to remove the precipitate of insoluble barium phosphates, the clear fluid being decanted into a measuring cylinder. The precipitate is washed with 2 c.c. of $N/4$ barium trichloroacetate neutral to phenolphthalein, centrifugalized, and the washing added to the soluble fraction. No further washing is usually made, for such washings merely extract minute quantities of some phosphoric ester which is almost but not quite insoluble in the reagent used. Water may not be used for this washing, since it dissolves appreciable quantities of orthophosphate.

Stage 4. The precipitate is dissolved in a drop of $8N$ HCl and washed into a second calibrated cylinder, the successive washings totalling about 10 c.c. of water. The volumes of both fractions are measured.

The use of measuring cylinders at this stage is very convenient and involves no loss of accuracy if the cylinders have been calibrated against a standard burette. We use 25 c.c. cylinders graduated in one-fifth c.c., calibrated at each 5 c.c. mark. Only cylinders with constant and small errors are retained and are used in conjunction with a calibration chart. We have satisfied ourselves that the use of these cylinders, although unorthodox, is not a source of error.

Stage 5. The acid-soluble phosphorus of the muscle is estimated in five fractions, of which two have soluble barium salts. The soluble fraction contains all the creatinephosphoric acid but no orthophosphate. This is shown by the following facts:

(i) Barium orthophosphate is found to be completely insoluble under the prescribed conditions: 10 c.c. of fluid in equilibrium with a barium phosphate precipitate contains no phosphorus that can be detected by the Briggs method.

(ii) The colour production curve of the soluble fraction, followed under the conditions described earlier in this paper for use with resting muscles, starts from the origin (Fig. 1).

(iii) A muscle in an advanced state of rigor which contains much orthophosphate gives no directly estimable phosphorus in the soluble fraction (Table XI). An acid extract, made from any muscle and allowed to stand until all the creatinephosphoric acid is hydrolysed, behaves similarly.

(iv) Orthophosphate added to a muscle extract is recovered quantitatively in the insoluble fraction (Table IV).

(v) Further extraction of the insoluble barium salts with the prescribed extracting fluid extracts no directly estimable phosphorus.

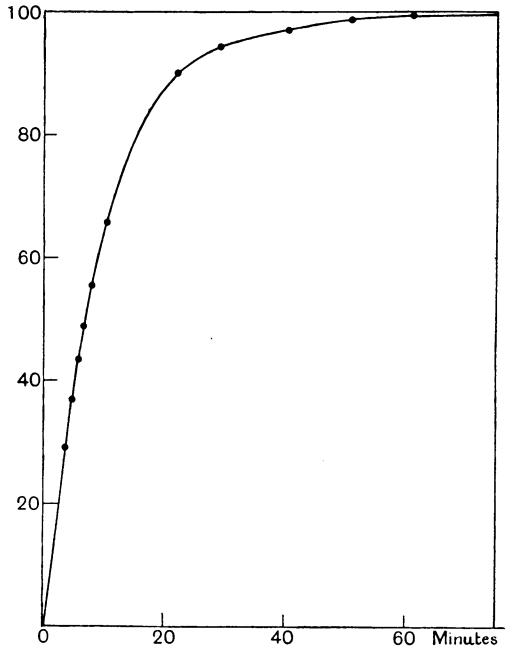

Fig. 1. The phosphagen fraction of a muscle extract prepared according to the text was compared with pure orthophosphate solutions in respect of the rate of blue colour production after the addition of Briggs reagents. The curve gives the change of colour intensity of the phosphagen solution relative to the inorganic phosphate standard as time progressed. The colour values are expressed as a percentage of the value finally attained (100 minutes). The first six points were obtained by a comparison with a phosphate standard having a value 37 p.c. on the above scale, the last five points with a standard of value 88 p.c. Note that the curve starts from the origin and is practically a straight line for the first 8 minutes.

The organic phosphorus in this fraction includes any barium hexose-monophosphate present in the muscle, together with any other phosphate esters with soluble barium salts. Whatever be the compounds included here, it seems justifiable to class them as a distinct fraction since the separation from the insoluble salts is so nearly complete.

With regard to the "pyrophosphate" fraction we have not ourselves attempted to isolate this compound, but we have found that:

(i) Pyrophosphate is completely hydrolysed by the treatment given.

(ii) A similar hydrolysis curve is given by the solution obtained

from muscle, when allowance is made for a very gradual hydrolysis of some other, relatively resistant, ester (Fig. 2 and Table III).

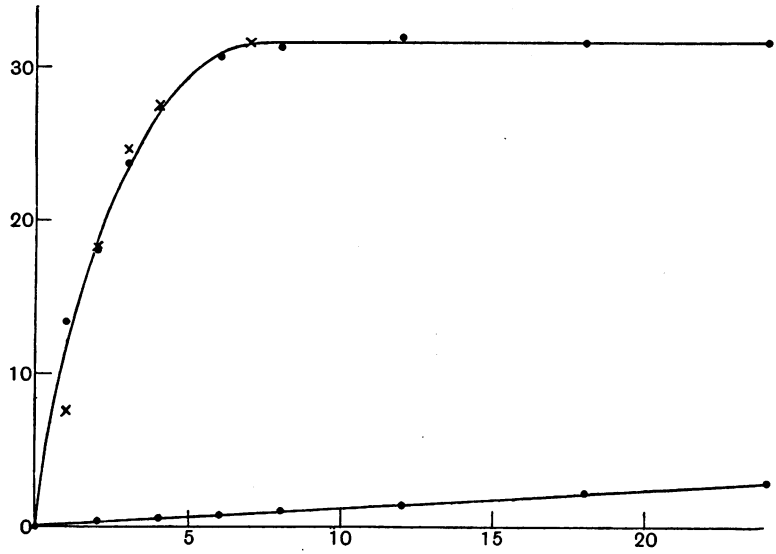

Fig. 2. The hydrolysis curve of the "pyrophosphate" fraction given in Table III is here reproduced graphically and compared with the hydrolysis curve of pyrophosphate under similar conditions. The points on the latter curve are marked with crosses. The lower almost horizontal line represents the slow hydrolysis of some phosphoric ester. The ordinates are mg. of phosphorus per 100 mg. of muscle, the abscissæ time in minutes.

TABLE III. Hydrolysis of fraction with insoluble barium salts in $N/1$ HCl at 100° C.

Time in minutes	Orthophosphate present	Orthophosphate produced	Analysis of compound hydrolysis curve: "Pyro-phosphate"	Analysis of compound hydrolysis curve: Other phosphorus
0	32·5	—	—	—
1	45·9	13·4	13·3	0·1
2	50·5	18·0	17·7	0·3
3	56·2	23·7	23·3	0·4
4	59·9	27·4	26·9	0·5
6	63·2	30·7	30·0	0·7
8	64·8	32·3	31·3	1·0
12	65·8	33·3	31·9	1·4
18	66·3	33·8	31·6	2·2
24	67·1	34·6	31·7	2·9

The experimental hydrolysis curve analyses directly into two curves, one of which is identical with that of pyrophosphate (Fig. 2). The other is practically a straight line, and registers the very slow hydrolysis of some phosphoric ester. The results are expressed in mg. of P per 100 g. of muscle. The total phosphorus in this fraction was 81·6 mg. per 100 g. of muscle.

(iii) Pyrophosphate added to the original muscle extract appears quantitatively in this fraction (Table IV).

TABLE IV. Recovery of ortho- and pyrophosphate added to trichloroacetic acid extracts of muscle.

Experiment	1	2	3	4	5
		Original analysis.			
Orthophosphate	515	595	406	475	491
Phosphagen	231	142	254	313	384
Pyrophosphate	313	292	236	268	317
Soluble esters	144	109	141	95	95
Insoluble esters	180	143	145	142	172
		Phosphorus added.			
Orthophosphate	188	188	218	220	227
Pyrophosphate	134	137	137	130	129
		Final analysis.			
Orthophosphate	701	795	620	696	720
Phosphagen	240	139	257	306	373
Pyrophosphate	452	431	378	401	450
Soluble esters	135	106	132	85	91
Insoluble esters	180	142	134	151	180
		Phosphorus recovered.			
Orthophosphate	186	200	214	221	229
Pyrophosphate	139	139	142	133	133

The extract from a muscle sample was divided into two parts, to one of which was added a known quantity of pyrophosphate and orthophosphate. The effect of this alteration in the distribution of the different phosphorus fractions is shown in the table. Results are given in micrograms of P per g. of muscle.

The five phosphorus estimations involved in a full muscle analysis are carried out as described below.

Fraction A.

(*a*) Directly estimable phosphorus (creatinephosphoric acid). A quantity of the solution expected to contain 0·08 mg. or less of labile phosphorus is taken in a 15 c.c. flask, made up to nearly 10 c.c., and treated with 2 c.c. each of the sulphuric acid and molybdate stock reagents. After 60 minutes, when the creatinephosphoric acid has been hydrolysed, 1 c.c. of the third reagent is added, the solution made up to 15 c.c., stirred, and left for 30 minutes. The precipitate of barium sulphate present is removed by centrifugalization, and the colour is compared with that of a phosphate standard of appropriate strength.

(*b*) A quantity of fraction *A* calculated to contain 0·08 mg. of total phosphorus is taken in a pyrex boiling tube and boiled down with 2 c.c. of the stock sulphuric acid reagent. When all water has been driven off the tube is allowed to cool, one or two drops of Merck's perhydrol are

added and the tube gently heated whilst the decomposing hydrogen peroxide oxidizes the organic matter present. After cooling again water is added and the fluid again boiled down to destroy the excess of hydrogen peroxide and any persulphate which may be present. The contents of the tube are washed with not more than 10 c.c. of water into a graduated cylinder. 2 c.c. of the stock molybdate reagent and 1 c.c. of the hydroquinone reagent are added, the volume is made up to 15 c.c. and measured. After 30 minutes the barium sulphate precipitate is removed by centrifugalization and the solution is estimated colorimetrically.

Fraction B.

(*c*) The orthophosphate is determined in the same manner as the labile phosphate (fraction *A*(*a*)) save that it is unnecessary to wait 60 minutes before adding the hydroquinone-reagent.

(*d*) A quantity of solution calculated to contain 0·08 mg. of ortho- and pyrophosphate is treated with 0·5 c.c. of 8*N* HCl and diluted to 4 c.c. with water (the resulting acidity being *N*/1), and the solution is kept in boiling water for 7 minutes. The tube is then chilled and the contents washed into a calibrated measuring cylinder. 1·3 c.c. of the stock sulphuric acid reagent are added so that the total acidity (sulphuric and hydrochloric) is equivalent to 2 c.c. of the stock sulphuric acid reagent, and the solution made up to 12 c.c. The remaining Briggs reagents are added, and the final volume (about 15 c.c.) measured. After standing 30 minutes, the barium sulphate precipitate is removed as before and the solution estimated colorimetrically. This simple technique is made possible by the fact that HCl can replace sulphuric acid in the Briggs solution equivalent for equivalent without any effect on the tint or intensity of the blue colour (Table V).

TABLE V. Effect of replacing sulphuric acid by hydrochloric acid in estimation of phosphorus by Briggs method.

Sulphuric acid	2	1·5	1	0·5	0 c.c.
Hydrochloric acid	0	1·5	3	4·5	6 c.c.
Recovery p.c.	100	100·3	99·8	100·3	100·1

4 c.c. standard phosphate solution (0·8 mg. P) was present in each case. The total acidity in all cases was equivalent to 2 c.c. 5·5*N* sulphuric acid.

(*e*) The total phosphorus in this fraction is estimated exactly as under (*b*).

COMPARISON OF NEW AND OLD METHODS.

The results obtained by the old direct method are usually 1 to 2 p.c. higher than those obtained by the new method on the same acid extract of muscle. The difference may in part be due to the presence of barium

in the fractions estimated in the second method since addition of barium to a trichloroacetic acid extract of muscle without any subsequent fractionation of the salts frequently produces the same very slight lowering of the result. The difference is sufficiently small to be unimportant for most purposes (Table VI).

TABLE VI. Comparison of phosphorus fractions in muscle extracts by the old and new methods.

Experiment	Orthophosphate and phosphagen	Pyrophosphate	Organic phosphate	Total phosphate
1. Old	95	26½	22½	144
New	92½	27½	21½	141½
2. Old	89½	32	26	147½
New	87½	32	26½	146
3. Old	85	32	26	143
New	83½	30	26½	140

The results quoted in the first column for the new method are the sum of the separately obtained values for the orthophosphate and phosphagen, and in the third column the sum of the two organic phosphorus fractions, also separately estimated. Results are given in mg. P per 100 g. of muscle.

RANDOM ERRORS.

In Table VII are reproduced the results of a quadruplicate analysis of a muscle sample. The total acid-soluble phosphorus was determined in five fractions exactly as described in the preceding section. The column "soluble" esters refers to the phosphoric esters with soluble barium salts and is the difference between the total phosphorus and the directly estimable phosphorus (creatinephosphoric acid) in the soluble fraction. The column "insoluble" esters is similarly derived: it is the phosphorus in the insoluble fraction not accounted for as ortho- or pyrophosphate. It will be seen that two-thirds of the values given lie within 0·5 mg. p.c. of the mean. The degree of freedom from random error is satisfactory.

TABLE VII. Four independent analyses of a trichloroacetic acid extract of a partially fatigued muscle.

Exp.	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters	Total P
1.	54·7	37·0	28·9	7·9	14·1	142·6
2.	55·0	36·2	28·2	7·9	15·7	143·0
3.	54·4	35·6	29·7	8·4	14·9	143·0
4.	55·3	35·4	29·7	7·9	13·9	142·2
Mean	54·9	36·0	29·1	8·0	14·7	142·7

The actual analyses made in each case were creatinephosphoric acid, and total phosphorus in soluble fraction; orthophosphate, ortho- and pyrophosphate, and total phosphorus in insoluble fraction. The above results have been obtained by differences and are given in mg. of P per 100 g. of muscle.

Some applications of the method.

The most obvious effect of rapidly induced fatigue in isolated muscles of the frog is a disappearance of phosphagen and an increase in the orthophosphate content, but the two changes are not equal in extent. As we have shown previously (Eggleton and Eggleton(9)) the increment in orthophosphate usually accounts for 60 to 80 p.c. of the phosphagen phosphorus which has gone: the remainder of this phosphagen phosphorus appears in the fraction defined by Embden as "lactacidogen" (Eggleton and Eggleton(10)), that is to say the group of phosphoric esters rapidly hydrolysed by the muscle enzymes when the cut-up muscle is incubated in a bicarbonate buffer. To be explicit on this point, some previously published results have been reproduced in Table VIII, from which it will be seen that the amount of organic phosphorus remaining after the incubation was the same for resting and fatigued muscles.

Table VIII. The effect of fatigue on the "lactacidogen" content of muscle.

	Orthophosphate and phosphagen	Organic phosphate	Total phosphate
Killed immediately			
Resting	85	76	161
Fatigued	65	93	158
Incubated			
Resting	116	39½	155½
Fatigued	115½	39	154½

The "non-lactacidogen" phosphoric esters are not altered in amount by fatigue. Results are given in mg. of phosphorus per 100 g. of muscle.

The new method of analysis yields further information on this point. From the results quoted in Table IX it is clear that the ester formed as a result of activity has a soluble barium salt: there is in general no change in the amount of pyrophosphate in the muscle (confirming Lohmann(14)) and only a slight rise, if any, in the amount of phosphoric esters with insoluble barium salts. There is no change in the total quantity of acid-soluble phosphorus. It may be noted in passing that the analytical figures supply what is probably a maximum value for the amount of hexosemonophosphoric ester in the resting muscle, for this ester if present would be found in the fraction with soluble barium salts. The amount is not greater than 5 to 7 mg. of phosphorus, which corresponds to something less than 40 mg. of hexose, per 100 g. of muscle.

The possibility was suggested in an earlier paper that since the phosphagen contents of resting muscles of different types appeared to be directly correlated to their normal ability for rapid energy output,

TABLE IX. The effect of rapidly induced fatigue on skeletal muscles of the frog.

		Total phosphate	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters
1.	Resting	142½	24	40	20	5	11
	Fatigued	142½	38	18	23½	12	8½
	Change	—	+14	−22	+ 3½	+ 7	− 2½
2.	Resting	105	20	39	21½	5½	14
	Fatigued	105½	37½	16½	24	10	12
	Change	—	+17½	−22½	+ 2½	+ 4½	− 2
3.	Resting	121½	19½	40	24	4½	12
	Fatigued	120½	40½	5	24	15	15½
	Change	—	+21	−35	0	+10½	+ 3½
4.	Resting	134	18½	49	20½	2	10
	Fatigued	125½	53½	4½	18½	11	12½
	Change	—	+35	−44½	− 2	+ 9	+ 2½
5.	Resting	138½	20½	47½	21	1	10
	Fatigued	138½	34½	26	21	7½	11
	Change	—	+14	−21½	0	+ 6½	+ 1
6.	Resting	112½	15	42½	22	9	11½
	Fatigued	111½	38	12	21½	15½	12
	Change	—	+23	−30½	− ½	+ 6½	+ ½
	Mean change	—	+21	−29½	+ ½	+ 7½	+ ½

The muscle pairs were in most cases the gastrocnemii of the Hungarian (giant) species of *R. temporaria*. The fatigued muscle had been tetanized directly without load for 1–2 minutes. The total phosphate values are given in mg. of phosphorus per 100 g. of muscle; the five fractions are expressed as percentages of the total.

the function of phosphagen might be that of an accelerator of muscular activity. The recent work of Nachmannsohn(11) has brought out clearly the existence of such a relationship, and it seemed possible that the phosphagen of a muscle might not be distributed evenly throughout its bulk, but might be absent from any part of the muscle which contained no nerves or motor end plates. Accordingly the distal ends of the sartorii and graciles (which are known to contain few if any nerve endings) of some giant frogs were cut off and estimated separately from the main mass of muscle. No difference was detectable however, either in the total phosphorus content or in the distribution of the five phosphorus fractions determined (Table X).

TABLE X. Comparison of muscle tissue free from motor end plates with remainder of muscle.

	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters	Total phosphate
Nerve-free	31·8	55·4	25·7	5·8	15·6	134·3
Remainder	32·2	55·4	25·4	5·3	16·1	134·4

The lower portions (about one-tenth) of the sartorii and graciles of several frogs were analysed separately from the main bulk of the muscle. The results are given in mg. of phosphorus per 100 g. of muscle.

Clearly the phosphagen of the muscle is associated with the fibres themselves and not with the nerve endings.

The slow death of an isolated muscle through oxygen lack is marked by a steady formation of orthophosphate, a process finishing only when some 90 p.c. of the muscle phosphorus has become inorganic. During the earlier part of the process the only phosphoric ester which can be shown to be irreversibly destroyed is phosphagen. This we demonstrated in the case of the coraco-mandibular muscle of the ray (1928). In the experiments then made a single muscle was cut into several pieces and kept in hydrogen, analyses being made by the old direct method. We have repeated the experiments, using in each experiment a number of uninjured frog muscles and performing the analyses by the new method. The results (Table XI) confirm the earlier ones and bring to light further interesting relationships which the older method failed to reveal. For 24 hours after the beginning of anærobiosis phosphagen steadily disappears and its phosphorus appears quantitatively as orthophosphate. The sum of the two does not change. Later, when almost all the phosphagen has gone, a breakdown of pyrophosphate begins, and continues till no pyrophosphate is left. Later still, the phosphoric esters with insoluble barium salts are attacked and ultimately are completely destroyed. But during the whole period of 50 to 60 hours there is no sign of a disappearance of the acid-stable group of esters with soluble barium salts: indeed they show a steady tendency to increase in amount throughout the whole time. The amount is more than doubled. It is not

TABLE XI. The effect of prolonged oxygen lack on the distribution of phosphorus in the skeletal muscles of the frog.

Time in hours	Muscle	Total	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"In-soluble" esters
0	1 Gr. L.	128·8	13·1	53·3	21·6	2·6	9·6
0	1 S. R.	128·0	15·1	49·4	28·5	4·3	2·7
4	1 G. L.	129·2	31·8	30·6	21·4	5·7	10·5
4	2 Gr. R.	135·1	28·2	36·3	24·0	5·5	5·8
19	2 S. L.	141·1	47·8	12·2	22·4	8·4	9·4
19	2 G. R.	148·4	46·2	14·2	22·5	6·6	10·6
26	1 Gr. R.	141·9	51·5	9·2	19·0	9·1	11·3
26	1 G. R.	129·5	62·6	6·2	14·4	7·3	9·4
43½	2 G. L.	133·0	65·1	3·0	13·4	7·5	10·9
43½	1 S. L.	136·2	83·7	0	2·9	8·9	4·4
50½	2 S. R.	144·0	90·6	0	2·8	9·4	0
50½	2 Gr. L.	133·0	89·7	0	0	10·3	0

The gastrocnemii (G.), graciles (Gr.) and semimembranosi (S). from the left (L.) and right (R.) legs of two frogs (1 and 2) were kept in moist hydrogen at room temperature for the stated time. The total acid-soluble phosphorus is given in mg. per 100 g. of muscle; the five fractions are expressed as a percentage of this figure. The frogs used were the giant species of *R. temporaria* from Hungary.

possible, nor fortunately is it necessary, to offer an "explanation" of these results. They are introduced here primarily as an illustration of the use of the new method of analysis.

A COMPARISON OF DIFFERENT TISSUES.

The technique outlined in this paper is applicable without modification to plain muscle, blood, and several other tissues, but cannot be applied to liver owing to the presence of some pigment which interferes with the estimation, and which we have not succeeded in removing by any simple treatment. The results that have been obtained illustrate the particular value of the method in detecting traces of phosphagen (Table XII).

TABLE XII. Phosphorus distribution in certain organs of some vertebrates.

Tissue	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Insoluble" esters	"Soluble" esters	Total P
Heart						
Guinea-pig	27·5	5·2	24·3	18·9	16·2	92·1
Stomach						
Rabbit	32·0	2·3	14·5	11·6	17·7	78·1
Rabbit	24·7	5·1	19·7	14·5	12·2	76·2
Frog	18·5	0·5	8·5	8·0	17·0	52·5
Uterus						
Rabbit	11·6	1·4	8·4	8·8	22·6	52·8
Guinea-pig	14·0	Trace	7·8	11·2	17·0	50·0
Testis						
Rat	10·7	0·9	11·0	8·7	42·7	74·0
Rat	11·7	0·7	12·9	11·8	40·0	77·1
Cat	9·1	0·6	5·3	5·0	29·2	49·2
Rabbit	10·6	2·0	5·6	7·6	32·8	58·6
Guinea-pig	7·6	2·6	17·0	13·0	37·2	77·4
Kidney						
Rabbit	18·0	0	10·3	9·6	50·3	88·2
Cat	10·1	0	8·3	6·4	36·0	60·8
Spleen						
Rabbit	13·7	0	13·2	17·8	42·8	87·5
Cat	9·8	0	10·9	7·9	29·1	57·7
Blood						
Dog	6·0	0	2·0	17·5	1·0	26·5
Human	3·0	0	3·8	12·7	1·0	20·5
Rabbit	5·1	0	(... 19·7 ...)		3·5	28·3
Cat	4·3	0	1·2	—	1·4	—

The figures in columns 4 and 5 refer to esters having insoluble and soluble barium salts respectively. All values are given in mg. of P per 100 g. of tissue (100 c.c. in the case of blood).

Analyses of blood from four different vertebrates reveal no trace of phosphagen. The fraction in which phosphagen would appear is completely blank. We cannot confirm the statement of Fiske and Subbarow(12) that the Briggs reagents develop a blue colour in the absence of phosphate. If clean glassware and pure reagents are used a "blank" test develops no blue colour. There is a small trace of pyrophosphate in blood according to our figures, and an even smaller amount of organic phosphate with a soluble barium salt—about 1 mg. per 100 c.c. Most of the blood phosphorus is in the form of esters with insoluble barium salts.

The spleen and the kidney of the rabbit and cat we have also found to contain no phosphagen, though according to Ferdmann and Feinschmidt(13) appreciable quantities of phosphagen can be found in the spleen. These two organs are alike in their content of the different phosphorus fractions, and are remarkable for the proportion and absolute amount of "soluble" esters: more than half the phosphorus present is in this group.

The uterus and stomach have been taken as examples of unstriated muscle. Both contain labile phosphorus in small amounts. We have previously reported failure to detect phosphagen in the stomach muscle of the frog when using the old technique. The new method reveals a trace of labile phosphorus in this organ such as certainly could not have been detected by the old method. In the thick muscular coat of the pyloric end of the stomach of the rabbit larger amounts are found, though the quantity is always less than in any striated muscle we have examined. Except for a rather high proportion of "soluble" phosphoric esters there is nothing remarkable in the rest of the analytical results of these organs.

In all cases where a tissue apparently contains a minute amount of labile phosphorus it is our custom to boil a separate sample of the original trichloroacetic acid extract for 2–3 minutes, a treatment which destroys any phosphagen present, and repeat the analysis for phosphagen. In all cases this has been found to give a clear negative result.

The most interesting results quoted in Table XII are the analyses of the testes of rabbit, guinea-pig, cat and rat. These organs all contained labile phosphorus in an amount comparable to the stomach muscle. The testes are said to contain very few muscle fibres: it is highly improbable that these few fibres could account for the presence of this "labile" phosphorus assuming that it represents phosphagen. It seems possible that this "labile" phosphorus is really a constituent of the spermatozoa.

Pyrophosphate.

Since this paper was written we have had the privilege of reading the manuscript of a paper by Dr Lohmann in which he states that the pyrophosphate of muscle exists in combination with adenylic acid in a molecule which therefore contains three phosphorus atoms. Two of these (linked as pyrophosphate) are easily hydrolysed by acid, whilst the third is an orthophosphate radicle relatively resistant to hydrolysis. Reference to Tables IV, VII and IX in this paper reveals two interesting facts which seem to be associated with this discovery. In the first place the amount of the "insoluble organic phosphate" in a voluntary muscle is almost exactly one-half of the amount of pyrophosphate. If we make the assumption (for which at present we have no direct evidence) that the adenylic acid phosphorus associated with the pyrophosphate is estimated in this fraction we can calculate the amount of phosphorus in this fraction in excess of that present as the pyrophosphoric-adenylic acid complex. It amounts to practically nothing. In the case of the twelve muscles in Table IX the "excess" is 1, − 3, 3, 0, 0, $3\frac{1}{2}$, $-\frac{1}{4}$, 3, $-\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$, and 1 p.c. of the total acid-soluble phosphorus. This suggests strongly that the fractions which we have called "pyrophosphate" and "insoluble esters" respectively measure together the pyrophosphate-adenylic acid complex of Lohmann. If this be so there remains practically none of the acid-soluble phosphorus of the muscle to be accounted for, since the group we have called "soluble esters" probably consists in the main of hexosemonophosphate.

As regards other tissues it will be seen in Table XII that the "insoluble ester" fraction is never less than half the pyrophosphate fraction. Indeed, the two fractions are usually about equal in amount. This suggests the presence of some compound in the "insoluble ester" fraction which is absent from voluntary muscle.

Summary.

1. The suggestion is made that the creatinephosphoric acid which can be isolated from voluntary muscles may not be identical with phosphagen. The latter may contain in addition some component of a colloidal nature.

2. Mention is made of some improvements which make the previously published method for the estimation of phosphagen more convenient and useful over a wider range. Resting frog muscles which contain 12–18 mg. of inorganic phosphorus and 55–70 mg. of phosphagen

phosphorus per 100 g. of muscle may be conveniently analysed by this improved technique.

3. A method is described for the separation and separate estimation of the inorganic (ortho)phosphate and phosphagen in 0·5 to 1 g. of muscle, based upon the different solubilities of the barium salts of orthophosphoric acid and creatinephosphoric acid. The method is primarily intended for use where the phosphagen content of the tissue is very low in comparison with the inorganic phosphate content; by it one can detect with certainty one part of phosphagen phosphorus in 200,000 parts of tissue, irrespective of the inorganic phosphate content. The use of the method need not, however, be restricted to such cases.

4. An easy extension of the technique permits of the additional estimation of pyrophosphate and of two distinct fractions of phosphoric esters, with soluble and insoluble barium salts respectively.

5. Analyses of resting voluntary muscles by the above method give the distribution of acid-soluble phosphorus as follows: orthophosphate 15 p.c., pyrophosphate 20 p.c., phosphagen 50 p.c., acid-stable phosphoric esters with soluble barium salts ("soluble" esters) 4 p.c., with insoluble barium salts ("insoluble" esters) 11 p.c.

6. Fatigue increases the orthophosphate content and the amount of "soluble" esters, diminishing the phosphagen content, but leaving unchanged the pyrophosphate content and the content of "insoluble" esters.

7. The lower end of the sartorius muscle of the frog, which contains no nerves or motor end plates does not differ from the remainder of the muscle in its analysis by the above method.

8. Prolonged oxygen lack produces no change in frog muscles in respect of the above fractions (other than the breakdown of phosphagen and the formation of orthophosphate), until almost all the phosphagen has disappeared. At this stage pyrophosphate and the "insoluble" esters begin to disappear, and after 50 to 60 hours of anærobiosis the muscle contains no phosphagen, pyrophosphate, or "insoluble" esters, though the quantity of "soluble" esters has, if anything, increased and amounts to some 10 p.c. of the acid-soluble phosphorus.

9. Blood from four different vertebrates when analysed by the new technique was found to contain no phosphagen; phosphagen was also found to be absent from the spleen and kidney of the rabbit and cat. The uterus of the rabbit and guinea-pig, and the stomach of the rabbit and frog were found to contain labile phosphorus in amount varying between a trace and 5 mg. per 100 g. The testes of the rabbit, guinea-pig,

cat and rat contain labile phosphorus, but it is uncertain whether this represents phosphagen and whether it is derived from the supporting tissue of the testis or from the spermatozoa.

10. It is suggested that there may be a close relationship between the amount of pyrophosphate in a voluntary muscle and the amount of orthophosphoric esters which have insoluble barium salts.

REFERENCES.

1. Meyerhof and Lohmann. Biochem. Zeit. 196. p. 49. 1928.
2. A. V. Hill. Proc. Roy. Soc. B, 103. p. 163. 1928.
3. Eggleton and Eggleton. Biochem. Journ. 21. p. 190. 1927.
4. Eggleton and Eggleton. Chem. and Ind. 46. p. 485. 1925.
5. Dulière and Horton. This Journ. 67. p. 152. 1929.
6. Sacks and Davenport. Journ. Biol. Chem. 79. p. 493. 1928.
7. Stella. This Journ. 66. p. 19. 1928.
8. Irving and Wells. Journ. Biol. Chem. 77. p. 97. 1928.
9. Eggleton and Eggleton. This Journ. 63. p. 155. 1927.
10. Eggleton and Eggleton. Ibid. 65. p. 15. 1928.
11. Nachmannsohn. Biochem Zeit. 208. p. 237. 1929.
12. Fiske and Subbarow. Journ. Biol. Chem. 81. p. 629. 1929.
13. Ferdmann and Feinschmidt. Zeit. Physiol. Chem. 178. p. 173. 1928.
14. Lohmann. Biochem. Zeit. 203. p. 172. 1928.