CI. THE CONDITION OF CREATINE IN AMPHIBIAN VOLUNTARY MUSCLE.

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It was shown by Eggleton and Eggleton [1928] in connection with a study of the "labile phosphorus" (phosphagen) of muscle that certain conditions leading to a disappearance of labile phosphate, with or without the appearance of free orthophosphate, gave rise also to the appearance of "free creatine," *i.e.* creatine determined by Walpole's method [1911] which does not estimate creatinine or the creatine combined in the phosphagen complex. No quantitative comparison was made between the free and labile phosphate on the one hand and free and combined creatine on the other. The results of such a comparative study are presented in this communication.

One object of this work was to determine whether the "combined" creatine (creatine estimated by the Folin method but not by the Walpole method) accounted for or exceeded in amount the creatine associated with the labile phosphorus. Each atom of labile phosphorus is thought to be combined with one molecule of creatine. It was clearly desirable to ascertain what other forms of combined creatine, if any, existed in the muscle. Another object was to determine the amount of free creatine in a resting oxygenated muscle.

Methods.

The Folin-Jaffé reaction for the estimation of creatine involves the conversion of the creatine to creatinine by boiling in acid, a process which hydrolyses creatinephosphoric acid. The method is therefore only of use to determine the total creatine of the muscle. Fresh muscle fortunately contains practically no creatinine. The reaction utilised by Walpole for the direct estimation of creatine in urine consists in the condensation of creatine with diacetyl in sodium carbonate solution to give a coloured product. Creatinephosphoric acid (the "labile phosphorus" of muscle) was shown by Eggleton and Eggleton not to give this reaction. This statement requires some modification, as will be shown in this paper.

The technique used by Walpole consisted in mixing 2 cc. urine, 2 cc. saturated sodium carbonate solution and three drops of a 1 % solution of diacetyl. The mixture gives on boiling a pink colour proportional in intensity

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to the amount of creatine present. Unfortunately it gives also a yellow colour, independently of the presence of creatine, which results in a variation of tint as well as of intensity with variation of the creatine content. An attempt was made to remove this disadvantage by introducing a phenyl group into the diketone molecule[1]. The resultant compound, methylphenyldiketone, which gave a violet condensation product with creatine, had no less tendency to the formation of this yellow pigment. Diphenyldiketone did not react at all. No further attempts at improvement along these lines were made, and diacetyl was used in the studies recorded here.

A more serious weakness of Walpole's technique, as applied to muscle analysis, is that the boiling of the alkaline solution results in a slight breakdown of creatinephosphoric acid, and the consequent development of a slight pink colour even in the absence of pre-existing free creatine. It was found impossible to overcome this by using less alkaline solutions such as bicarbonate and phosphate buffers, but when the colour development was allowed to take place at 28° instead of 100° very little liberation of creatine from phosphagen occurred during the 60 minutes necessary for the reaction. On account of the yellow colour of the blank it was necessary to match the unknown with a standard (prepared from pure creatine) differing by less than 10 % in its colour intensity. As finally used the method was as follows.

The trichloroacetic acid filtrate of the muscle was neutralised as rapidly as possible with *N* NaOH. A suitable quantity of the neutral extract (2–5 cc.) was added to an equal volume of saturated carbonate solution and two drops of a 1 % diacetyl solution (freshly prepared from dimethylglyoxime) were added. The mixture was incubated with a set of standards similarly prepared from pure creatine solutions for 1 hour at 28°. The creatine standards ranged from 0·2 to 1·5 mg. Finally comparison was made between the unknown and the most suitable standard. In another portion of the neutralised muscle filtrate the orthophosphate and phosphagen were determined by the Eggleton method [Eggleton and Eggleton, 1927]. Recovery of added creatine from muscle extracts by this technique was good (Table I).

Table I. *Recovery of creatine added to neutralised trichloroacetic acid extract of muscle. No phosphagen present (modified Walpole method).*

		Creatine mg.
1.	1 cc. muscle extract	0·920 0·931
	1 cc. „ +0·2 mg. creatine	1·125
	1 cc. „ +0·4 mg. „	1·325
	1 cc. „ +0·6 mg. „	1·525
	1 cc. „ +1·0 mg. „	1·925
2.	2 cc. muscle extract	0·164
	1 cc. „ +0·1 mg. creatine	0·182

[1] The synthesis of methylphenyldiketone was accomplished by condensing benzene with propionyl chloride to give propiophenone. This was converted into the nitrosoketone by means of amyl nitrite in hydrochloric acid. The oxime was finally converted into the diketone by distillation with dilute sulphuric acid.

The results obtained by the above modification of Walpole's method are not of a very high order of accuracy, particularly when large amounts of phosphagen are present, owing to slight breakdown of the latter; but they are confirmed by a more accurate technique, based on quite different principles, which has been used by Eggleton and Eggleton in studying the phosphorus compounds of muscle. In this method the trichloroacetic acid extract of the muscle is treated with two volumes of alcohol and immediately neutralised with powdered baryta. The solution is not only neutralised but saturated with baryta, which is soluble in 66 % alcohol to the extent of about $N/70$. In this condition practically all the phosphorus in the extract is precipitated whilst the creatine, other than that combined with phosphorus, remains in solution. The precipitate of barium salts, if extracted with water made faintly alkaline with baryta, yields up its content of water-soluble barium creatinephosphate, retaining the insoluble inorganic phosphate. The free creatine (in the alcohol) and the phosphagen-creatine (in the aqueous extract) may be estimated separately by the Folin method, whilst orthophosphate and phosphagen may be estimated separately by the Briggs colorimetric technique without the necessity for following colour production curves. As used in the experiments reported in this paper the exact technique was as follows.

1. The muscle was ground in ice-cold 4 % trichloroacetic acid (20 cc. per g. tissue) and centrifuged.

2. An aliquot part of the extract was treated with 2 volumes of alcohol and shaken with powdered baryta until alkaline, avoiding large excess.

3. The alcohol containing the flocculent phosphate precipitate was decanted from the small excess of baryta and centrifuged. Both baryta and precipitate were washed with 66 % alcohol which was added to the alcoholic fluid containing the creatine.

4. The alcoholic solution was treated with sulphuric acid to remove barium and "total creatinine" estimated in the filtrate by the Folin technique.

5. The phosphate precipitate was extracted three times in succession with 4, 2 and 2 cc. of a faintly alkaline 0·1 N barium acetate solution and the collected extracts, after removal of the barium as above, were used for the estimation of total creatine and phosphagen-phosphate.

6. The residue of insoluble phosphates was dissolved in 0·1 N HCl and freed from barium, and a suitable quantity was used for the estimation of free orthophosphate.

Results.

The analyses made by the first method are given in Table II. They are divided into two groups, and the experiments in each group are arranged in ascending order of orthophosphate content; *i.e.* with the more resting muscles first. In the first group the free creatine bears to the free phosphate a ratio of unity, but in the second there is an excess of free creatine. In all cases, however, there is an equivalence between the combined creatine and the

labile phosphorus such as would be expected if all the combined creatine were present as phosphagen.

The analyses made by the second method are contained in Table III. They are arranged similarly, and display the same relationships.

Table II. *The free phosphate, free creatine, phosphagen-phosphate, and combined creatine in the leg muscles of the frog (modified Walpole method).*

	Free phosphate	Free creatine	Ratio	Phosphagen-phosphate	Combined creatine	Ratio
1	5·40	6·00	0·90	16·9	13·8	1·22
2	5·65	5·65	1·00	14·0	15·3	0·91
3	6·60	6·80	0·97	19·4	18·8	1·03
4	7·95	7·75	1·03	22·4	20·3	1·10
5	9·00	10·00	0·90	11·0	13·2	8·30
6	9·10	10·00	0·91	22·7	19·7	1·15
7	10·70	12·10	0·89	20·0	18·9	1·06
8	13·10	10·80	1·21	13·5	14·7	0·92
		Mean	0·975		Mean	0·97
9	3·20	4·90	0·65	23·0	23·00	1·00
10	4·35	6·45	0·67	11·0	0·95	1·16
11	4·55	6·00	0·76	17·1	14·20	1·20
12	7·20	19·30	0·37	15·4	11·30	1·36
13	10·20	16·10	0·63	15·7	11·80	1·33
14	11·00	18·00	0·61	—	—	—
		Mean	0·62		Mean	1·2

The results are expressed as mg. atoms P and mg. mols creatine per kg. muscle.

Table III. *The free phosphate, free creatine, phosphagen-phosphate, and combined creatine in the leg muscles of the frog.*

	Free phosphate	Free creatine	Ratio	Phosphagen-phosphate	Combined creatine	Ratio
1	3·0	3·5	0·86	9·9	9·0	1·10
2	8·1	9·2	0·88	11·0	10·6	1·04
3	11·0	11·5	0·96	9·7	8·8	1·10
4	11·4	11·2	1·02	7·9	7·8	1·01
5	11·8	11·9	0·99	8·2	7·2	1·14
6	12·3	13·9	0·89	—	—	—
7	12·9	13·0	0·99	5·7	5·7	1·00
8	12·9	14·1	0·92	1·5	8·8	0·85
9	13·5	14·5	0·93	4·6	5·6	0·82
10	14·1	14·9	0·94	6·2	6·6	0·94
11	14·2	17·0	0·84	5·5	7·0	0·79
12	19·4	20·4	0·95	0	0	—
13	20·0	19·7	1·02	0·7	0·8	0·88
		Mean	0·94		Mean	0·97
14	3·7	8·1	0·46	17·20	17·80	0·97
15	5·7	11·5	0·50	4·65	5·55	0·84
16	6·8	14·8	0·46	2·85	2·55	1·12
17	7·9	14·1	0·56	7·50	8·80	0·85
18	8·8	16·0	0·55	7·90	8·25	0·96
19	12·6	24·0	0·52	8·50	8·90	0·96
20	14·0	27·0	0·52	2·70	2·80	0·96
21	14·5	29·0	0·50	—	—	—
22	14·8	24·0	0·62	12·80	14·80	0·87
23	15·5	29·5	0·53	5·80	8·40	0·69
24	18·7	29·0	0·64	5·90	5·90	1·00
		Mean	0·53		Mean	0·97

The results are given in mg. atoms P and mg. mols creatine per kg. muscle.

SUMMARY.

1. In many cases of resting muscles, the ratio of free creatine to free orthophosphate is practically unity, but exceptions to this rule are not infrequent, the ratio reaching sometimes a value of two.

2. The combined creatine and the labile phosphate occur in the ratio of unity.

3. The total creatine and the total "directly estimatable phosphate" (orthophosphate and labile phosphate) are present consequently in many cases in approximately equimolecular amounts.

4. In resting, well oxygenated muscles, the free creatine may be found to be no more than 50 mg./100 g. fresh muscle.

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