Different lots of the same dyes may vary in purity, and therefore each new lot should be evaluated in terms of solutions of pigments of known concentrations. The color standards fade slowly when exposed to light and should be made up fresh from the one half per cent. aqueous solutions every few weeks. The one half per cent. solutions should be discarded if they become turbid or if sediments appear.

Full details regarding the preparation and use of these color standards, as well as the results of the studies on relations between chloroplast pigments and growth of maize, will be published in the near future.

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SPECIAL ARTICLES

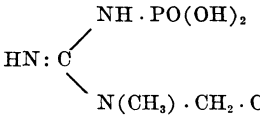
THE ISOLATION AND FUNCTION OF PHOSPHOCREATINE

I. ISOLATION

IN a previous communication[1] we have offered indirect proof of the presence in voluntary muscle of a compound containing one molecule each of creatine and phosphoric acid. The amount of this substance in muscle is considerable (0.4 to 0.5 per cent.); in fact it (and not free creatine) is the principal "extractive" as long as the muscle has not been stimulated or otherwise disturbed. During muscular contraction the compound undergoes hydrolysis, and the same change occurs outside the body under the influence of an enzyme in the muscle or of acid, whereas resynthesis takes place when fatigued muscle is permitted to recover.

At the time of our first report, the leading evidence for the existence of a creatine-phosphoric acid compound depended upon its separation from free creatine by precipitation with copper in very slightly alkaline solution. Under all the conditions enumerated above the precipitate so formed contains creatine and a peculiarly unstable form of phosphoric acid in equimolecular proportions, excepting when—in consequence of stimulation or some other cause—complete hydrolysis of the substance has occurred, and then the copper precipitate is free from both the named constituents. In view of the quantitative nature of the evidence, and of the variety of conditions under which the test has been applied, a different explanation of the results described is hardly possible, and we felt no hesitation therefore in stating that such a compound actually exists. The precise nature of the substance, however—in particular the question whether it contains anything besides creatine and phosphoric acid—can hardly be settled with certainty except by its isolation in the pure state.

Within a few days of the publication of the paper mentioned, we succeeded in isolating a barium salt in crystalline form, but the method of preparation was unsatisfactory and the yields were very poor. After many variations of the original procedure had been tried, the conclusion was finally forced upon us that the use of barium for this purpose is successful only when preceded by a series of preliminary separations (with other metals) in the course of which a large amount of material is lost. By using calcium in place of barium, however, most of the phosphocreatine in protein-free muscle filtrates can readily be separated, in a crystalline condition, from all the other organic phosphoric acid compounds present, but in order to remove these impurities without hydrolysis of the desired product it must be crystallized from alkaline solution. Under these circumstances the result is not a single substance. It contains both secondary and tertiary salts, and (partly because carbonate is present) the carbon content is too high. To obtain the pure secondary salt, having the composition required by theory, special measures must be taken, for this salt in aqueous solution is acid and therefore unstable. The final product crystallizes in spherulites, and has the composition $C_4H_8O_5N_3PCa.4H_2O$. The most probable structure of the new substance is hence the following:



and its most characteristic chemical property, *viz.*, marked instability in acid solution, is in fact characteristic also of the few other known compounds which contain the group $-NH.PO(OH)_2$. This is the first substance containing phosphorus attached to nitrogen to be isolated from natural sources, and the instability of the phosphamic group marks it as one of considerable biological importance, as will be seen in the next section. The details of preparation will be published elsewhere.

II. FUNCTION

In spite of much investigation, the function of creatine in muscle has remained as much a mystery as it was at the time of the discovery of this substance 97 years ago. Having found that most of the creatine in normal resting muscle is combined with phosphoric acid, and that the compound is destroyed during contraction at a rate which rivals that of glycogenolysis and lactic acid production, we naturally anticipated

[1] C. H. Fiske and Y. Subbarow, SCIENCE, Vol. 65 (403)—1927.

that some light might at last be thrown on this time-honored question. Among the possibilities which suggest themselves is a change in hydrogen ion concentration accompanying the hydrolysis of phosphocreatine, and either augmenting or opposing the increase in acidity associated with the formation of lactic acid. The determination of the direction and magnitude of this effect becomes therefore a matter of some consequence.

From experiments with a preparation of the crystalline calcium salt which we had in our possession several months ago it was evident that hydrolysis in slightly acid solution resulted in a very marked decrease in acidity. This was one of the preparations to which we have referred in the first section as being a mixture of two salts, containing too much carbon, consequently the presence of organic impurities which might account for the observed effect could not be excluded. Moreover, the existence of a special neutralizing mechanism has been denied on the ground that muscle acquires the same pH whether lactic acid is produced within it as a result of stimulation or whether the same amount of lactic acid is added artificially to a muscle suspension in which enzyme action has presumably been stopped.[2] For this reason, and because of the importance of the question in relation to the chain events occurring during muscular contraction, we regarded our observations as uncertain until they could be confirmed with material that was analytically pure. While engaged in collecting a fresh supply of phosphocreatine for this and other uses we found that our first method of preparation could not be relied upon, for if it fails to yield an essentially pure product on the first crystallization the substance is largely decomposed, and any that remains intact is less pure than before. The consequence is that the confirmation of our earlier results has been delayed still further through the necessity of developing an entirely new process free from this element of risk.

The second dissociation constant (k_2') of phosphocreatine, determined by the titration of a 0.005 M solution of the pure secondary calcium salt with acid, is about 2.5×10^{-5}, or roughly 250 times as great as the second constant of o-phosphoric acid at the same ionic strength. This result, which is presumably to be attributed to the "unmasking" of the carboxyl group, establishes the function of phosphocreatine in muscle—or one function, since there may be others—as that of neutralizing a considerable part of the lactic acid formed during muscular contraction.

The other dissociation constants have not been determined, but on the addition of alkali to a solution of the secondary salt no marked evidence of buffer action appears until well beyond the turning point of phenolphthalein. It follows that the third constant is much less than 10^{-7}, so in resting muscle,—which according to the most recent evidence[2] is practically neutral—phosphocreatine exists wholly as the secondary salt.

Calculations based on these facts show that the hydrolysis of phosprocreatine (taking an average figure of 0.45 per cent. for the amount in resting cat muscle) liberates sufficient base under optimum conditions (pH 6)[3] to neutralize the lactic acid formed up to a concentration of about 0.23 per cent.[4] Approximately half of this amount of lactic acid, moreover, can be neutralized at pH 7, *i.e.*, without the development of any acidity at all. Since the lactic acid maximum for complete fatigue, at least in isolated frog muscle, is 0.4–0.5 per cent. (or even more under special circumstances), it appears that this new mechanism is peculiarly designed for the neutralization of the lactic acid formed in muscular exercise of moderate intensity. Finally, it should be noted that the occurrence in contracting muscle of a reaction by which fixed base is set free necessarily detracts to some extent from the importance of protein[5] in the neutralizing process. The hydrolysis of phosphocreatine seems now to be the principal factor permitting contraction to take place to a limited extent without the appearance of fatigue, if it is true—as has been claimed[2]—that the main restriction on muscular performance is the accumulation of acid in the cells.[6]

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[2] O. Meyerhof and K. Lohmann, *Biochem. Z.*, Vol. 168 (128)—1926.

[3] *I.e.*, the maximum amount of base is released (in dilute solution) at pH 6, which is roughly the acidity of completely fatigued muscle.[2]

[4] This figure is necessarily a rough one, including as it does the supposed "physiological minimum" (0.06 per cent. lactic acid) for mammalian muscle (W. M. Fletcher, *J. Physiol.*, Vol. 47 (361)—1913; G. Embden, E. Schmitz, and P. Meincke, *Z. Physiol. Chem.*, Vol. 113 (10)—1921).

[5] O. Meyerhof, *Arch. ges. Physiol.*, Vol. 195 (22)—1922.

[6] The existence in muscle of a special device for neutralizing acid raises a number of interesting questions which can not be answered without further experimental data. For example, judging from some recent observations made by Meyerhof and Lohmann (Naturwissenschaften, Vol. 15 (670)—1927), the hydrolysis of phosphocreatine in brief periods of stimulation proceeds more rapidly than the production of lactic acid. This is difficult to reconcile with the prevailing view that contraction is a response to increased acidity, but further investigation may show that the inconsistency is only an apparent one.