I. PHOSPHOROUS COMPOUNDS IN HEART

II. ANAEROBIC METABOLISM OF MUSCLE

Manoukian

I. HIGH-ENERGY PHOSPHORUS COMPOUNDS OF MAMMALIAN HEART-PHOSPHOCREATINE

II. THE ANAEROBIC METABOLISM OF SKELETAL MUSCLE

by

Elizabeth Mamas Manoukian

"Submitted in partial fulfillment of the requirements
of the degree of Master of Science
in the Pharmacology Department of the
American University of Beirut
Beirut, Lebanon"
1962

Acknowledgment

The writer wishes to express her thanks and gratitude to Professor George Fawaz, Chairman of the Pharmacology Department of the American University of Beirut, who suggested the problem and whose constant advice and help made this work possible. Thanks also go to Mrs. E. N. Fawaz for her advice and help concerning the second part of this problem.

Abstract

This paper is divided into two parts. The first part describes the procedure for estimating phosphocreatine in mammalian hearts, whereby the highest values of phosphocreatine are reported due to the application of better methods, not only in the analysis of the extracts of cardiac muscle, but also in quick-freezing of the tissues. It also contains a modification of the original method of synthesizing phosphocreatine whereby the yield is almost doubled.

The second part of this paper is on anaerobic metabolism of rat skeletal muscle especially the inhibition of glycolysis by malonate whereby lactate formation is inhibited and 3-phosphoglycerate, α-glycerophosphate, and phosphopyruvate accumulate. Addition of phosphopyruvate to malonate-treated suspension gave rise to 3-phosphoglycerate but not to pyruvate and lactate as in the untreated suspension. The inhibition by malonate of pyruvic kinase resulted in a reversal of the glycolytic reactions which could not proceed beyond 3-phosphoglycerate owing to lack of ATP. Addition of pyruvate to malonate-blocked suspension resulted in stoicheiometric formation of lactate with increased production of 3-phosphoglycerate but no α-glycerophosphate.

TABLE OF CONTENTS

LIST OF TABLES

PART I

HIGH-ENERGY PHOSPHORUS COMPOUNDS OF MAMMALIAN HEART-PHOSPHOCREATINE

A. Introduction.

The energy utilized by muscle in performing its work is derived from the chemical reactions which take place within the muscle itself.[1]

The search for the nature of these reactions led to much experimentation. At first it was thought that in the protoplasm of the muscle there was a giant molecule called inogen which on stimulation took up oxygen, gave off carbon dioxide and contracted.[2] In 1907 Fletcher and Hopkins[3] disproved this theory by showing that with the onset of fatigue and rigor there was a production of lactic acid. Parnas and Wagner[4] in 1914 showed that this lactic acid was derived from the breakdown of muscle glycogen, and Meyerhof[5] in 1920 confirmed this fact by demonstrating that during anaerobic contraction, lactic acid formation was proportional to the work performed. So in normal anaerobic contraction the major overall supply of energy comes from glycolytic reactions.

A. V. Hill (1928)[6] in his measurements of the initial heat production in muscle, indicated that the chemical reactions underlying contraction must be non-oxidative, since the size of the initial heat, its time relations and its proportionality to the production of tension are identical when muscle is allowed to contract either under oxygen or nitrogen. Later D. K. Hill[7] proved by his experiments on frog muscle at 0° C with a short tetanus, that even if the conditions from the start are aerobic, increase of oxygen consumption starts only after the mechanical activity is over.

Contraction and Phosphocreatine.

This idea that lactic acid formation from carbohydrate is the only energy-providing reaction prevailed till 1930 when Lundsgaard[8] showed that lactic acid production could be stopped by iodoacetate, whereas muscle would continue to contract. (Iodoacetate acts by inhibiting the action of glyceraldehyde phosphate dehydrogenase). He also showed that the tension produced in this poisoned muscle is proportional to the breakdown of phosphocreatine. This substance, at first called phosphagen was discovered independently by Eggleton and Eggleton,[9] and Fiske and Subbarow.[10] Eggleton and Eggleton connected the metabolism of phosphocreatine with contraction because they found it to decrease when muscle contracted and be resynthesized during recovery. Fiske and Subbarow[10] in their classical paper on phosphocreatine elucidated its structure and described a method for obtaining it from muscle. At the same time, Meyerhof and Lohmann[11, 12] found that the hydrolysis of phosphocreatine was an exothermic reaction producing 12,000 cal. per gram molecule of inorganic phosphate liberated.[1]

In 1930 Lipmann and Meyerhof[13] observed that in the experiments on frog's sartorii, there was a change of pH of the muscle towards the alkaline side during the first few series of short tetani. This finding was correlated with those of Meyerhof and Lohmann[12] who studied the titration curves of phosphocreatine and of an equimolar mixture of creatine and inorganic phosphate. These experiments also proved the breakdown of phosphocreatine in muscle. In the light of the above experiments and those of his own with iodoacetate, Lundsgaard[8,14] suggested

that the breakdown of phosphocreatine supplied also the energy for contraction even in the unpoisoned muscle and that the role of carbohydrate breakdown was to supply the energy for the resynthesis of phosphocreatine.

The Discovery of Adenosine Triphosphate.

After the discovery of phosphocreatine there was also discovered in muscle by Lohmann[15, 16] as well as by Fiske and Subbarow[1] a substance called adenosine triphosphate (ATP). Two significant observations were made on this compound by Lohmann.[15] One is that ATP acts as a coenzyme of glycolysis, although the details of this action was not found until later, and second that the hydrolysis of the two phosphate groups liberated 12,000 cal. per gram molecule of phosphate. The significance of ATP hydrolysis for the contraction came later through the work of Lohmann[17] on dialyzed cell-free muscle extracts. These extracts did not cause the hydrolysis of phosphocreatine unless adenosine diphosphate was present. He explained this fact according to the following reaction

$$\text{Phosphocreatine} + \text{ADP} \longrightarrow \text{Creatine} + \text{ATP}$$

$$\text{ATP} \longrightarrow \text{ADP} + H_3PO_4$$

This discovery of Lohmann led to consequences of great importance, because he suggested that before phosphocreatine is hydrolyzed to yield energy, ATP must dephosphorylate, and furthermore, these reactions were the first observation made on phosphate transfer between two compounds containing an energy rich phosphate bond.[18]

Lundsgaard[19] had found that for every molecule of lactic acid formed during the anaerobic breakdown of glycogen, about two molecules of phosphocreatine were resynthesized. This resynthesis occurs first through

the formation of ATP which later transfers its phosphate to creatine. One molecule of phosphocreatine comes from an exothermic reaction taking place in glycolysis and this is the oxidoreduction between glyceraldehyde phosphate and pyruvate giving as an end product phosphoglyceric and lactic acids. It is now known that if adenylic acid is added to this mixture, one molecule of ATP is synthesized for every molecule of lactic acid formed.[20] In this coupled esterification 1,3-diphosphoglyceric acid is formed and acts as a phosphate donor to ADP.[21] This involves reaction of the aldehyde group of glyceraldehyde phosphate with SH group of glyceraldehyde phosphate dehydrogenase which results to the formation of an "energy-rich" bond. This is followed by the phosphorolysis of the enzyme-acyl compound in presence of inorganic phosphate forming diphosphoglyceric acid which transfers its acyl group to ADP.

1,3 diphosphoglyceric acid + ADP $\longrightarrow$ 3-phosphoglyceric acid + ATP

The second molecule of phosphocreatine comes also via ATP, through the dephosphorylation of phosphoenol pyruvic acid.[22]

Phosphoenol pyruvate + ADP $\rightleftharpoons$ pyruvate + ATP

In the presence of creatine and catalytic amount of ADP, phosphocreatine is formed, but there is no direct evidence of the formation of phosphocreatine directly. This reaction together with the above reaction explain the findings of Lundsgaard.

In all the above reactions the synthesis of phosphocreatine was secondary to the formation of ATP. But in 1956 Cori et al.[23] working with skeletal muscle described a direct path for the formation of phosphocreatine and back form 1:3 diphosphoglyceric acid that did not need ATP.

1:3 diphosphoglyceric acid + creatine $\rightleftharpoons$ phosphocreatine + 3-phosphoglyceric acid.

This finding contradicted those of Lohmann because phosphocreatine could break down while reacting with 3-phosphoglyceric acid and this breakdown does not need the presence of ADP.

The synthesis of phosphocreatine was described for the first time in 1938 by Zeile and Fawaz[24] who obtained a crystalline calcium salt. Ennor and Stocken,[25] and Peanasky, Kuby and Lardy[26] modified the original procedure (without improving the yield) in order to prepare the sodium salt and thus avoid traces of calcium that might remain after converting the calcium salt into the sodium salt by sodium oxalate. At present it is possible to prepare a pure sodium or potassium salt simply by shaking the calcium salt with the sodium or potassium form of Dowex 50 resins.

This paper describes the procedure for estimating phosphocreatine in mammalian hearts, whereby the highest values for phosphocreatine are reported. It also contains a modification of the original method of Zeile and Fawaz for synthesizing phosphocreatine whereby the yield is almost doubled. It is now possible to obtain 4-5 grams of crystalline phosphocreatine calcium salt from 10 gms. of creatine on a laboratory scale in about 48 hours making this compound more available for experimentation. (Present price for 1 gm. of sodium salt of phosphocreatine is sold by Light and Co. for $250.00).

B. The Steady-State Level of Phosphocreatine in the Heart

In 1953 a figure of 20 mg% phosphorus (P) was reported as the steady-state value for phosphocreatine (PC) in the left ventricle of the dog.[27]

Attention was also called to the precautions that should be taken in preparing the tissue for analysis, among other things, that it should be finely powdered in the frozen state before treatment with perchloric or trichloroacetic acid. An explanation was offered for the low values (4-8 mgs% P) for dog hearts reported in the literature theretofore.[28-30] Later it was shown[31] that a few minutes of hypoxia were sufficient to almost deplete the heart of its PC. An increase in the oxygen consumption of the dog heart-lung preparation of up to 76% for instance by a toxic dose of ouabain[32] or up to 200% by epinephrine or norepinephrine[33] does not reduce the PC of the heart provided no hypoxia develops as can be judged by the oxygen content of coronary venous blood.

Low steady-state values for PC continue to be reported in the literature. Most recently[34] a figure of 4.65 mg% P was reported for rat heart. Here the thorax was opened and the heart removed, trimmed, blotted free of blood and frozen. In another paper[35] where a value of 5.3 mg% PC-P is given, the rat ventricle was removed while still beating, frozen, crushed and homogenized with trichloroacetic acid. In an investigation[36] designed to study the effect of anoxia on the phosphorus compounds of the dog heart the control hearts contained 3.8 mg% PC-P. In this case, three minutes were allowed to elapse between opening the chest and homogenization of heart tissue in trichloroacetic acid. In another article[37] where a figure of 9.1 mg% PC-P is reported for dog left ventricle, the whole heart was plunged in a freezing mixture, and the frozen specimen was not powdered before deproteinization. No mention is made of the use of artificial respiration in any of these four investigations.

At the other extreme, a figure of 39.7 mg% P is reported as the "real" PC content of dog left ventricle.[38] Here the heart was frozen in situ by means of two aluminum blocks pre-cooled in liquid air. This procedure is expected to effect a quicker freezing of the tissue than dipping in liquid air. In the experiments to be described in this paper the PC content of dog, rat and rabbit hearts was determined using this novel procedure of quick freezing in situ.

Methods

Intraperitoneal pentobarbital anesthesia 40 mg/kg was used in all experiments. The chest was opened under artificial respiration, and the heart tissue—left ventricle in the case of dogs and whole hearts in rats and rabbits—was gripped between two pre-cooled aluminum blocks, which formed the head of a pair of pliers. (A picture of these pliers is given by Wollenberger et al.[39]). Freezing of the tissue is complete in a fraction of a second. Samples from the atria and the right ventricle of the dog were not frozen by the metal block technique. After the sample from the left ventricle was frozen, the right and left atria were cut and blotted free of blood and dipped in liquid air. After that, a piece of the right ventricle was cut with a razor and frozen in liquid air. The frozen tissue was, in all cases, prepared for analysis of the phosphorus compounds as described previously[27] except that the time chosen for hydrolising the labile nucleotide-P was seven minutes instead of ten. In some experiments PC and ATP were also determined enzymatically.[40] The values obtained by both methods were in agreement.

Results

The experimental results are summarized in Table 1. It can be seen that quick freezing with pre-cooled metal blocks, as compared with plunging the tissue in liquid air, results in a slightly higher PC value for dog left ventricle and rabbit heart but a relatively much higher value for rat heart. The results of analysis of samples from the atria and right ventricle of the dog are included in order to present the sum of PC and inorganic P (Pi) and the labile nucleotide-P, which is not sensitive to such short periods of anoxia. It can be seen that the right ventricle is probably not different from the left, for although the PC value is lower owing to hypoxia, yet the sum of PC and Pi is the same in both. The labile nucleotide-P is slightly less in the right ventricle as is the total acid-soluble P—about 96% of that of the left ventricle. The right and left atria, on the other hand, contain less total acid-soluble P—about 60% of that of the ventricles—and almost the same ratio applies for the labile nucleotide-P and the sum of PC + Pi.

Discussion

The use of cooled metal blocks attached to anatomical forceps for freezing tissues was introduced in 1954 by Ernkö,[41] who stressed the advantages of this method over freezing by plunging in liquid air. This procedure was used in Bücher's laboratory,[42] and by Wollenberger et al.[43] The latter authors used pliers instead of forceps, a modification that is of advantage especially for handling tough and large organs such as a dog

heart. In the present experiments, pliers kindly supplied by Professor Wollenberger were used for dog hearts. Smaller pliers were made for use with smaller animals.

In this investigation the method of freezing used by Wollenberger et al.[38] was followed. However, values for the steady-state level of PC in the left ventricle, although somewhat higher than those reported by Fawaz and Hawa[27] are much lower than those reported by Wollenberger et al. (24 mg% P vs. 40). We are not in a position to explain this discrepancy. However, Wollenberger et al. use their own method for estimating Pi and PC, which involves cooling below 0° C and extraction of Pi as dodecamolybdate-phosphate with organic solvents. We have used the method of Fiske and Subbarow[10] which had recently been checked against an enzymatic method developed in this laboratory[40] and found satisfactory. There is no need to go below 0° C to avoid the splitting of PC for there is no measurable breakdown of PC after 10 minutes at 0° C in perchloric or trichloroacetic acid. In fact, Furchgott and De Gubareff[44] find no significant splitting of PC in perchloric acid even after one hour at 0° C. It should be emphasized, however, that the present results do not exclude the possibility of the presence of a phosphorus compound more labile than PC (see Seraydarian et al.[45]). The results merely indicate that we have accounted for all the PC found in our frozen specimens.

Michal and Lamprecht,[46] offer an explanation for the high PC values reported by Wollenberger et al. They compared the method of the latter authors with that of Fiske and Subbarow and found that according to the method of Wollenberger et al. the amount of Pi does not increase

measurably after 30 min. of heart anoxia, —a finding contrary to all experience, —whereas it doubles if the method of Fiske and Subbarow is employed. Michal and Lamprecht's explanation for this discrepancy is that the Pi is not completely extracted by the method of Wollenberger et al. and since PC is determined as the difference between the value of Pi and that for the sum of PC and Pi, a high apparent PC value is obtained. On the other hand, Michal and Lamprecht, who use the metal block technique of freezing, and powder the tissue before fixation, still get low PC values —11.3 mg% P —for dog heart. They do not state whether or not artificial respiration was used.

The low phosphorus values in the atria of dogs are in harmony with the observations of Mulder et al.[37] As they point out, this does not indicate that atrial muscle cells necessarily contain less high-energy P-compounds. Histological examination reveals considerable amounts of connective and adipose tissue in the atria. More refined methods are needed to settle this point.

As can be seen from Table 1, the PC value for rabbits is also slightly higher with the new freezing procedure than with the old. The value for the rat heart, on the other hand, is considerably higher with the new procedure. We had previously attempted to freeze the rat heart <u>in situ</u> by pouring liquid air into the thoracic cavity under artificial respiration but failed to obtain values higher than 9-10 mg% P. This finding should be taken into consideration by investigators who freeze the rat heart by dipping the whole animal in liquid air. Why this freezing procedure should make so much more difference for the rat than for the dog and rabbit

remains to be investigated. Sambhi and White[47] report a heart rate of 300 for the rat under ether anesthesia, and according to a communication from Dr. White, the rat heart under anesthesia is extremely sensitive to hypoxia as can be judged by EKG changes.

Another method for the determination of PC which has found acceptance by some workers is that of Ennor and Rosenberg[48] which is based on the estimation of the so-called bound creatine. The most striking thing about this method is the extreme scattering in the PC values obtained by its use. Ennor and Rosenberg[48] report PC values of 0-2.1 mg% P for rabbit heart, 0.7-5.6 for cat heart and 6.6 for one dog heart. It is clear that such values are among the lowest recorded in the literature, although the authors used artificial respiration and took precautions to avoid hypoxia of the tissues.

Summary

Freezing the heart *in situ* by means of metal blocks pre-cooled in liquid air, compared with excision of the tissue and plunging it in liquid air, yields slightly higher values for phosphocreatine in dog (24.2 mg% P vs. 19.9) and rabbit (16.8 vs. 14.2) hearts but considerably higher values in the rat heart (17.3 vs. 9). A discussion and evaluation of some of the techniques and methods employed in the estimation of phosphocreatine are given.

Table 1. The Effect of Freezing Technique on the High-Energy Phosphate Content of Cardiac Tissue.

Species	No. of Animals	Method of Freezing	Phosphocreatine-P ± S.E.	Inorganic-P ± S.E.	Labile Nucleotide-P ± S.E.
Rat	6*	Excision	9.0 ± 1.0	23.6 ± 0.33	29.9 ± 1.0
"	11	In Situ	17.3 ± 1.2	16.2 ± 1.1	28.6 ± 0.74
Rabbit	5*	Excision	14.2 ± 1.7	19.3 ± 1.2	33.9 ± 1.2
"	10	In Situ	16.8 ± 0.65	12.7 ± 0.9	28.8 ± 1.0
Dog left ventricle	18*	Excision	19.9 ± 0.84	20.1 ± 0.72	39.5 ± 0.98
" " "	25	In Situ	24.2 ± 1.0	15.0 ± 0.8	34.6 ± 0.8
Dog right ventricle	17	Excision	12.0 ± 1.4**	26.5 ± 1.1**	30.5 ± 1.0
" " atrium	18	Excision	7.1 ± 0.4**	16.0 ± 0.6**	18.0 ± 0.6
Dog left atrium	18	Excision	8.8 ± 0.6**	17.8 ± 0.7**	19.1 ± 0.84

* Data taken from Fawaz and Hawa[27]

** Figures for PC are low and those for Pi are high due to hypoxia

"Excision" means sample was cut with the razor and dipped in liquid air. "In situ" means sample was frozen between metal blocks pre-cooled in liquid air. All values are expressed as mg phosphorus per 100 g fresh tissue.

C. Preparation of Phosphocreatine

10 gm creatine (Merck) are shaken in a 500 cc Erlenmeyer flask with 24 cc of 17 N NaOH (50% by weight) until almost complete solution. Add 40 cc of H_2O. This results in a clear solution. Cool thoroughly by immersing in ice water. Add from a burette 4 cc of pure $POCl_3$ at once and continue swirling the flask which is kept immersed in the ice-water till the end of the experiment. The temperature rises after the $POCl_3$ addition, but it should not go above 40° C. It is important to wait before the next addition of $POCl_3$ until the reaction mixture is again cold. The best yields are obtained between temperatures of 10°-40° C.

Na_3PO_4 usually precipitates shortly after the addition of the first portion of $POCl_3$. This does not interfere with the reaction. Just enough water should be added to the mixture, that it does not get too viscous. Now add 15 cc of 17 N NaOH followed by 4 cc $POCl_3$ and the mixture is stirred with a glass rod while the flask is swirled in ice-water. This procedure is repeated three more times, water being added from time to time. In all, 84 cc 17 N NaOH and 20 cc $POCl_3$ are added, with the NaOH being added before the $POCl_3$ in each case. The total volume of the mixture at the end of the reaction should not exceed 170 cc or else the yield would decrease. The phosphorylation takes not more than 20-30 minutes and it should be performed under a well-ventilated hood. Keep the reaction mixture in ice-water for another 15 minutes. Now filter with suction in the cold (funnel and filter plate) and rinse the pressed precipitate of Na_3PO_4 with just enough ice-cold water to displace all mother liquor. Discard the precipitate after making sure that all the mother liquor has

been displaced i.e. that analysis of the precipitate shows no significant amounts of phosphocreatine.

The mother liquor is now neutralized in the cold with concentrated HCl to bring the pH to 8.5 (phenolphthalein). The volume of the solution is measured and a PC determination is performed. The yield should be at least 25%. If less, it does not pay to proceed further and one should investigate the reasons for the low yield, such as inefficient stirring and improper cooling during the phosphorylation.

The inorganic phosphate is precipitated by the addition of 10% $CaCl_2$ under continuous stirring. Precipitation of $Ca_3(PO_4)_2$ is accompanied by acidification, hence NaOH is constantly added to keep the mixture just alkaline to phenolphthalein. If too much alkali is added $Ca(OH)_2$ precipitates and this adsorbs a considerable part of the phosphocreatine. The end of the precipitation is recognized when the phenolphthalein is not decolorized upon further addition of $CaCl_2$. However, to make sure, filter a portion of the fluid and test for inorganic phosphate. If after addition of Molybdate I and reducer no blue color develops in a minute it means inorganic phosphate is absent. The blue color due to PC develops slowly and reaches its maximum in about 15-20 minutes depending on the temperature.

Centrifuge. Keep supernatant in a well stoppered flask in the ice-box. The voluminous calcium phosphate precipitate is treated with an equal volume of water, stirred and vigorously shaken in the same centrifuge bottle and centrifuged. This process of washing is repeated until the last washings contain insignificant amounts of PC. (PC is slightly adsorbed on calcium phosphate).

The clear supernatant is now measured and cooled to 0° C. Add 20 gm $CaCl_2$ dissolved in the minimum amount of water. Add 3 volumes of cold 95% alcohol slowly and under constant stirring and cooling. During the precipitation, 10% NaOH should be added to keep the suspension just alkaline to phenolphthalein.

Keep in the ice-box overnight. Siphon off the clear supernatant, which contains very little PC (less than 10%). The residue which consists of PC Ca-salt and NaCl, is centrifuged. NaCl can be removed as follows and without loss of PC: Add to the residue in the centrifuge bottle an equal volume of water. Stir with a glass rod and you can feel that the NaCl goes into solution. Now add with stirring 3 times as much neutralized 95% alcohol as the volume of water added, and centrifuge. Repeat this procedure until the residue is free of NaCl. This can be tested by immersing a glass-rod into the residue, where the NaCl settles below the PC.

Now extract the residue by shaking with small portions of water at room temperature for about 10 minutes. This extraction is repeated four or five times until the last extracts contain insignificant amounts of PC. The volume of the combined extracts is around 500-700 cc. Filter, and evacuate, using the ice desiccator, to a volume of about 40-50 cc or until the solution just starts to crystallize. Keep in the ice-box for several hours for complete crystallization.

The precipitate is collected by centrifugation washed with 50% alcohol about 4 times and dried in a vacuum desiccator over $CaCl_2$. Yield 4-4.5 gm of analytically pure Ca-salt.$4H_2O$ containing practically no inorganic PO_4. The aqueous mother liquor after the evacuation may be precipitated with 1 volume of alcohol and a further 1-1.5 gm of a less pure Ca salt is obtained. However, this can be further purified as above.

REFERENCES

1. Needham, D. M., "Biochemistry of Muscular Action," The Structure and Function of Muscle, ed. G. H. Bourne (New York: Academy Press, 1960), II, Ch. II, 55.

2. Davies, R. E., Cain, D., and Delluva A. M., The Energy Supply for Muscle Contraction. Ann. N. Y. Acad. Sci. 81: 468, 1959.

3. Fletcher, W. M., and Hopkins, F. G., Lactic Acid in Amphibian Muscle. J. Physiol. 35: 247, 1906.

4. Parnas, J. and Wagner, R., Über den Kohlenhydratumsatz isolierter Amphibien-muskelin und über die Beziehungen zwischen Kohlenhydratschwund und Milchsäurebildung im Muskel. Biochem. Z. 61: 387, 1914.

5. Meyerhof, O., Die Energieumwandlungen in Muskel. I. Über die Beziehungen der Milchsäure zur Wärmebildung und Arbeitsleistung des Muskels in der Anaerobiose. Arch. ges. Physiol. Pflüger's 182: 232, 1920.

6. Hill, A. V., The Recovery Heat-Production in Oxygen After a Series of Muscle Twitches. Proc. Roy. Soc. London, B103: 183, 1928.

7. Hill, D. K., The Time Course of Oxygen Consumption of Stimulated Frog's Muscle. J. Physiol. 98: 207, 1940.

8. Lundsgaard, E., Untersuchungen über Muskelontraktionen ohne Milchsäurebildung. Biochem. Z. 217: 162, 1930.

9. Eggleton, P., and Eggleton G. P., The Physiological Significance of "Phosphagen." J. Physiol. 63: 155, 1927.

10. Fiske, C. H., and Subbarow, Y., Phosphocreatine. J. Biol. Chem. 81: 629, 1929.

11. Meyerhof, O., and Lohmann, K., Über die natürlichen Guanidinophosphorsäuren (Phosphagene) in der quergestreiften Muskulatur. Mitteilung I, Biochem. Z. 196: 22, 1928.

12. _____, and _____, Über die natürlichen Guanidinophosphorsäuren (Phosphagene) in der quergestreiften Muskulatur. Mitteilung II, Biochem. Z. 196: 49, 1928.

13. Lipmann, F., and Meyerhof, O., Über die Reaktionsänderung des tätigen Muskels. Biochem. Z. 227: 84, 1930.

14. Lundsgaard, E., Weitere Untersuchungen über Muskelkontraktionen ohne Milchsäurebildung. Biochem. Z. 227: 51, 1930.

15. Lohmann, K., Zuschriften. Naturwissenschaften 17: 624, 1929.

16. _____, Darstellung der Adenylpyrophosphorsäure aus Muskulatur. Biochem. Z. 233: 460, 1931.

17. _____, Über die enzymatische Aufspaltung der Kreatinphosphorsäure; zugleich ein Beitrag zum Chemismus der Muskelkontraktion. Biochem. Z. 271: 264, 1933.
1934

18. Lipmann, F., "Metabolic Generation and Utilization of Phosphate Bond Energy," Advances in Enzymology and Related Subjects, ed. F. F. Nord, and C. H. Werkman, (New York: Interscience Publishers, Inc., 1941), I, 99.

19. Lundsgaard, E., Über die Energetik der anaeroben Muskelkontraktion. Biochem. Z. 233: 323, 1931.

20. Meyerhof, O., and Kiessling, W., Die Umesterungsreaktion der Phosphobrenztraubensäure bei der alkoholischen Zuckergärung. Biochem. Z. 281: 249, 1935.

21. Racker, E., and Krimsky, I., The Mechanism of Oxidation of Aldehydes by Glyceraldehyde-3-Phosphate Dehydrogenase. J. Biol. Chem. 198: 731, 1952.

22. Needham, D. M., and van Heyningen, W. E., The Linkage of Chemical Changes in Muscle Extract. Biochem. J. 29: 2040, 1935.

23. Cori, O., Abarca, F., Frankel, R., and Traverso-Cori, A., Synthesis of Phosphocreatine by Enzymes from Heart and Skeletal Muscle, Nature 178: 1231, 1956.

24. Zeile, K., and Fawaz, G., Synthese der natürlichen Kreatinphosphorsäure. Z. Physiol. Chem. 256: 193, 1938.

25. Ennor, A. H., and Stocken, L. A., The Preparation of Sodium Phosphocreatine. Biochem. J. 43: 190, 1948.

26. Lardy, H. A., "Crystalline Sodium Phosphocreatine," Manometric Techniques by Umbreit, W. W., Burris, R. H., and Strauffer, J. F., (Minn. Burgess Publishing Co. 1957) Ch. 17, 288.

27. Fawaz, G., and Hawa, E. S., Phosphocreatine Content of Mammalian Cardiac Muscle. Proc. Soc. Exper. Biol. & Med. 84: 277, 1953.

28. Pollack, H., Flock, E., and Bollmann, J. L., Compounds of Phosphorus in the Heart and Striated Muscles of the Dog. Am. J. Physiol. 110: 105, 1934.

29. Cruickshank, E. W. H., McClure, G. S., and Machintosh, D. C., Cardiac Metabolism. Physiol. Rev. 16: 623, 1936.

30. Wollenberger, A., Energy-Rich Phosphate Supply of the failing Heart. Am. J. Physiol. 150: 733, 1947.

31. Fawaz, G., Hawa, E. S., and Tutunji, B., The Effect of Dinitrophenol, Hypoxaemia and Ischaemia on the Phosphorus Compounds of the Dog Heart. Brit. J. Pharmacol. 12: 270, 1957.

32. _____, and Tutunji, B., Ouabain-induced Ventricular Tachycardia and its Effect on the Performance and Metabolism of the Dog Heart. Brit. J. Pharmacol. 14: 355, 1959.

33. _____, and _____, The Effects of Adrenaline and Noradrenaline on the Metabolism and Performance of the Isolated Dog Heart. Brit. J. Pharmacol. 15: 389, 1960.

34. Gangloff, E. C., Hemmings, I. L., and Krause, R. F., Creatine, Creatinine and Creatine Phosphate in Normal and Hypertrophied Rat Hearts. Am. J. Physiol. 201: 363, 1961.

35. Minton, P. R., Zoll, P. M. and Norman, L. R., Levels of Phosphate Compounds in Experimental Cardiac Hypertrophy. Circulation Research 8: 924, 1960.

36. Michal, G., Naegle, S., Danforth, W. H., Ballard, F. B., and Bing, R. J., Metabolic Changes in Heart Muscle During Anoxia. Am. J. Physiol. 197: 1147, 1959.

37. Mulder, A. G., Omachi, A., and Rebard, B. T., Content of Inorganic and High Energy Phosphates, Potassium, Sodium, Lactate and Glycogen in Different Areas of the Dog Heart. Am. J. Physiol. 186: 309, 1956.

38. Wollenberger, E., Krause, G., and Wahler, B. E., Über den tatsächlichen Orthophosphat - und N-Phosphoryl-kreatingehalt des Säugetierherzens. Archiv. ges. Physiol. 270: 413, 1960.

39. Wollenberger, A., Ristau, O., and Schoffa, G., Eine einfache Technik der extrem schnellen Abkühlung grösserer Gewebestücke. Archiv. ges. Physiol. 270: 399, 1960.

40. Fawaz, E. N., Fawaz, G. and v. Dahl, K., The Enzymatic Estimation of Phosphocreatine. Proc. Soc. Exper. Biol. & Med. 109: 38, 1962.

41. Eränkö, O., Quenching of Tissues for Freeze-drying. Acta Anatomica 22: 331, 1954.

42. Bücher, T. and Klingenberg, M., Wege des Wasserstoffs in der lebendigen Organisation. Angew. Chemie. 70: 552, 1958.

43. Wollenberger, A., Krause, E. G., Wahler, B. E., Orthophosphate und Phosphokreatingehalt des Herzmuskels. Naturwiss. 45: 294, 1958.

44. Furchgott, R. F., and DeGubareff, T., The High Energy Phosphate Content of Cardiac Muscle under Various Experimental Conditions Which Alter Contractile Strength. J. Pharmacol. & Exper. Therap. 124: 203, 1958.

45. Seraydarian, K., Mommaerts, W. F. H. M., Wallner, A., and Guillory, R. J., An Estimation of the true Inorganic Phosphate Content of Frog Sartorius Muscle. J. Biol. Chem. 236: 2071, 1961.

46. Michal, G., and Lamprecht, W., Methoden zur Bestimmung von Metaboliten des Kohlenhydratstoffwechsels im Herzmuskel. Z. Physiol. Chem. 324: 170, 1961.

47. Sambhi, M. P. and White, F. N., The Electrocardiogram of the Normal and Hypertensive Rat, Circulation Research 8: 129, 1960.

48. Ennor, A. H., and Rosenberg, H., The Determination and Distribution of Phosphocreatine in Animal Tissues. Biochem. J. 51: 606, 1952.

PART II

THE ANAEROBIC METABOLISM OF SKELETAL MUSCLE

A. Introduction

Muscle metabolizes carbohydrate in 2 phases: the anaerobic phase which involves the breakdown of carbohydrate to small fragments like lactic or pyruvic acid and for which no oxygen is needed, and the aerobic phase during which pyruvic acid is oxidized to carbon dioxide and water. The first phase is called glycolysis or the Embden-Meyerhof pathway and the second the citric acid or Krebs cycle, the name cycle indicating the cyclic nature of this pathway. Both phases require a multiple of enzymatic reactions. One of the reactions of the citric acid cycle is inhibited by malonate and this inhibition was first described by Krebs and Eggleston.[1] A great deal of work has been done since then on this subject and it is now known that malonate inhibits competitively succinic dehydrogenase. In this paper it is shown that the classical inhibitor of Krebs cycle also inhibits glycolysis. This was found accidentally in this laboratory during the studies of phosphocreatine splitting in rat skeletal muscle. Kuby, Noda and Lardy[2] crystallized creatine phosphokinase, the enzyme that catalizes the following reaction: ATP + creatine $\rightleftharpoons$ ADP + phosphocreatine. They also observed that malonate inhibits this enzyme.[3] We have utilized their findings and applied malonate to a muscle suspension that actively breaks down glycogen to lactic acid, a suspension that is very rich in ATP-ase thus causing a rapid breakdown of phosphocreatine. Malonate in 0.06 M concentration inhibited lactic acid formation but had little affect on

phosphocreatine breakdown. The inhibition of lactic acid formation was accompanied by accumulation of some acid-resistant phosphorous compound. It was assumed that this compound must be an intermediate of the glycolytic pathway and that it accumulates due to malonate inhibition of one of the enzymatic steps of glycolysis. In this study the exact location of the malonate block was established and the phosphorus acid-resistant fraction was identified.

B. Methods and Materials

Preparation of Muscle Suspensions and Extracts

Adult non-starved albino rats were anaesthetized with pentobarbitone (40 mg/kg intraperitoneally). The thigh muscles were removed and immediately immersed in a mixture of dry ice and ether (-60°). The frozen muscle was quickly blotted with filter paper to remove the ether and weighed without delay in a closed weighing bottle. It was then ground in a mortar to a fine powder with frequent additions of dry ice to prevent thawing. The powder was quantitatively transferred by means of dry ice-cooled spatula and wide-necked funnel to a flask containing an amount of perchloric acid or incubation medium equal to 9.2 times the volume of the tissue. This procedure is used routinely in this laboratory (Fawaz and Hawa 1953)[4] to estimate the steady-state levels of phosphorus compounds in tissues. Another variation which proved to be equally satisfactory is to omit the weighing of the tissue before powdering. Samples of the powder were transferred to pre-weighed flasks containing the medium and their weight determined by difference. The latter procedure is advantageous in that

the same powder can be used for both control and malonate suspensions, as well as the determination of the steady-state levels.

The incubation medium consisted of 0.1 M glycine buffer, pH 8.5, in the preliminary experiments but was later replaced by 0.0025 N KOH. Malonate was dissolved in the medium before the tissue powder was added. The pH of the medium at the end of the incubation period was 7.05 for the malonate experiments and 6.65 for the controls. The corresponding pH figures when glycine buffer was used were 7.6 and 7.3. The accumulation of lactic acid and precursors occurred at the same rate in the two media.

The suspension was deproteinized with one-tenth volume of 6.6 N perchloric acid to give a final tissue dilution of 1:11. Malonic acid was removed by subjecting the perchloric acid filtrate to exhaustive ether extraction with a Kutscher - Steudel apparatus, but this procedure was found later to be unnecessary. The perchloric acid extract was neutralized with KOH solution, cooled in ice-water and centrifuged.

Analytical Methods

Lactate was estimated by the method of Barker and Summerson,[5] phosphocreatine and inorganic phosphate were determined by the method of Fiske and Subbarow.[6] In preliminary experiments (Table 1), acid hydrolysis (1 N HCl at 100°) for varying periods was used to identify some of the phosphate esters. For instance, the 7-minute value (H7) minus the sum of phosphocreatine and inorganic phosphate is taken, at least in fresh muscle, as a measure of the "labile" nucleotide phosphorus or 2/3 of the ATP phosphorus. In all other experiments enzymatic methods were used for the estimation of the intermediates of glycolysis. These are based upon the

DPN $\rightleftharpoons$ DPNH or TPN $\rightleftharpoons$ TPNH reaction and the corresponding change in optical density at 340 mμ first observed by Warburg. Most of these methods have been described in the literature.[7-9] We have followed directions given to us by the C.F. Boehringer Biochemical Laboratory and which will appear shortly in a book entitled: "Methoden der enzymatischen Analyse" (Verlag Chemie) by H.U. Bergmeyer. In order to shorten the reaction time or counteract any possible inhibitory effect of malonate remaining in the test samples the enzyme concentrations mentioned below are in some cases greater than those given by Bergmeyer. A Beckman spectrophotometer model B was used in all enzymatic tests. The semi-micro-cuvettes had a light path of 1 cm. and a width of 0.4 cm. All measurements were made at a wave length of 340 mμ and a temperature of 25-26°. The calculations are based on the assumption that a solution containing one micromole of DPNH or TPNH per ml. has an extinction of 6.22 at 340 mμ (light path 1 cm.). In general, each enzymatic reaction took 5-10 minutes to go to completion except the reactions for 3-phosphoglycerate and α-glycerophosphate where more time was allowed.

Enzymes--All enzymes were obtained from C.F. Boehringer and Soehne, Mannheim-Waldhof, Germany. Proper refrigeration was provided for during air-transport and subsequent storage (2-4°). The enzymes - mostly crystalline - were obtained as suspensions in ammonium sulfate solution. The concentration of the enzyme protein in the suspension as well as the approximate activity per mg. protein (specific activity) were given. Boehringer expresses activity in terms of Bücher units (for definition of Bücher unit see Beisenherz et al. 1953).[10] We have always checked the

activity of these enzymes at regular intervals and observed, as a rule, no significant loss even after several months of storage. Prof. Bücher kindly supplied us with a sample of glucose 6-phosphate dehydrogenase (Zwischenferment) with very low glutathione reductase activity.

Reagents and Buffers--The concentrations of nucleotides used for the estimation of substrates were the following: DPNH 10 mM (7.1 mg. $DPNHNa_2$/ml), DPN 60 mM (40 mg. DPN/ml.), TPN 13 mM (10 mg. TPNNa/ml.), ADP 20 mM (10 mg. $ADPNa_3.H_2O$/ml.), ATP 16 mM (10 mg. $ATPNa_2.H_2O$/ml.). All nucleotides were dissolved in water except DPNH which was dissolved in 0.05 M triethanolamine pH 7.6. Boehringer supplied the above reagents as well as sodium pyruvate, phosphoenolpyruvate (tricyclohexylammonium salt), 3-phosphoglycerate (tricyclohexylammonium salt) and 2-phosphoglycerate (barium salt).

0.05 M triethanolamine buffer pH 7.6 (9.3 gm. amine.HCl + 22 ml. N NaOH diluted to 1 liter) was used in all estimations of substrates except α-glycerophosphate. It was pipetted into the cuvette first in such quantities that the final volume amounted to one ml. For measuring pyruvic kinase activity 0.5 ml. of 0.1 M triethanolamine containing 5 mM EDTA was used per test.

Glycine-EDTA-hydrazine buffer pH 9.5 was used for the determination of α-glycerophosphate. 1.89 gm. glycine, 1.3 gm. hydrazine sulphate and 46.8 mg. EDTA (disodium salt) were dissolved in water and diluted to 20 ml. This stock solution is stable if kept refrigerated. Just before use a portion is brought to pH 9.5 with 5 N NaOH. The dilution factor for the stock solution due to pH adjustment should be 1.4. If less, a calculated amount of water is added.

Glass redistilled water was used all through this study.

Estimation of metabolites

a) 2-Phosphoglycerate, Phosphoenolpyruvate and Pyruvate were estimated simultaneously according to the following reactions:

$$\text{2-Phosphoglycerate} \xrightarrow{\text{Enolase}} \text{Phosphoenolpyruvate} + H_2O$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Pyruvic kinase}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{DPNH} + H^+ \xrightarrow{\text{Lactic dehydrogenase}} \text{Lactate} + \text{DPN}^+$$

To the cuvette are added enough 0.05 M triethanolamine buffer so the total volume without enzymes = 1 ml., 0.05 ml. 0.2 M $MgSO_4$ in 2 M KCl, 0.04 ml. 20 mM ADP, 0.012 ml. 10 mM DPNH. The volume of the solution being assayed should be such that the total change in extinction does not exceed 0.4. The addition of 0.005 ml. lactic dehydrogenase (around 500 units) leads to a change in extinction (ΔE) which measures pyruvate. When E becomes steady 0.005 ml. pyruvic kinase (50 units) is added: ΔE now measures phosphoenolpyruvate. Finally 0.01 ml. enolase (20 units) is added when ΔE measures 2-phosphoglycerate.

b) 3-Phosphoglycerate and 1,3-Diphosphoglycerate were estimated simultaneously as follows:

$$\text{3-Phosphoglycerate} + \text{ATP} \xrightarrow{\text{Phosphoglyceric kinase}} \text{1,3-Diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-Diphosphoglycerate} + \text{DPNH} + H^+ \xrightarrow{\text{Glyceraldehyde phosphate dehydrogenase}} \text{3-Phosphoglyceraldehyde} + \text{phosphate} + \text{DPN}^+$$

To the cuvette are added buffer as in (a), 0.008 ml. 0.2 M $MgSO_4$, 0.1 ml. 16 mM ATP, 0.012 ml. 10 mM DPNH and test solution. 0.005 ml. glyceraldehyde phosphate dehydrogenase (70 units) is added when $\Delta \underline{E}$ measures 1,3-diphosphoglycerate. Finally 0.01 ml. phosphoglyceric kinase (200 units) is added when $\Delta \underline{E}$ measures 3-phosphoglycerate.

c) Hexosediphosphate, Glyceraldehyde phosphate and Dihydroxyacetone phosphate were estimated simultaneously as follows:

Hexosediphosphate $\xrightarrow{\text{Aldolase}}$ Glyceraldehyde phosphate + Dihydroxyacetone phosphate

Glyceraldehyde phosphate $\xrightarrow{\text{Triosephosphate isomerase}}$ Dihydroxyacetone phosphate

Dihydroxyacetone phosphate + DPNH + H^+ $\xrightarrow{\text{Glycerophosphate dehydrogenase}}$ α-Glycerophosphate + DPN^+

To the cuvette are added buffer, DPNH and test solution as in (a). The successive additions of glycerophosphate dehydrogenase (0.01 ml. = 60 units), triosephosphate isomerase (0.005 ml. = 50 units) and aldolase (0.005 ml. = 50 units) give rise to change in $\underline{E}$ which measure respectively dihydroxyacetone phosphate, glyceraldehyde phosphate and hexose diphosphate.

d) Glucose 6-Phosphate was estimated as follows:

Glucose 6-Phosphate + TPN^+ $\xrightarrow{\text{Glucose 6-phosphate dehydrogenase}}$ 6-Phosphogluconate + TPNH + H^+

To the cuvette are added buffer as in (a), 0.008 ml. 0.2 M $MgSO_4$,

0.02 ml. 13 mM TPN and solution to be analysed. Glucose 6-phosphate dehydrogenase (0.01 ml. = 40 units) is added. Increase in E measures glucose 6-phosphate. If the enzyme contains glutathione reductase, TPNH is reoxidised when extracts contain much glutathione. This "back run" can be detected if readings are taken every 2 minutes and can be prevented by addition of 0.01 ml. 0.8 mM $ZnSO_4$ to the reaction mixture. $ZnSO_4$ is a powerful inhibitor of glutathione reductase.

e) α-Glycerophosphate was estimated as follows:

$$\alpha\text{-Glycerophosphate} + DPN^+ \xrightarrow{\text{Glycerophosphate dehydrogenase}} \text{Dihydroxyacetone phosphate} + DPNH + H^+$$

To the cuvette are added 0.5 ml. glycine - EDTA - hydrazine buffer pH 9.5, water to give a final volume of 1 ml. before addition of enzyme, 0.05 ml. 60 mM DPN and the solution to be analysed. α-Glycerophosphate is determined from the increase in E following the addition of 0.015 ml. (300 units) glycerophosphate dehydrogenase. The reaction time is around 15 minutes. The reaction mixture should contain no phenolphthalein.

f) Adenosinediphosphate (ADP) was estimated as follows:

$$ADP + \text{Phosphoenolpyruvate} \xrightarrow{\text{Pyruvic kinase}} ATP + \text{Pyruvate}$$

$$\text{Pyruvate} + DPNH + H^+ \xrightarrow{\text{Lactic dehydrogenase}} \text{Lactate} + DPN^+$$

To the cuvette are added buffer as in (a), 0.04 ml. 0.2 M $MgSO_4$, 0.05 ml. 0.2 M KCl, 0.02 ml. 0.02 M phosphoenolpyruvate, 0.008 ml. 10 mM DPNH and solution to be assayed. Lactic dehydrogenase (0.005 ml. = 200 units) is added first and when the extinction has reached a steady value, 0.005 ml. (50 units) pyruvic kinase is added. ΔE now measures ADP.

g) Adenosinetriphosphate (ATP) was estimated as follows:

$$ATP + 3\text{-Phosphoglycerate} \xrightarrow{\text{Phosphoglyceric kinase}} ADP + 1,3\text{-diphosphoglycerate}$$

$$1,3\text{-Phosphoglycerate} + DPNH + H^+ \xrightarrow{\text{Glyceraldehydephosphate dehydrogenase}} \text{Glyceraldehydephosphate} + DPN^+ + \text{Phosphate}$$

To the cuvette are added buffer as in (a), 0.04 ml. 0.2 M $MgSO_4$, 0.07 ml. 0.1 M 3-phosphoglycerate, DPNH as in (f) and the solution to be assayed. Glyceraldehydephosphate dehydrogenase (0.01 ml. = 40 units) is added followed immediately by 0.01 ml. (200 units) phosphoglyceric kinase. ΔE measures ATP.

Assay of Pyruvic Kinase

To the cuvette are added 0.5 ml. 0.1 M triethanolamine buffer pH 7.6 containing 10 mM EDTA and water to give a final volume of 1 ml. after adding the following: 0.015 ml. 0.5 M $MgSO_4$, 0.04 ml. 2 M KCl, 0.02 ml. 0.02 M phosphoenolpyruvate, 0.025 ml. 20 mM ADP, 0.015 ml. 10 mM DPNH and 0.001 ml. lactic dehydrogenase (100 units). Limiting amount (around 1 unit) of diluted pyruvic kinase is now introduced and after a short run the time needed to give a decrease in extinction of 0.1 is measured. The same experiment is repeated in presence of malonate or NaCl. Here solutions of malonate or NaCl are substituted for part of the water added to the cuvette to give a final concentration in the mixture of 0.12 and 0.06 M with respect to malonate and 0.12 M with respect to NaCl.

In all preceeding photometric work blank cuvettes were filled with the same buffer as in the reaction tests. Enzymes were omitted.

C. Results and Discussion

The sequence of enzymatic steps involved in glycogen breakdown is presented in the accompanying scheme. It is assumed that every molecule of sugar metabolised by animal tissue proceeds through at least part of the pathway here presented. We are mainly interested in the fate of glycogen since it is the only source of carbohydrate used by our preparation for lactic acid formation.

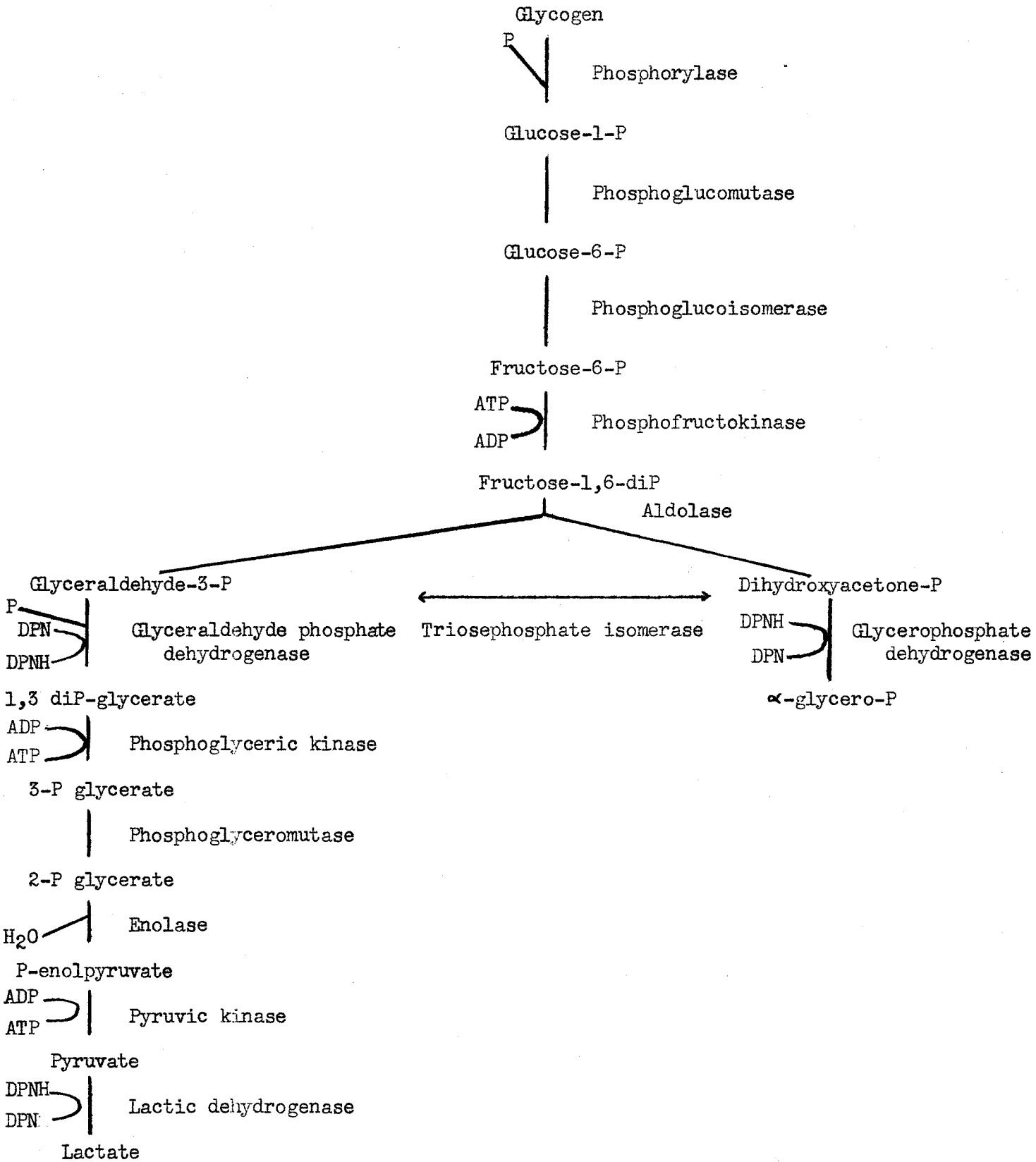
Glycogen
P
Phosphorylase
Glucose-1-P
Phosphoglucomutase
Glucose-6-P
Phosphoglucoisomerase
Fructose-6-P
ATP
ADP
Phosphofructokinase
Fructose-1,6-diP
Aldolase
Glyceraldehyde-3-P
Dihydroxyacetone-P
P
DPN
DPNH
Glyceraldehyde phosphate dehydrogenase
Triosephosphate isomerase
DPNH
DPN
Glycerophosphate dehydrogenase
1,3 diP-glycerate
∝-glycero-P
ADP
ATP
Phosphoglyceric kinase
3-P glycerate
Phosphoglyceromutase
2-P glycerate
H2O
Enolase
P-enolpyruvate
ADP
ATP
Pyruvic kinase
Pyruvate
DPNH
DPN
Lactic dehydrogenase
Lactate

Table 1 shows the effect of malonate on a glycolysing muscle suspension. Here, the usual methods of acid hydrolysis were applied to make a preliminary study of the phosphorus compounds. Phosphorus values for fresh muscle without incubation are also given. It can be seen that during incubation without malonate most of the labile phosphorus appears as inorganic phosphate. In the malonate-treated suspension, however, about half of the total phosphorus appears in a bound form which is not hydrolysed after 3-hours contact with N HCl at 100°. Since the malonate-treated suspension formed very little lactate as compared with the control it was concluded that the accumulating acid-resistant phosphate fraction must be an intermediate in the glycolytic pathway.

The nature of this fraction was determined enzymatically. Since it was feared that malonate might inhibit some of the enzymes, it was removed bofore starting the enzymatic tests. Table 2 shows that more than one phosphorus compound accumulates: 3-phosphoglycerate and α-glycerophosphate being formed in about equal quantities. In addition to these acid-resistant compounds, the more labile phosphoenolpyruvate is formed although in smaller amount. The latter must be responsible for the higher "labile-phosphorus" value observed in the malonate-treated suspension as compared with the control suspension (Table 1), since at the end of the incubation period both suspensions contained only traces of ATP and small amounts of ADP. Table 2 also shows that the presence of malonate in the test samples did not interfere with the enzymatic tests. This is not surprising as the inhibitor was diluted many-fold by the reaction medium and an excess of the necessary enzymes was used in each test. The ether extraction step was thus abandoned in all subsequent experiments.

Table 1. Effect of Malonate on Phosphorus Compounds of Rat Skeletal Muscle Suspension and Lactate Production.

Frozen skeletal muscle was powdered as described in Methods. A portion of powder was treated with $HClO_4$ to obtain "resting" values. Remaining powder was used for preparing control and malonate suspensions. To get maximum effect the suspensions were incubated for 5 hrs. at 6°, protein-free extracts were then prepared and analysed for acid-soluble phosphorus. All values are expressed in mg of P per 100g of wet tissue except for lactate which is given in mg lactic acid per 100g wet tissue. The terms "Hydrolysable-P" 7 min. and 180 min. denote inorganic phosphate liberated after treatment with N HCl at 100° for 7 and 180 min. respectively in addition to inorganic phosphate found before hydrolysis.

	Unincubated Control Muscle	Control Suspension	Malonate Suspension
Lactic acid	42.2	617	96.7
Phosphocreatine	49.8	1.0	1.3
Phosphocreatine + Inorg. Phosphate	79.8	134.0	58.5
"Hydrolysable-P".7 min. (H_7)	122.6	136.5	65.8
"Labile"-P	42.8	2.5	7.3
Total-P (TP)	163.4	167.5	170.5
Non-labile-P ($TP-H_7$)	40.8	31.0	104.7
"Hydrolysable-P".180 min.		145.0	85.0
Resistant-P.(to 180 min. Hydrol.)		22.5	85.5

Table 2. Glycolytic Intermediates in Extracts of Rat Skeletal Muscle as Assayed before and after Ether Extraction.

Portions of perchloric acid extracts previously analysed for acid-soluble phosphorus compounds (see Table 1) were used directly for enzymatic estimation of intermediates. Remaining portions were freed of malonate by 3-hour ether extraction and then analysed. In both cases extracts were neutralized prior to assay. The values are expressed in mg of phosphorus and are calculated back to 100g of tissue (wet weight).

	Unincubated Control Muscle	Control Suspension		Malonate Suspension	
		Before ether ext.	After ether ext.	Before ether ext.	After ether ext.
3-P-glycerate	---*	---	---	36.30	36.00
2-P-glycerate	---	---	---	3.59	3.32
α-glycerophosphate	0.73	1.81	1.95	30.30	29.40
Glucose 6-phosphate	6.33	4.93	4.72	2.54	2.60
Hexosediphosphate	0.25	---	---	---	---
Dihydroxyacetonephosphate	---	---	---	1.71	1.04
Phosphoenolpyruvate	---	---	---	12.07	10.25
ATP(2P)	39.2	---	---	---	---
ADP(1P)	2.25	1.76	1.62	1.60	1.16

* None or within experimental error.

The maximum yield of intermediates was obtained if the suspension had been allowed to stand at room temperature for a short period (about 3 min.) and at a lower temperature for about 2 hours (Table 3). If the incubation is carried out only at room temperature the yield is small owing presumably to inactivation of some enzymes, and if low temperature is maintained throughout, at least five hours of incubation would be required for maximum effect. Under all conditions all intermediates appeared simultaneously. Addition of 3-phosphoglycerate, 2-phosphoglycerate or phosphoenolpyruvate to the control suspension resulted in their breakdown to inorganic phosphate and pyruvate or lactate. When, however, these esters were added to the malonate-treated suspension the greater part of each of them was found as 3-phosphoglycerate at the end of the incubation period. The fact that phosphoenolpyruvate gave rise to 3-phosphoglycerate but not to lactate or pyruvate implies that the block must lie at the pyruvic kinase level. The inhibition of pyruvic kinase must have resulted in a reversal of the glycolytic reactions which could not proceed beyond 3-phosphoglycerate due to lack of ATP.

Table 4 summarizes the results obtained after the addition of various substances to malonate-treated muscle suspensions. The figures in Exp. (i) show the effect of 15 min. incubation with malonate in the cold. A significant amount of phosphate esters accumulates and the process continues, although at a lower rate, during the next hour (Exp. ii). The rate of accumulation of esters increases if inorganic phosphate is present (Exp. iii). If inorganic phosphate is added at the beginning along with malonate and the incubation period prolonged, its effect is more pronounced than in Exp. iii. The stimulating effect of inorganic phosphate can be

Table 3. Accumulation of Glycolytic Intermediates.

Frozen muscle powder was transferred into pre-weighed malonate medium kept at room temp. After shaking and reweighing which took 3 min. the suspension was cooled in ice-water. Volume was adjusted to obtain 1:10 dilution of muscle, and 10 min. from the time of powder transfer the suspension was placed in a refrigerator. (Temp. 6°). At various intervals portions were withdrawn, deproteinized and analysed enzymatically for intermediates. All values are given in mg. of P calculated back to 100g tissue (wet weight).

Incubation time with malonate	Intermediates Formed			
	Phosphoenol-pyruvate	2-Phosphoglycerate	3-Phosphoglycerate	α-Glycerophosphate
6 min	6.12	1.84	19.00	22.30
30 min	8.60	2.45	29.10	30.80
120 min	10.90	3.18	45.60	33.40

Table 4. Effect of Addition of Phosphate, Pyruvate, Phosphate + Pyruvate, and Phosphoenolpyruvate on Accumulation of Glycolytic Intermediates in Malonate-Blocked System.

Malonate muscle-suspension (1:10 dilution) was prepared as in Table 3. 7 portions of 10 ml each were pipetted into separate flasks and kept in ice. At 15 min. (counting from time of powder transfer) one portion was deproteinized. To other portions different solutions were added in total volume of 1 ml. All flasks were then kept at 6° for 1 hour. After deproteinization the neutralized filtrates were analysed. Values were calculated back to 100g of wet tissue and expressed in mg P. Lactate and pyruvate values are given in mg% of the corresponding acids.

	Amounts added per 100g tissue	Lactate	Pyruvate	Phosphoenol-pyruvate	2-Phospho-glycerate	3-Phospho-glycerate	α-Glycero-phosphate
(i) Analysis at time of additions (15 min after preparation of suspension).	---	84	---	6.8	2.1	23.1	24.4
(ii) Analysis 1 hour later. No additions. (control).	---	95.7	---	10.6	2.8	33.6	31.6
(iii) Analysis after 1 hour of incubation with inorg. phophate.	101 mg P of inorg. phosphate	85.2	---	12.5	3.2	38.2	38.6
(iv) Analysis after 1 hour of incubation with phosphoenolpyruvate.	92.2 mg P of phosphoenol-pyruvate	91.0	---	34.4	8.5	106.2	33.9
(v) Analysis after 1 hour of incubation with pyruvate	285 mg pyruvate	176	191	14.3	4.7	43.2	27.1
(vi) Analysis after 1 hour of incubation with inorg. phosphate + pyruvate	101 mg P + 285 mg pyruvate	233	136	15.7	6.4	62.2	26.6

reproduced by phosphocreatine, ATP and other esters that can yield inorganic phosphate notwithstanding the block. These findings suggest that in this system inorganic phosphate is a limiting factor, since the inorganic phosphate, or its precursors originally present in muscle are trapped in the form of the accumulating esters. If phosphoenolpyruvate is added (Exp. iv) there is an increased formation of 3-phosphoglycerate, which accounts almost quantitatively for the decrease in phosphoenolpyruvate, but the increase in lactate is insignificant. When pyruvate is added (Exp. v) lactate is formed, the increase in the latter being equivalent to the decrease in the former. However, very little glycerophosphate is formed. It is clear that in the malonate experiments without pyruvate, α-glycerophosphate is a secondary product. In other words, in the absence of pyruvate the DPNH formed in the "oxidation reaction of fermentation," was reoxidized through the reduction of triosephosphate. Since the lactic dehydrogenase-catalysed reaction is more efficient in regenerating DPN it is clear that addition of pyruvate to the malonate-treated suspension (Exp. v) results in a greater formation of total esterified phosphorus than when no pyruvate is added (Exp. ii). Simultaneous addition of pyruvate and inorganic phosphate (Exp. vi) produces an additive effect. Here again lactate is formed at the expense of pyruvate.

Hitherto, malonate was used in a concentration of 0.12 M. However, 0.06 M malonate is as effective as 0.12 M if the accumulation of phosphoric esters is taken as a criterion, although there is incomplete (90%) inhibition of lactate formation. With 0.03 M malonate the inhibition of lactate production is about 60% and the accumulation of phosphoric esters is around

60% of that observed with the higher concentrations.

Finally, the effect of malonate was tested on crystalline pyruvic kinase. In a system where the enzyme was limiting a decrease in extinction of 0.1 at 340 mu required a reaction time of 51 seconds. With 0.12 M malonate the reaction was too slow for accurate measurement, with 0.06 M malonate the reaction time was 365 sec. and with 0.03 M it was 153 sec. In presence of 0.12 M NaCl the reaction time was 83 sec. indicating that the inhibition by malonate is predominantly a malonate and not a sodium ion effect. Thus the effect of malonate on the pure enzyme system is not unlike that on the muscle suspension.

The results reported in this paper are consistent with the interpretation that malonate in concentration exceeding 0.06 M almost completely inhibits the reaction involving pyruvic kinase. The formation of phosphoglycerate (from diphosphoglycerate) requires the presence of ADP and is accompanied by ATP formation. It can be seen from Table 2 that ADP is available all through, its concentration after incubation with malonate does not differ markedly from that found in the unincubated control. The fact that ATP is found only in traces implies that the ATP is hydrolysed as soon as it is formed.

Malonate at 0.03 M causes only partial blockage of glycolysis in our muscle suspension, and only partly inhibits pyruvic kinase in a pure system. At 0.06 M, malonate blocks glycolysis almost completely and the same is true of its action on the pure enzyme system.

Summary

1. A rat muscle suspension prepared from frozen muscle powder is used.
2. Malonate in a concentration above 0.06 M inhibits lactate formation almost completely and causes accumulation of 3-phosphoglycerate, α-glycerophosphate and phosphoenolpyruvate.
3. Addition of phosphoenolpyruvate to the malonate-treated suspension gave rise to 3-phosphoglycerate but not to pyruvate and lactate as in the untreated suspension. The inhibition of pyruvic kinase resulted in a reversal of the glycolytic reactions which could not proceed beyond 3-phosphoglycerate owing to lack of ATP.
4. Addition of pyruvate to the malonate-blocked system results in a stoicheiometric formation of lactate. 3-phosphoglycerate production is increased, but no α-glycerophosphate is formed.
5. 0.03 M malonate only partly inhibits glycolysis in our system. Experiments on the pure enzyme system also showed that while 0.06 M malonate strongly inhibits pyruvic kinase, 0.03 M malonate is only partially effective.

REFERENCES

1. Krebs, H. A., and Eggleston, L. V., The Oxidation of Pyruvate in Pigeon Breast Muscle. Biochem. J. 34: 442, 1940.

2. Kuby, S. A., Noda, L., and Lardy H. A., Adenosinetriphosphate-Creatine Transphosphorylase. J. Biol. Chem. 209: 191, 1954.

3. _____, _____, and _____, Adenosinetriphosphate-Creatine Transphosphorylase. J. Biol. Chem. 210: 65, 1954.

4. Fawaz, G., and Hawa, E. S., Phosphocreatine Content of Mammalian Cardiac Muscle. Proc. Soc. Exper. Biol. & Med. 84: 277, 1953.

5. Barker, S. B., and Summerson W. H., The Colorimetric Determination of Lactic Acid in Biological Material. J. Biol. Chem. 138: 535, 1941.

6. Fiske, C. H., and Subbarow, Y., Phosphocreatine. J. Biol. Chem. 81: 629, 1929.

7. Slatter, E. C., Spectrophotometric Determination of Fructose-1:6-Diphosphate, Hexosemonophosphates, Adenosinetriphosphate and Adenosinediphosphate. Biochem. J. 53: 157, 1953.

8. Scholz, R., Schmitz, H., Bücher, Th., and Lampen, J. O., Über die Wirkung von Nystatin auf Bäckerhefe. Biochem. Z. 331: 71, 1958.

9. Hohorst, H. J., Kreutz, F. H., and Bücher, Th., Über Metabolitgehalte und Metabolit-Konzentrationen in der Leber der Ratte. Biochem. Z. 332: 18, 1959.

10. Beisenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G., Diphosphofructose-Aldolase, Phosphoglyceraldehyde-Dehydrogenase, Milchsäure-Dehydrogenase, Glycerophosphat-Dehydrogenase und Pyruvat-Kinase aus Kaninchenmuskulatur in einem Arbeitsgang. Z.f. Naturforsch, 8b, 555, 1953.