CXCIX. PHOSPHAGEN FORMATION AND OXIDATION OF TRIOSEPHOSPHATE IN MUSCLE EXTRACT

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THE anaerobic synthesis of creatinephosphate in unpoisoned, dialysed muscle extract was demonstrated by Ostern *et al.* [1935], Needham & van Heyningen [1935] and Lehmann [1935]. The mechanism suggested for this reaction is the transfer of organic phosphate from phosphopyruvic acid to adenylic acid and hence to creatine according to the following scheme:

In the present work, the formation of creatinephosphate under aerobic conditions has been studied. In the absence of inorganic phosphate, and with hexosediphosphate as substrate, any creatinephosphate formed must arise by transfer of organic phosphate from phosphopyruvate. This substance may be formed from the phosphoglycerate arising by dismutation or, aerobically, it is possible that the triosephosphate is oxidized to phosphoglycerate by means of a dehydrogenase system. This oxidation was suggested by von Euler *et al.* [1936] for yeast juice in which they found an enzyme which caused decoloration of methylene blue in the presence of triosephosphate and cozymase. The scheme would then be:

It was found that the muscle extracts were capable of oxidizing triosephosphate and that iodoacetate had little effect on the oxygen uptake. Investigations were therefore made of the creatinephosphate formation by extracts in presence of iodoacetate to poison the mutase system. The energy relations of these changes are discussed later (p. 1592). The aerobic formation of creatinephosphate in the presence of free inorganic phosphate was also studied in the present case, as well as the effect of fluoride and iodoacetate upon it.

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During the course of this work a comparison was made between the oxido-reduction activity of the muscle extracts and the activity of the triosephosphate dehydrogenase, in an attempt to investigate the suggestion that oxido-reduction activity is due to the linked reaction of two dehydrogenases. It is known that muscle extract can bring about the dismutation:

and can also effect an oxido-reduction between triosephosphate and pyruvate with the formation of phosphoglycerate and lactate. It is therefore possible that such an oxido-reduction is due to the combination of the lactic and triosephosphate dehydrogenases working together, and that in the presence of iodoacetate the linking between them is inhibited in some way; or it may be that a mutase, itself poisoned by iodoacetate, is concerned.

TECHNIQUE

The enzyme preparations used in this work were made from fresh ice-cooled rabbit muscle, passed twice through a cooled mincer. Two preparations were used.

(a) An extract of the muscle prepared according to the directions of Meyerhof [1936] with a volume of ice-cold water of 1.5 times the weight of the muscle. The extracting mixture was stirred for 30–60 min. and strained through muslin. After standing for 1 hr. at room temperature for breakdown of substrates, the extracts were dialysed for times varying from 5 to 40 hr. against ice-cooled 0.45 % KCl solution, the solution being changed several times according to the length of dialysis. These extracts contained little or no carbohydrate or carbohydrate breakdown products.

(b) An acetone powder prepared from the above extracts either before or after dialysis. The extract was precipitated with three times its volume of ice-cold acetone, the precipitate being spun off, washed with acetone and ether and dried *in vacuo* over sulphuric acid. A suspension of this powder was made by grinding a weighed amount with a known volume of distilled water. If the original muscle extract had been precipitated undialysed, the extract of the acetone powder was dialysed against distilled water.

These extracts were buffered either with bicarbonate, bicarbonate + phosphate or phosphate buffer only. In the respiration experiments the pH was maintained at 7.2 by means of phosphate buffer, whereas in the creatinephosphate formation work a pH of 8 was used.

Cozymase was prepared from yeast according to the directions of Green [1936].

Sodium hexosediphosphate was prepared from the calcium salt by precipitation of the calcium in the form of carbonate.

Analysis. Estimation of phosphate was done by the method of Fiske & Subbarow [1929].

The methylene blue, which was present in the aerobic experiments, was removed by filtration through kieselguhr on a Büchner funnel. The creatinephosphate was separated from the phosphates having insoluble calcium salts by addition of Fiske & Subbarow's $\text{CaCl}_2\text{-Ca(OH)}_2$ reagent. After standing for 20 min. the precipitate was centrifuged off and the amount of creatinephosphate in the centrifugate was estimated by standing for 40 min. with the Fiske & Subbarow reagents.

The inorganic phosphate was estimated in the precipitate after solution in dilute hydrochloric acid.

Lactic acid was estimated by the method of Friedemann & Graeser [1933], the protein of the extracts being precipitated by phosphotungstic acid and the soluble carbohydrates by the $\text{CuSO}_4\text{-Ca(OH)}_2$ reagent.

FORMATION OF CREATINEPHOSPHATE

All extracts were allowed to stand for 5 days in the ice-chest to destroy adenylyl-pyrophosphatase, the creatinephosphate-forming capacity being unaffected. Methylene blue was added in aerobic experiments to act as an oxygen carrier.

(1) *By transfer of organic phosphate*

Hexosediphosphate was used as substrate as it is rapidly transformed into triosephosphate by the extracts. Sodium iodoacetate in a concentration of 1 : 850 was added to poison the mutase. Experiments were therefore set up as follows, anaerobic unpoisoned and poisoned controls being run simultaneously with the aerobic experiments.

Exp. 1. Suspension of acetone powder 80 mg./ml. 5 days old, 24 hr. dialysed. 2 ml. extract + 3 ml. additions. Aerobic samples contained 0.4 ml. of 0.5% methylene blue. Each sample contained 0.15 mg. Mg as MgCl_2 , 0.2% NaHCO_3 and 0.5 ml. *M/5* sodium hexosediphosphate solution. The samples were shaken at 37.5° for 10 min.

Sample	Additions			Phosphate	
	Adenylic acid mg.	Creatine mg.	Cozymase (0.15%) ml.	Creatine P mg.	Inorganic P mg.
Aerobic poisoned	0.2	8	0.8	0.2	Unreadable trace
Aerobic unpoisoned	0.2	—	0.8	—	Unreadable trace
Anaerobic unpoisoned	0.2	8	0.8	0.36	Unreadable trace
Anaerobic poisoned	0.2	—	0.8	—	Unreadable trace
Anaerobic unpoisoned	0.2	8	—	—	Unreadable trace
Anaerobic poisoned	0.2	8	0.8	—	0.1
Blank (ppt. immediately)	0.2	8	0.8	—	Unreadable trace

From these results it can be seen that the unpoisoned extract can form creatinephosphate anaerobically provided that adenylic acid, creatine and cozymase are present. Addition of iodoacetate appears to inhibit the formation completely under anaerobic conditions, whereas aerobically there is a formation of creatinephosphate up to 55% of the unpoisoned anaerobic yield. The phosphorylation must occur at the expense of the hexosediphosphate as the inorganic phosphate is very low. The rise in inorganic phosphate observed in the poisoned anaerobic sample is probably due to decomposition of some of the triosephosphate formed and trapped by inhibition of the mutase. As triosephosphate is hydrolysed by alkali some of the amount present in the sample will be decomposed during the 20 min. standing with the alkaline $\text{CaCl}_2\text{-Ca(OH)}_2$ precipitating reagent.

The effect of fluoride in a concentration of *M/50* on the formation of creatinephosphate was then observed. It appears from *Exp. 2* that fluoride inhibits the formation of creatinephosphate both aerobically and anaerobically from phosphate combined organically. The same results were obtained using another preparation of muscle extract, dialysed for 24 hr. and 5 days old.

Exp. 2. Acetone powder suspension 5 days old, 15 hr. dialysed. 2 ml. extract + 3 ml. additions. Each sample contained 0.25 mg. adenylic acid, 10 mg. creatine, 0.25 mg. Mg, 0.2% bicarbonate, 0.5 ml. 0.15% cozymase and 0.5 ml. *M/5* hexosediphosphate.

Sample	Addition		Phosphate	
	Iodoacetate	Fluoride	Creatine P mg.	Inorganic P mg.
Aerobic	1 : 850	—	0.19	0.05
Aerobic	—	M/50	—	0.05
Anaerobic unpoisoned	—	—	0.32	0.06
Anaerobic	1 : 850	—	—	0.08
Anaerobic	—	M/50	—	0.05

(2) *From inorganic phosphate*

Experiments were then carried out to see whether the presence of free inorganic phosphate altered the results in any way.

Exp. 3. Muscle extract, 5 days old, dialysed for 7 hr. 5 ml. + 5 ml. of additions, 0.15 % NaHCO_3 . Each sample contained 12 mg. creatine, 0.3 mg. Mg, 1 ml. cozymase, 0.8 ml. M/5 hexosediphosphate, 0.75 mg. adenylic acid and 2 mg. of P as Na_2HPO_4 . All samples were incubated for 10 min. at 37.5° .

Sample	Addition		Phosphate	
	Iodoacetate	Fluoride	Creatine P mg.	Inorganic P mg.
Aerobic	1 : 850	—	0.5	2.5
Aerobic	—	M/50	0.5	2.05
Anaerobic unpoisoned	—	—	1.2	2.45
Anaerobic	1 : 850	—	0.03	2.9
Anaerobic	—	M/50	0.29	1.99
Blank	1 : 850	M/50	—	2.4

From these results it appears that the creatinephosphate formed aerobically in the presence of iodoacetate is not produced at the expense of the free inorganic phosphate, whereas that formed in extracts poisoned with fluoride corresponds with the decrease in free inorganic phosphate. The increase in inorganic phosphate observed in the anaerobic iodoacetate sample is due to trapping of triosephosphate and its subsequent alkaline hydrolysis. Anaerobically with fluoride the creatinephosphate formation is at the expense of inorganic phosphate. In other experiments it was found that no creatinephosphate was formed in the absence of hexosediphosphate or in the presence of lactic acid as substrate for oxidation.

(3) *Comparison with oxygen uptake*

The next series of experiments were designed to show whether creatinephosphate formation under aerobic conditions was of the order of magnitude to be expected from the oxygen uptake, assuming that the triosephosphate was being oxidized to phosphoglycerate and the phosphate transferred to creatine from phosphopyruvate via adenylic acid. It had previously been noted that the activities of the two reactions appeared to be similar and to vary with the individual enzyme preparations.

The respiration was measured in Warburg manometers at 37.5° . Each cup contained:

0.2 ml. of 0.5 % methylene blue.

0.25 ml. 0.4 M phosphate buffer pH 7.2.

0.5 ml. cozymase 0.15 %.

0.15 ml. iodoacetate solution making concentration 1 : 850.

1.5 ml. of enzyme preparation.

0.5 ml. hexosediphosphate was introduced from a side bulb after 10 min. equilibration in the bath. The experiments on formation of creatinephosphate

were carried out as before and the amount formed aerobically in the presence of iodoacetate was compared with the amount calculated from the oxygen uptake in the same time.

A typical experiment of this kind is as follows:

Exp. 4. Muscle extract 5 days old, dialysed for 5 hr., 5 ml. + 5 ml. additions. Each sample contained 0.75 mg. adenylic acid, 15 mg. creatine, 0.8 ml. 0.15 % cozymase, 0.4 mg. Mg, 0.2 % NaHCO_3 and 2 mg. phosphate P. Samples were incubated at 37.5° for 15 min.

Sample	Iodoacetate	Phosphate		
		Creatine P mg.	Inorganic P mg.	P $\equiv$ of oxygen uptake (mg.)
Aerobic	1 : 850	0.76	2.60	0.61
Anaerobic	1 : 850	0.18	2.99	—
Anaerobic	—	1.5	2.60	—
Blank	1 : 850	—	2.60	—

From these results it is evident that the creatinephosphate formed aerobically, less the small amount present in the anaerobic poisoned sample, is of the order to be expected if the phosphate is being transferred from phosphopyruvate formed by oxidation of triosephosphate. On the other hand it is seen that there is some formation of creatinephosphate in the anaerobic poisoned sample suggesting incomplete inhibition of the dismutation. This was tested by the capacity of the same extract to form lactic acid both from hexosediphosphate alone and from an equivalent mixture of hexosediphosphate and pyruvate in the presence of fluoride and iodoacetate anaerobically.

Exp. 5. 2 ml. extract + 2 ml. additions. Each sample contained 0.3 mg. Mg, 0.8 ml. 0.15 % cozymase and 0.25 ml. of 0.4 *M* phosphate buffer. The flasks were filled with nitrogen and incubated at 37.5° for 15 min.

Sample poisoned with	Addition		Lactic acid formed mg.
	<i>M</i> /5 Hexose- diphosphate ml.	<i>M</i> /5 Sodium pyruvate ml.	
(1) Iodoacetate	0.5	0.5	0.31
(2) Fluoride	0.5	0.5	1.6
(3) Iodoacetate	0.8	—	—
(4) Fluoride	0.8	—	0.08

It therefore appears that the dismutation is completely poisoned for hexosediphosphate alone both by iodoacetate and fluoride and only 80 % inhibited by iodoacetate when pyruvate is present. This being so it was necessary to see whether the respiration in the presence of hexosediphosphate was really due to the oxidation of triosephosphate to phosphoglycerate or to the sum of the oxidations of α -glycerophosphate and lactic acid formed in dismutation and oxidized by the dehydrogenases for these substances. Respiration experiments were carried out in the presence of iodoacetate using α -glycerophosphate, lactate and hexosediphosphate in equivalent amounts as substrates. The extracts used were not very active but in every case the oxidation of hexosediphosphate was more than twice as great as the control whereas the oxygen uptake with the glycerophosphate and lactate was never greater than the control respiration. It follows that the oxygen uptake in the presence of iodoacetate cannot be due to oxidation of dismutation products.

FORMATION OF LACTIC ACID

During early respiration experiments on these extracts a comparison of the aerobic oxygen uptake in the presence of iodoacetate and the anaerobic lactic acid formation in the presence of fluoride was made in an attempt to discover whether the oxido-reduction depended on the lactic and triosephosphate dehydrogenases working in conjunction. If the formation of lactic and phosphoglyceric acids from pyruvic acid and triosephosphate anaerobically involves the triosephosphate dehydrogenase, then one might expect the phosphoglyceric acid formed anaerobically to be equivalent to the oxygen uptake with triosephosphate aerobically (one molecule of phosphoglyceric acid to one atom of oxygen). As the lactic acid formed is equivalent in this oxido-reduction to the phosphoglyceric acid formed, the matter was more readily investigated by comparing the anaerobic lactic acid formation with the oxygen uptake. Two examples of these comparisons are given below:

Exp. 6. Suspension of acetone powder, 5 hr. dialysed. Aerobic samples as in respiration experiments above. Anaerobic samples as in *Exp. 5* (2) above. 1 ml. extract + 2.3 ml. additions.

	Oxygen uptake/hr. μ l.	Oxygen $\equiv$ of lactic acid formation/hr. μ l.
(1)	65	117
(2)	60	108

In each experiment the oxygen equivalent of the lactic acid formed from hexosediphosphate and pyruvate in the presence of fluoride was greater than the oxygen uptake aerobically in the presence of iodoacetate. As the respiration in the latter case falls off sharply after 15–20 min., the rates of formation of lactic acid and uptake of oxygen were compared and here it was found that the bulk of the lactic acid was also formed within the first 15–20 min.

Exp. 7. Suspension as in *Exp. 6.*

Oxygen uptake/15 min. μ l.	Oxygen $\equiv$ of lactic acid formed/15 min. μ l.
50	94

It would appear from these results that, although the speeds of the reactions are different, there is a similarity in their falling off with time. It is interesting that the iodoacetate, which inhibits the oxido-reduction almost completely, affects the dehydrogenase only to the extent of 15–30 %, the preparations used showing an increase in respiration of this degree when unpoisoned with iodoacetate.

During the course of these experiments it was noticed that the presence of cozymase greatly increased the yield of lactic acid, adenylypyrophosphate having no such effect. This was also observed by Meyerhof [1936] who published the observation about the same time. Lack of cozymase in aerobic experiments causes a 50 % decrease in respiration.

Exp. 8. Suspension of acetone powder from 30 hr. dialysed extract. 2 ml. + 1.3 ml. of additions.

Sample	Oxygen uptake/30 min. μ l.	Lactic acid forma- tion/30 min. mg.
(1) + Cozymase	105	6.7
No cozymase	50.4	0.84
(2) + Cozymase	90.5	6.5
No cozymase	45.0	0.72

It appears from this that the two systems are again affected to different degrees by the absence of cozymase.

DISCUSSION

The formation of creatinephosphate under aerobic conditions is of interest owing to the important role assigned to this substance in the contracting muscle. As the muscle probably works normally under more or less aerobic conditions it is to be expected that some system of oxidation should be involved in the formation of creatinephosphate.

In the present work where there is no free inorganic phosphate creatinephosphate is formed aerobically in the presence of iodoacetate but not anaerobically. The phosphate can only arise from organically combined phosphate and, as dismutation is inhibited, the phosphopyruvate cannot be formed by this reaction. It would appear, therefore, that the following reaction is taking place:

and that the energy for this formation of adenylpyrophosphate is obtained from the breakdown of the phosphopyruvate.

In the presence of fluoride, which does not inhibit dismutation but prevents further breakdown of the products of this reaction, no creatinephosphate is formed and it would therefore appear that the energy of dismutation cannot be utilized for this formation as the organically combined phosphate cannot be transferred either from phosphoglycerate or glycerophosphate to the adenylic acid.

In the case where free inorganic phosphate is present it is noticeable that creatinephosphate formation aerobically in presence of iodoacetate does not appear to be at the expense of the inorganic phosphate. It would therefore appear that the reactions

are not coupled under these conditions.

In the presence of fluoride, creatinephosphate formation occurs both aerobically and anaerobically at the expense of the inorganic phosphate so that the reactions

appear to be coupled. This reaction will be described in detail by Needham & Pillai elsewhere.

The coupling of oxidation and esterification of inorganic phosphate was suggested by Lennerstrand & Runnström [1935] in their system—apozymase + cozymase + hexosediphosphate + glucose + methylene blue in the presence of fluoride to inhibit fermentation. This coupling is again suggested by Lennerstrand [1937] for haemolysed horse blood corpuscles using hexosediphosphate as substrate and phosphorylating adenylic acid in the presence of free phosphate. In his earlier work [Lennerstrand & Runnström, 1935] he found that inorganic phosphate was necessary for respiration and that respiration and esterification by the horse blood corpuscles were completely inhibited by the presence of iodoacetate. In the present study of the muscle system free inorganic phosphate in

the concentration suggested by Lennerstrand did not appear to increase the oxygen uptake, whilst iodoacetate merely inhibited it 15–30 % and did not inhibit the phosphorylation of adenylic acid. It would therefore appear possible that the source of energy for the phosphorylations observed by Lennerstrand might be dismutation of triosephosphate and not its oxidation as he suggests. This would account for the need of inorganic phosphate and for the inhibition of phosphorylation by iodoacetate and not by fluoride. The observed oxygen uptake in his system may be due to oxidation of α -glycerophosphate by its dehydrogenase and this again would be inhibited by iodoacetate as formation of α -glycerophosphate by dismutation would cease. On the other hand the suggestion that in Lennerstrand's experiments the source of energy is dismutation leaves unexplained the necessity which he observed for the presence of the oxygen carrier.

Needham & Pillai [1937] and Meyerhof [1937] have shown that the energy of certain anaerobic dismutations and oxido-reductions can be used in the synthesis of adenylypyrophosphate or creatinephosphate from free phosphate.

It would therefore appear that the aerobic phosphorylation of creatine in muscle extract in the presence of iodoacetate and in the presence or absence of free phosphate is accomplished by transfer of phosphate from phosphopyruvate (reaction 1) and not by coupling of oxidation of triosephosphate and esterification of free phosphate. In the presence of fluoride however phosphorylation appears to be at the expense of the free inorganic phosphate. This occurs anaerobically by reaction (3), but aerobically either by reaction (3) alone or possibly by this and some reaction coupling oxidation and phosphorylation, as it will be seen from Exp. 3 that the amount of creatinephosphate formed aerobically is twice as great as that formed anaerobically in the presence of fluoride.

The above experiments also suggest that the triosephosphate dehydrogenase plays no part in the anaerobic oxido-reduction process of lactic and phosphoglyceric acid formation. It is interesting to compare with this the results of Dixon & Lutwak-Mann [1937] who proved that the acetaldehyde mutase and dehydrogenase of horse liver are two separate enzymes. It is also interesting to note that Needham & Pillai [1937], working with a similar aqueous extract of muscle acetone powder, find good triosephosphate dismutation activity in the clear centrifugate of this extract, whereas in the present work such a centrifugate was found to be practically inactive in the aerobic oxidation of triosephosphate.

The effect of cozymase on lactic acid formation in this system is greater than its effect on the oxidation of triosephosphate, and this again suggests that the two mechanisms are distinct.

SUMMARY

1. Aerobic formation of creatinephosphate in absence of free inorganic phosphate and presence of hexosediphosphate and iodoacetate is demonstrated.

2. Aerobic formation of creatinephosphate in presence of free inorganic phosphate, hexosediphosphate and iodoacetate or fluoride is also shown, the formation being at the expense of inorganic phosphate in the fluoride experiments but not in the iodoacetate experiments.

3. From these results it is deduced that the energy necessary for phosphorylation in the presence of iodoacetate is not obtained by oxidation of triosephosphate but by breakdown of the phosphopyruvate formed by such an oxidation, and that coupling of the oxidation with phosphorylation of creatine by free inorganic phosphate does not occur. It is also deduced that the energy for phosphorylation of creatine by fluoride-poisoned extracts is obtained either from coupled dismutation alone, or possibly from coupled dismutation and a coupled oxidation.

4. Results were obtained suggesting that, in the oxido-reduction reaction of triosephosphate with pyruvic acid, triosephosphate dehydrogenase is not concerned.

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