XXXIII. ENZYMIC PHOSPHORYLATIONS IN INVERTEBRATE MUSCLE

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It has been shown by Lehmann [1935] that the enzymic transfer of phosphate from phosphopyruvic (or phosphoglyceric) acid to arginine, in crab's muscle extract, is readily brought about by adenosine-diphosphate or -triphosphate but not by equivalent amounts of adenylic acid. In rabbit's muscle extract, however, where phosphate is transferred to creatine, adenylic acid is also active.

The following reactions are known to occur in vertebrate muscle (see the reviews by Parnas [1937] and Needham [1937]):

The sum of reactions (1) and (2) gives:

By studying the dephosphorylation of phosphoarginine in crab and octopus muscle, Lohmann [1935; 1936] obtained evidence for the occurrence of the following reaction:

Phosphoarginine was not dephosphorylated by adenylic acid.

These findings made likely the occurrence of the following reaction (5), in invertebrate muscle, in the place of reaction (1):

If adenylic acid could not be phosphorylated by phosphopyruvic acid, its ineffectiveness in activating the synthesis of phosphoarginine would be understandable. This point, however, had not been hitherto investigated although Lohmann [1936] adduced indirect evidence to show that phosphorylation of adenylic acid may occur in octopus muscle. This is able to dephosphorylate phosphopyruvic acid in presence of adenylic acid, a reaction which requires preliminary phosphorylation of the latter with subsequent breakdown of the adenosine-polyphosphate by the specific phosphatase.

I have now found, working with lobster muscle, that although phosphopyruvic acid readily phosphorylates adenylic acid in the absence of arginine, this is not the case when the latter is present.

The phosphorylations of cozymase and alkali-inactivated cozymase have also been studied. It has been found that manganese can replace magnesium in the transfer of phosphate from phosphopyruvic acid to arginine as observed by Ohlmeyer & Ochoa [1937] for the transfer of phosphate to glucose in yeast.

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METHODS

Acetone powder preparations from the tail muscle of the common lobster (*Homarus vulgaris*) have been used. These were obtained, according to Meyerhof & Kiessling [1935], from distilled water extracts (4 parts water to 3 parts muscle). The acetone powders have, among others, the advantage of being practically free from adenosine-polyphosphatase, so that no significant liberation of inorganic phosphate took place in these experiments. Extracts were prepared from the acetone powder by dissolving it in distilled water (40 mg./ml.). The extracts were dialysed at 0° in collodion tubes against distilled water, usually for 13–16 hr.

For the experiments on the synthesis of phosphagen, 0.8 ml. samples of the dialysed extract were made up, with additions, to 2 ml. and incubated for 15 min. at 30°. For those on the phosphorylation of adenylic acid and cozymase, 1.7 ml. samples of the dialysed extract were made up to 3 ml. and incubated for 5 min. at 30°. The additions included phosphate buffer pH 6.8 (final concentration $M/150$) and, occasionally, NaHCO_3 . After incubation the samples were deproteinized with trichloroacetic acid and the phosphate fractions determined in the filtrates as described by Lehmann [1935] and Lohmann [1936].

I am indebted to Prof. O. Meyerhof for kindly supplying adenosine-diphosphate and triphosphate and Kiessling's synthetic phosphopyruvic acid, to Dr P. Ohlmeyer for a supply of pure cozymase.

Since adenosine diphosphate, adenosine-triphosphate and cozymase contain adenylic acid in their molecules, the amounts of these substances are expressed throughout in mg. P_2O_5 of their adenylic acid equivalent.

Transfer of phosphate from phosphopyruvic acid to arginine

While Lehmann [1935], in comparing the activities of adenylic acid and adenosine triphosphate, only tried equivalent amounts of these compounds, it has been found here that the former compound, at a concentration 60 times higher, is still less effective than the latter (see Table I). In view of the findings of Ohlmeyer & Ochoa [1937] on the influence of metallic ions on the transfer of phosphate from phosphopyruvic acid to glucose in yeast maceration juice, Mn^{++} (as MnSO_4) has been added in some cases or else the Na^+ , used for neutralization and buffering, has been substituted by NH_4^+ , with no significant differences. The experiment of Table I was performed with an extract dialysed for 15 hr. Each sample had, besides phosphate, 0.1 mg. Mg^{++} (as MgCl_2), 1.7 mg. phosphopyruvate P_2O_5 and 14 mg. neutralized arginine. As alkaline cations

Table I. *Transfer of phosphate from phosphopyruvic acid to arginine in lobster muscle extract*

Incubation 15 min. at 30°				
Alkaline cation besides K ⁺	mg. adenylic acid equivalent P ₂ O ₅ added		mg. Mn added	mg. phospho- arginine P ₂ O ₅ synthesized
	Adenosine triphosphate	Adenylic acid		
Na ⁺	—	—	—	0.03
Na ⁺	0.02	—	—	1.30
Na ⁺	0.02	—	0.06	1.31
Na ⁺	—	0.30	—	0.02
Na ⁺	—	0.30	0.06	0.16
NH ₄ ⁺	—	—	—	0.09
NH ₄ ⁺	0.005	—	—	0.40
NH ₄ ⁺	0.01	—	—	0.53
NH ₄ ⁺	0.015	—	—	1.02
NH ₄ ⁺	0.02	—	—	1.32
NH ₄ ⁺	—	0.30	—	0.26

K^+ (0.17×10^{-2} mol.) and either Na^+ (6.5×10^{-2} mol.) or NH_4^+ (7×10^{-2} mol.). The Na^+ samples also had 0.13 % bicarbonate.

As in the transfer of phosphate from phosphopyruvic acid to glucose in yeast [Ohlmeyer & Ochoa, 1937], Mn^{++} can be substituted for Mg^{++} in the similar transfer to arginine in lobster muscle extract. For this purpose an extract was used which was dialysed for 38 hr., changing the water at 12 hr. intervals, as after the ordinary dialysis period of 15 hr. enough Mg^{++} is apparently present to allow the reaction to proceed on addition of adenosine triphosphate only. Each sample contained besides phosphate, 1.9 mg. phosphopyruvate P_2O_5 and 14 mg. arginine, as alkali cations only K^+ and NH_4^+ in concentrations as before. Table II shows that the ratio of the molar concentrations of Mn^{++} and Mg^{++} producing a similar effect is approximately $Mn^{++} : Mg^{++} = 1 : 2$ to $1 : 1$ under the experimental conditions.

Table II. *Substitution of Mg^{++} by Mn^{++} in the transfer of phosphate from phosphopyruvic acid to arginine in lobster muscle extract*

Incubation 15 min. at 30°			
Adenosine triphosphate added (in mg. adenylic acid equivalent P_2O_5)	Mg added mol. $\times 10^{-6}$	Mn added mol. $\times 10^{-6}$	mg. phospho-arginine P_2O_5 synthesized
—	2	—	0.13
0.02	—	—	0.25
0.02	1	—	0.50
0.02	2	—	0.94
0.02	4	—	1.33
0.02	6	—	1.43
0.02	—	0.5	0.63
0.02	—	1	0.87
0.02	—	2	1.05

Transfer of phosphate from phosphopyruvic acid to adenylic acid and cozymase

In vertebrate muscle phosphate is readily transferred from phosphopyruvic acid to adenylic acid which is thereby transformed into adenosine triphosphate (reaction (1)). The phosphorylation of cozymase, on the other hand, is very limited and only some 10 % of the theoretical pyrophosphate formation is obtained [Meyerhof & Ohlmeyer, 1937; Ohlmeyer & Ochoa, 1937]. If cozymase be heated with alkali the resulting products, which are inactive in oxidoreductions, yield a pyrophosphate fraction just as adenylic acid does. Von Euler has shown that, on alkaline inactivation, adenylic acid and probably some adenosinediphosphate are set free from cozymase [Vestin & Euler, 1936; Schlenk *et al.* 1937]. In lobster muscle extracts cozymase is also phosphorylated to a very small extent, whereas adenylic acid and alkali-inactivated cozymase are readily phosphorylated (Table III). Provided that the acetone preparation is not too old and the dialysis not very long, the phosphorylation of adenylic acid proceeds practically quantitatively to adenosine triphosphate. In Exp. 2 of Table III the formation of pyrophosphate clearly surpasses theory for adenosine diphosphate which would be 1.2 mg. of easily hydrolysable P_2O_5 (formed 1.9). There is therefore no doubt that reaction (1) can also take place in lobster muscle.

Owing to the equilibrium reaction

the disappearance of phosphopyruvic acid is always greater than could be accounted for by pyrophosphate formation, the rest being converted into phosphoglyceric acid. Inorganic phosphate is not liberated.

The experiments of Table III were performed with extracts dialysed for 16 hr. (Exp. 1) and 14 hr. (Exp. 2). Each sample contained phosphate, Mg (0.1 mg.), phosphopyruvate and the additions indicated in the table. Alkali cations K⁺ and Na⁺.

Table III. *Transfer of phosphate from phosphopyruvic acid to adenylic acid, cozymase and alkali-inactivated cozymase in lobster muscle extract*

Incubation at 30°				
No.	Min.	Nucleotide added (1.2 mg. adenylic acid equivalent P ₂ O ₅)	mg. phosphopyruvate P ₂ O ₅	mg. pyrophosphate P ₂ O ₅
1	0	—	2.60	0.00
	5	Adenylic acid	1.01	1.06
	5	Cozymase	1.64	0.17
	5	Alkali-inactivated cozymase 1*	1.18	1.27
	5	Alkali-inactivated cozymase 1†	1.11	0.91
2	0	—	2.50	0.00
	5	Adenylic acid	0.18	1.90

* 5 min. 100° in 0.05 N NaOH.

† 6 min. 100° in 0.06 N NaOH.

The ineffectiveness of adenylic acid and the effectiveness of adenosine-diphosphate and adenosine-triphosphate in the transfer of phosphate to arginine in invertebrate muscle provides a reliable method of testing the liberation even of very small amounts of adenosine-diphosphate on alkaline hydrolysis of cozymase. If a sample of the hydrolysate proves effective in activating the transfer of phosphate to arginine, adenosine diphosphate must have been set free since adenylic acid and cozymase are themselves inactive. That such is the case, thus confirming von Euler's results, is shown in Table IV. Besides the two samples of alkali-inactivated cozymase, adenylic acid, adenosine-diphosphate and cozymase itself have been tested for comparison. The extract was dialysed for 15 hr. Each sample contained phosphate, 0.13 % NaHCO₃, 0.1 mg. Mg, 1.7 mg. phosphopyruvate P₂O₅ and 14 mg. arginine.

Table IV. *Transfer of phosphate from phosphopyruvic acid to arginine with adenosine-diphosphate, cozymase and alkali-inactivated cozymase in lobster muscle extract*

Incubation 15 min. at 30°					
Nucleotide added (in mg. adenylic acid equivalent P ₂ O ₅)					mg. phospho-arginine P ₂ O ₅ synthesized
Adenosine triphosphate	Adenosine diphosphate	Adenylic acid	Cozymase	Alkali-inactivated cozymase (No.)	
—	—	—	—	—	0.05
0.02	—	—	—	—	1.37
—	0.02	—	—	—	0.93
—	0.04	—	—	—	1.36
—	—	0.4	—	—	0.16
—	—	—	0.4	—	0.08
—	—	—	—	0.4 (1)	1.23
—	—	—	—	0.4 (2)	0.40

Whilst both samples of alkali-inactivated cozymase showed a good phosphate uptake (Table III) sample 2 has practically no effect on the arginine-phosphate synthesis. The phosphate acceptor must have been mainly adenylic acid.

Sample 1, however, has a marked effect, 0.4 mg. adenylic acid equivalent P_2O_5 being approximately as effective as 0.04 mg. adenosine-diphosphate. The milder alkaline hydrolysis would thus have set free some 10% of the theoretical yield of adenosine-diphosphate.

The fact that adenylic acid is readily phosphorylated by phosphopyruvic acid in lobster muscle extract makes it difficult to see why, when arginine is present, no synthesis of phosphoarginine takes place. If, after phosphorylation, part of the sample be heated at 100° for 2 min., cooled and centrifuged, the resulting solution proves quite effective in activating the synthesis of phosphoarginine, which makes it very likely that the easily hydrolysable phosphate formed is linked as adenosine-polyphosphate. The same experiment can be performed in a different way which, in fact, gives the explanation for the inactivity of adenylic acid. If adenylic acid be incubated with phosphopyruvic acid and lobster muscle extract for 5 min. and arginine then added, a good synthesis of phosphoarginine takes place on incubating for a further 15 min. in the usual way. The following typical example may be quoted. 0.04 mg. adenylic acid P_2O_5 was incubated at 30° with 0.8 ml. of dialysed lobster muscle extract and 0.2 mg. phosphopyruvate P_2O_5 . The sample contained, as usual, phosphate and Mg (0.1 mg.). After 5 min., 14 mg. arginine, 2 mg. phosphopyruvate and $NaHCO_3$ to make 0.13% concentration were added and incubated for a further 15 min. The synthesis of phosphoarginine was 0.23 mg. P_2O_5 in a blank sample which contained no adenylic acid and 1.33 mg. P_2O_5 in the sample which contained adenylic acid.

The data here presented suggest that phosphate is not transferred from phosphopyruvic acid to adenylic acid, in lobster muscle extract, in presence of arginine and this accounts for the inactivity of the nucleotide when all the three are added together. Under these conditions, therefore, and probably in the normal muscle, reaction (5), but not reaction (1), would take place. The combination of reactions (4) and (5) would give here the equation of the synthesis of phosphoarginine from phosphopyruvic acid and arginine:

Some experiments have also been performed with fish muscle. Acetone powders from the dorsal muscle of the ray (*Raja clavata*) were used. Phosphate is readily transferred from phosphopyruvic to adenylic acid but only to a very slight extent to cozymase. The transfer of phosphate from phosphopyruvic acid to creatine is also easily obtained. As in other vertebrates, adenylic acid is effective in this reaction although less so than adenosine triphosphate. It is interesting that the activity of adenylic acid does not seem to be limited to the transfer of phosphate from phosphopyruvic acid to creatine in vertebrate muscle. Baldwin & Needham [1937] have found it to be active in the transfer of phosphate to the two guanidine bases in echinoid jaw muscle and to arginine in holothurian muscle.

In agreement with previous findings as to the specificity of the enzymes catalysing the esterification of arginine and creatine in muscle extracts from invertebrates and vertebrates, it has been found that creatine is not esterified by lobster muscle extracts nor is arginine by those from ray muscle.

SUMMARY

In extracts of lobster muscle adenylic acid does not activate the transfer of phosphate from phosphopyruvic acid to arginine although it can be readily phosphorylated in absence of the latter. Alkali-inactivated cozymase is also easily phosphorylated but cozymase only to very small extent. It is suggested that the presence of arginine inhibits the phosphorylation of adenylic acid.

In the transfer of phosphate from phosphopyruvic acid to arginine manganese can be substituted for magnesium.

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