CCLXV. THE SPECIFICITY OF COUPLED ESTERIFICATION OF PHOSPHATE IN MUSCLE

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It has been shown that, while a number of oxidoreductions involving oxidation of triosephosphate to phosphoglyceric acid can be coupled with synthesis of adenylypyrophosphate from adenylic acid and free phosphate, similar oxidoreductions involving oxidation of glyceraldehyde to glyceric acid are not so coupled [Needham & Pillai, 1937]. Further experiments, in which a variety of conditions have been tried, have confirmed this lack of coupling. From these observations the inference may be drawn that the essential energy-providing reaction in the coupled mechanism is the reduction of coenzyme I by glyceraldehydephosphate; for the oxidation of reduced coenzyme by pyruvate goes on (when glyceraldehyde is the reductant in the oxidoreduction) without coupled esterification. As this inference must be important in the elucidation of the mechanism of the coupling, it was decided to test it further.

This has been done by separating the oxidoreduction into its two constituent reactions:

- (1) glyceraldehydephosphate + coenzyme
→ phosphoglyceric acid + reduced coenzyme,
- (2) pyruvate + reduced coenzyme → lactic acid + coenzyme,

and testing each separately for its effect on esterification. The experiments with reaction (2) are quite straightforward, but with reaction (1) a complication arises. In muscle extract it has not so far been possible to separate the glycerophosphate dehydrogenase from the triosephosphate dehydrogenase; this means that, in aqueous muscle extracts or in extracts of acetone powder, triosephosphate acts as oxidant as well as reductant, and the coenzyme reduced during phosphoglyceric acid formation is oxidized by more triosephosphate, which is itself reduced to glycerophosphate. We have therefore studied the triosephosphate oxidation to phosphoglycerate under conditions where the reduced coenzyme was largely, if not entirely, reoxidized by atmospheric O_2 through the cytochrome system. To enable O_2 uptake to go on, it was necessary to add to the extract of muscle acetone powder ordinarily used for study of oxidoreductions, a small amount of an enzyme preparation from heart made according to the method of Keilin & Hartree [1938]. This preparation contains (1) coenzyme factor, the enzyme catalysing the transfer of hydrogen from reduced coenzyme I or II to a carrier such as cytochrome [Dewan & Green, 1938]; (2) cytochromes *a* and *b*; (3) cytochrome oxidase, the enzyme catalysing the oxidation of reduced cytochrome by atmospheric O_2 [Keilin & Hartree, 1938]. When this heart

preparation is added to muscle extract, the following series of reactions can take place:

The amount of cytochrome present in our preparations was not optimal, and greater O_2 uptake was always obtained by adding methylene blue. Then

EXPERIMENTAL DETAILS

Enzyme preparations. The aqueous extract of dried acetone muscle powder (used in all the experiments to provide the oxidoreduction and phosphorylation mechanisms) was prepared in the same way as described by Needham & Pillai [1937]. Before use the extracts were dialysed 18 hr.

The heart preparation (added when interaction with atmospheric O_2 was needed) was made from pig's heart in the following way. Two hearts were freed from fat and minced twice through an ordinary mincer; the mince was thoroughly washed by suspending (with mechanical stirring) in about 5 l. tap water; this was repeated ten times, the muscle mass being squeezed in muslin each time before resuspension. It was then ground with fine sand and 250 ml. $M/25$ phosphate buffer (pH 7) for $1\frac{1}{2}$ hr. in a mechanical mortar. The thick paste was mixed with 350 ml. more buffer solution and centrifuged. The cloudy fluid was poured off, and acetate buffer pH 4.6 was added until the pH of the whole had fallen to 4.6. The mixture was again centrifuged; the clear fluid was discarded, and the residue was suspended in 30 ml. phosphate buffer ($M/10$ pH 7.2). Finely powdered Na_2HPO_4 was added until the pH was 7.0. The preparation was stored at 0° in this form; at the end of a week its activity was about 60% of the initial. Before use, portions of the preparation were dialysed 18 hr. in cellophane bags suspended in 3 l. 0.5% KCl .

It was already known that the extract of acetone muscle powder contained no adenylypyrophosphatase and no deaminase. As the heart preparation also was to be used in experiments where adenylypyrophosphate synthesis from adenylic acid was to be looked for, it was necessary to find out in this case too whether enzymes were present which could dephosphorylate synthesized adenylypyrophosphate, or deaminate added adenylic acid.

As regards possible destruction of added adenylic acid, the position appeared satisfactory; for under the conditions of the experiments, not more than 5% of the possible NH_3 was set free in 15 min.

On the other hand, unfortunately, it seemed to be impossible to free the heart preparation from adenylypyrophosphatase. Even after some days at 0° and several hr. dialysis added adenylypyrophosphate was broken down to a considerable extent. In order in these circumstances to demonstrate disappearance of inorganic phosphate, we tried adding to the experimental mixture glycogen as well as adenylic acid, in the hope that phosphate would be transferred to glycogen from synthesized adenylypyrophosphate more rapidly than it was split

off again from the adenylypyrophosphate to give free phosphate. Experiments in which adenylypyrophosphate+glycogen were added to the enzyme mixture showed that, even in the presence of glycogen, rather more than half the pyrophosphate disappearing was found as inorganic phosphate; thus the adenylypyrophosphatase appeared to be more active than the enzyme concerned with phosphate transfer. Creatine (at pH 9.0) instead of glycogen was tried, but with even less success. In absence, therefore, of any better way of demonstrating esterification of free phosphate, glycogen+adenylic acid were used in the O_2 uptake experiments.

The use of glycogen in this way led to the possibility of another source of error: the Parnas esterification of inorganic phosphate with glycogen might take place, leading to disappearance of inorganic phosphate quite independently of the coupled mechanism. Tests such as the following showed, however, that with the concentrations of phosphate, glycogen and enzymes used in the experiments, no Parnas esterification was observable.

Three mixtures were prepared. Each contained glycogen 3.6 mg., adenylic acid 0.6 mg., phosphate (0.066 *M*, pH 7.2) 0.3 ml., $MgSO_4$ (0.006 *M*) 0.05 ml. and NaF (0.5 *M*) 0.1 ml.; water to 2.4 ml. In addition the following were added:

	1	2	3
Heart preparation	0.75 ml.	0.75 ml.	0 ml.
Muscle extract	0.25 ml.	0 ml.	0.25 ml.

An initial sample of 0.5 ml. was removed from each, then all were incubated at 37°. Samples were again removed at the end of 15 min. and 30 min. Only in no. 3 was any change in free phosphate observed, and here the decrease was only 0.09 mg. out of 0.6 mg.

Finally, tests were made to see whether the hexosediphosphatase activity discussed by Pillai [1938, 1] was of importance under our conditions.

Three mixtures were prepared and incubated at 37°. Each contained hexosediphosphate (0.14 *M*) 0.15 ml., phosphate (0.066 *M*, pH 7.2) 0.3 ml., $MgSO_4$ (0.006 *M*) 0.1 ml. and NaF (0.5 *M*) 0.1 ml.; water to 2.2 ml. In addition the following were added:

	1	2	3
Heart preparation	0.75 ml.	0.75 ml.	0 ml.
Muscle extract	0.25 ml.	0 ml.	0.25 ml.

Samples were removed at 0, 15 and 30 min. The increase in inorganic phosphate found was within the limits of experimental error.

Summing up this preliminary examination of the properties of the enzyme preparations to be used, we may therefore say that no destruction of adenylic acid by deaminase, no significant removal of free phosphate by the Parnas esterification, and no significant increase in free phosphate by hexosediphosphatase activity is to be expected. On the other hand, owing to the persistence of adenylypyrophosphatase in the heart preparation, some hydrolysis of adenylypyrophosphate, even in presence of glycogen, will take place, and one can hope to observe only a fraction of the adenylypyrophosphate synthesis actually occurring.

Chemical preparations and methods. These were for the most part the same as those used in previous work [e.g. Needham & Pillai, 1937]. For estimation of phosphoglyceric acid the method of Rapoport [1937] was followed and for O_2 uptake the method of Warburg, using 15 ml. conical cups with two side-bulbs.

LACK OF ESTERIFICATION WITH GLYCERALDEHYDE OXIDOREDUCTION

In the preliminary experiments of Needham & Pillai [1937] it was found that oxidoreduction between glyceraldehyde and pyruvate went on unaccompanied by esterification of free phosphate when adenylic acid was present as phosphate acceptor. About 1.5 mg. lactic acid per ml. extract were formed; when similar amounts of lactic acid were formed by reduction of pyruvate by triosephosphate, about 0.5 mg. inorganic P disappeared. In the present work these results were confirmed. It seemed possible that the high adenylic acid concentration used (about 3 mg./ml. final concentration) might have some deleterious effect upon the coupled mechanism with glyceraldehyde as a component, although not with triosephosphate. Some experiments were therefore done with glycogen and creatine provided as possible acceptors of phosphate from adenylypyrophosphate if formed, only small amounts of adenylic acid being added.

Typical experiments are given below.

1 ml. extract was made up to a final volume of 3 ml. The final concentrations were: pyruvate, 0.026 *M*; hexosediphosphate, 0.013 *M*; inactive glyceraldehyde, 0.052 *M* (to allow for the *l*-component, which does not react); phosphate, 0.03 *M*; NaF, 0.025 *M*; NaHCO₃, 0.01 *M*. Crude coenzyme was added, 0.3 ml. of a solution containing 150 mg. per 100 ml. All samples were incubated 30 min. at 37°, pH 8.0. The results are expressed in mg. per ml. extract.

Table I

	Lactic acid mg.	Inorganic P mg.	Creatine- phosphate P mg.
Adenylic acid, 0.01 <i>M</i> (3.4 mg. per ml.)			
Hexosediphosphate + pyruvate	+ 1.2	- 0.5	—
Glyceraldehyde + pyruvate	+ 1.5	0	—
Adenylic acid, 0.002 <i>M</i> (0.7 mg. per ml.)			
Hexosediphosphate + pyruvate + glycogen (6 mg.)	+ 0.94	- 0.27	—
Glyceraldehyde + pyruvate + glycogen (6 mg.)	+ 0.90	0	—
Hexosediphosphate + pyruvate + creatine (10 mg.)	+ 1.31	—	+ 0.17
Glyceraldehyde + pyruvate + creatine (10 mg.)	+ 1.10	—	0

In the case of glyceraldehyde oxidoreduction with pyruvate, it was noticed that the lactic acid formation was much greater in a sample to which no adenylic acid or phosphate had been added. Further experiment showed that addition of adenylic acid has a marked inhibitory effect on the oxidoreduction, while addition of phosphate has a stimulating effect.

Table II

	Lactic acid formed (mg.)	
	Exp. 45 A	Exp. 45 B
No adenylic acid and no phosphate added	0.43	0.37
Both added	0.84	1.05
Phosphate added, no adenylic acid	1.44	1.24

For hexosediphosphate oxidoreduction with pyruvate the following results were found:

Table III

	Lactic acid formed (mg.)		
	Exp. 46	Exp. 48	Exp. 59 .
No adenylic acid and no phosphate added	0.84	0.64	—
Both added	1.28	2.4	1.19
Phosphate added, no adenylic acid	1.09	1.54	0.39

Here again the stimulating effect of phosphate addition is seen, but in this case adenylic acid shares in the stimulation instead of opposing it.

In the previous work of Needham & Pillai [1937] a stimulatory effect of higher adenylic acid concentrations (0.02 and 0.03 *M*) upon the oxidoreduction with triosephosphate was noticed; and Pillai [1938, 2] has found that with very thoroughly dialysed extracts (90 hr.), though oxidoreduction can go on without addition of phosphate or adenylic acid, activation follows on addition of these substances. Meyerhof *et al.* [1937; 1938] have observed the very marked stimulating effect of adenosinediphosphate upon reduction of coenzyme I by triosephosphate in presence of B protein from yeast, and this has been confirmed by Adler & Günther [1938]. Meyerhof and his collaborators consider that only when the esterification process is going on can the oxidoreduction between triosephosphate and pyruvate proceed. In view of all these facts the inhibitory effect of adenylic acid upon the uncoupled glyceraldehyde oxidoreduction is interesting.

ESTERIFICATION ACCOMPANYING TRIOSEPHOSPHATE OXIDATION

In these experiments 1 ml. heart preparation and 0.3 ml. acetone powder extract were used in each Warburg cup, with 0.1 ml. 5% methylene blue. The amount of heart preparation was the limiting factor in the O_2 uptake, which was not increased by using more muscle extract or carrier. The total volume was 3–4 ml. The concentration of phosphate was about 0.01 *M* and its *pH* 7.2. Crude coenzyme I was added, 0.4 ml. of a solution containing 150 mg. per 100 ml. NaF 0.025 *M* was always present to prevent breakdown of the phosphoglyceric acid formed. As explained in an earlier section, adenylic acid was used in low concentration as transporter in these experiments, and glycogen was present to accept the phosphate. About 1 mg. adenylic acid and 6 mg. glycogen were present in each cup. To provide the triosephosphate for oxidation, hexosediphosphate was added in amount to give a final concentration of 0.01 *M*. The neutralized solutions of hexosediphosphate and of adenylic acid were placed in the two side-bulbs, and were tipped in only after temperature equilibration.

Controls showed that (a) without added hexosediphosphate no O_2 uptake occurred; (b) addition of methylene blue increased the O_2 uptake by about 50%; (c) it was very low without acetone powder extract; (d) with acetone powder extract but no heart preparation it was about 20% of the amount when both were present. Iodoacetate (0.003 *M*) completely inhibited the O_2 uptake with hexosediphosphate.

All samples in Table IV were kept 30 min. at 37°; the results are given in μ l. and mg. per cup.

In most of these experiments, no dismutation of the hexosediphosphate has taken place. This is shown by the fact that the increase in difficultly hydrolysable P and the increase in phosphoglyceric acid P are about equivalent to each other and to the O_2 uptake. In a minority of the experiments, however, dismutation as well as oxidation of the triosephosphate took place. This is shown by the fact that the increase in difficultly hydrolysable P is much more than the equivalent of the O_2 uptake. In Exp. 30, for example, where the phosphoglyceric acid P was measured as well, this was found to be greater than the P equivalent of the O_2 uptake by 0.5 mg. This amount therefore must have been formed by dismutation, and the presence of 0.5 mg. glycerophosphate P was to be expected. 2.24 mg. difficultly hydrolysable P were indeed found.

Table IV

Exp.	O ₂ uptake μl.	P equiv. of O ₂ uptake mg.	Increase in diff. hydr. P mg.	Phospho- glyceric acid P formed mg.	Change in inorganic P mg.
24	210	0.6	0.52	—	-0.7
25	153	0.43	0.55	—	-0.66
27	468	1.3	1.7	—	-0.71
27	384	1.1	1.41	—	-0.89
31	231	0.64	0.52	0.748	-0.20
31	198	0.55	0.70	0.746	-0.21
31	249	0.68	0.72	0.702	-0.29
32	172	0.48	0.43	0.62	-0.19
32	181	0.50	0.64	0.71	-0.22
35	252	0.73	0.82	1.00	-0.46
37	335	0.93	0.87	1.1	-0.25
37	310	0.86	0.97	1.1	-0.22
37	300	0.84	0.99	1.1	-0.30
64	267	0.74	0.63	—	-0.4
64	429	1.2	0.99	—	-0.75
26	380	1.13	2.205	—	-1.02
26	263	0.77	2.17	—	-0.98
30	389	1.07	2.24	1.54	-0.71

These results on muscle may be compared with those obtained by Lennerstrand & Runnström [1935] and Lennerstrand [1936] on apozymase from dried yeast. Adenylpyrophosphatase seems to be absent from such preparations, for there was good agreement between inorganic phosphate disappearing and phosphoglyceric acid formed. Lennerstrand found that, using phosphate concentrations of about 0.03 *M*, the O₂ uptake was much less than with higher P concentrations. At a P concentration of nearly 0.3 *M* it had increased to tenfold. This effect was caused by long persistence of the power of O₂ uptake, the initial rate being about the same at all P concentrations. No correspondingly large effects were observed with muscle. Raising the P concentration from 0.01 to 0.03 *M* caused a 50% rise in O₂ uptake; but further increase to 0.1 *M* caused no further rise.

Meyerhof *et al.* [1938], using "B protein" from yeast (which contains no glycerophosphate dehydrogenase), have recently shown that when triose-phosphate reacts with coenzyme I to form phosphoglyceric acid and reduced coenzyme there is equivalent phosphate esterification.

Some experiments were done in which lactate and succinate were added as substrate for oxidation. The experimental conditions were as usual, except that, with lactate, HCN was added to remove the pyruvate formed [Green & Brosteaux, 1936]. No esterification accompanied O₂ uptake in these cases.

Table V

Substrate	O ₂ uptake μl.	Change in inorg. P mg.
Lactate	190	-0.06
Succinate	240	-0.10

LACK OF ESTERIFICATION WITH OXIDATION OF REDUCED COENZYME

In these experiments excess of pyruvate together with a known amount of reduced coenzyme I were added to the extract of muscle acetone powder, in presence of phosphate and adenylic acid. The amounts of reduced coenzyme I, of lactic acid and of inorganic P were estimated at the beginning and end of the incubation time.

The estimation of reduced coenzyme was made by measurement of the height of the absorption curve at 345 m μ , using a Spekker photometer.

The absorption coefficient $\beta = \frac{1}{d} \times \frac{1}{c} \times \ln \frac{I_0}{I}$,

where d = length of tube in cm.

c = concentration of absorbing solute in g. mol. per ml.

$\frac{I_0}{I}$ = $\frac{\text{intensity of light entering tube}}{\text{intensity of light leaving tube}}$.

Warburg *et al.* [1935; 1938] have shown for the dihydro bands at 345 m μ , characteristic of the nucleotides coenzymes I and II, that

$$\ln \frac{I_0}{I} = 1.3 \times 10^7,$$

when $d=1$ and $c=1$ g. mol. per ml. When $d=2$ (as in our experiments) and $c=0.1$ mg. per ml.

$$\ln \frac{I_0}{I} = \frac{1.3 \times 10^7 \times 2}{663 \times 10,000}.$$

As the drum readings on the Spekker instrument are engraved to give $\log \frac{I_0}{I}$, not $\ln \frac{I_0}{I}$, the reading to be expected for 0.1 mg. reduced coenzyme I per ml. in the 2 cm. tube is

$$\log \frac{I_0}{I} = \frac{1.3 \times 10^7 \times 2}{663 \times 10,000} \times \frac{1}{2.3} = 1.7.$$

Solutions of coenzyme I of unknown concentration were diluted to give a reading of about this value, and the original concentration could then be calculated.

The coenzyme I preparation used was made in the usual way [see Needham & Pillai, 1937]. Tests showed that it contained about 25% of the coenzyme. The reduced coenzyme was prepared from it by treatment with Na₂S₂O₄, excess Na₂S₂O₄ was oxidized by vigorous aeration and the Na₂SO₃ was removed according to the method of Euler & Hellström [1938]. One experiment is described in detail below.

300 mg. crude coenzyme and 40 mg. Na₂S₂O₄ were dissolved in 6.6 ml. 1.3% NaHCO₃ which had previously been gassed with a stream of N₂+5% CO₂. The mixture stood at room temp. 1 hr. with the gas stream running. The N₂-CO₂ stream was then exchanged for an air current, which ran for 2 hr. At the end of this time, the mixture was warmed to 80°, a slight excess of BaCl₂ was added and it was filtered through a heated funnel. The excess Ba was removed with Na₂SO₄. The final volume was 8.4 ml. Two mixtures were made up in Warburg cups, which were then filled with N₂-5% CO₂ mixture and shaken at 18°, until the gas absorption (due to the decrease in acidity when reduced coenzyme is oxidized) was finished.

Each contained reduced coenzyme solution 2 ml., adenylic acid (0.1 *M*) 0.2 ml., phosphate (0.05 *M*, pH 7.2) 0.5 ml., NaHCO₃ (3.3%) 0.4 ml. and muscle extract 0.3 ml.; to cup no. 1 pyruvate (0.25 *M*) 0.2 ml. was also added, while 0.2 ml. water was added to cup no. 2.

At the end of the time the cups were emptied into test tubes and washed out with three successive lots of 2 ml. water. The contents of the tubes were coagulated by placing in a boiling water bath and were then mixed with a small quantity of kieselguhr (*ca.* 20 mg.) and filtered through paper. The volume of each was made up to 20 ml. 2 ml. were used for phosphate determination, 15 ml. for lactic acid and 1 ml. was diluted to 5 ml. for measurement of the absorption spectra (Table VI).

Table VI

	Reduced coenzyme mg.	Lactic acid mg.	Phosphate mg.
1	0	1.83	0.87
2	9.4	0.69	0.87

Thus 1.14 mg. lactic acid have been formed from pyruvate, while 9.4 mg. reduced coenzyme (equivalent to 1.25 mg. lactic acid) have been oxidized. The inorganic phosphate remained absolutely unchanged.

In order to make sure that no substance capable of preventing phosphorylation was present in the reduced coenzyme solution used, the following control experiment was carried out. The "control solution" was made by carrying out all the processes involved in reduction of coenzyme, and removal of the $\text{Na}_2\text{S}_2\text{O}_4$ and Na_2SO_3 , but without adding coenzyme. Three mixtures were made up. Each contained hexosediphosphate (0.14 *M*) 0.2 ml., pyruvate (0.25 *M*) 0.2 ml., adenylic acid (0.1 *M*) 0.2 ml., phosphate (0.066 *M*) 0.5 ml., NaHCO_3 (1.3 %) 1.0 ml., NaF (*M*) 0.1 ml., coenzyme solution (0.15 %) 0.2 ml. and muscle extract 0.3 ml. No. 1 contained also 2.0 ml. water, nos. 2 and 3 contained each 2.0 ml. control solution. Nos. 1 and 2 were incubated 10 min. at 37°; then coagulated by placing in boiling water. In the case of No. 3, the tube was placed in boiling water before addition of the enzyme solution, so that the latter was coagulated at once. All were mixed with kieselguhr and filtered as usual, and the inorganic P was estimated (Table VII).

Table VII

No.	Inorganic P mg.	P esterified mg.
1	0.55	0.22
2	0.52	0.25
3	0.77	—

The control solution has thus had no inhibitory effect upon the phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate.

Meyerhof *et al.* [1938] reached the same conclusion that, in yeast, oxidation of reduced coenzyme by acetaldehyde is not connected with phosphorylation. In their experiments the phosphate disappearance was equivalent to the phosphoglyceric acid formed, as already mentioned, and this was the case whether or not acetaldehyde was introduced to reoxidize the reduced coenzyme.

At the beginning of this work the attempt was made to use the reduced coenzyme solution for experiments like that in Table VI without removing the Na_2SO_3 formed on aeration. But experiments on the lines of Table VII showed that these "control solutions" containing Na_2SO_3 had a very marked inhibitory effect upon phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate; the actual oxidoreduction, as measured by lactic acid formation, was unaffected.

Table VIII shows a comparison of the effects of the "control solution" (10 mg. $\text{Na}_2\text{S}_2\text{O}_4$ in 8 ml. 0.1 % NaHCO_3 ; well aerated), Na_2SO_3 solution (0.014 *M*) and arsenate solution (0.014 *M*). All the samples contained coenzyme, pyruvate, hexosediphosphate, adenylic acid, phosphate, muscle extract and NaF as in the experiment of Table VII.

Table VIII

	Lactic acid formed mg.	P esterified mg.
1. +3 ml. 1 % NaHCO_3	1.2	0.35
2. +3 ml. control solution	0.94	0.19
3. +3 ml. 0.02 <i>M</i> arsenate in 1 % NaHCO_3	1.50	0
4. +3 ml. 0.02 <i>M</i> Na_2SO_3 in 1 % NaHCO_3	1.09	0.19

This effect of sulphite is being further investigated.

SUMMARY

1. Further evidence has been obtained showing that in muscle extract oxidoreductions involving glyceraldehyde are not accompanied by coupled esterification of phosphate.

2. The oxidoreduction between triosephosphate and pyruvate has been separated into its two constituent reactions. The oxidation of triosephosphate to phosphoglyceric acid by coenzyme is accompanied by esterification; the oxidation of reduced coenzyme thus formed by pyruvate is not accompanied by esterification.

3. Adenylic acid has an activating effect on oxidoreduction involving triosephosphate, but an inhibitory effect on oxidoreduction involving glyceraldehyde.

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