CXLIV. DEPHOSPHORYLATION IN MUSCLE EXTRACTS

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THE dephosphorylating action of muscle extract on various substrates all of which are not necessarily intermediate products formed during normal glycolysis is very well known. The presence of the phosphatases, inorganic pyrophosphatase, adenylypyrophosphatase and hexosediphosphatase has been demonstrated [Lohmann, 1928, 2; 1933] and the enzymes catalysing the transfer of phosphate from creatinephosphate and phosphopyruvic acid to adenylic acid have been studied in greater detail. The dephosphorylation of phosphopyruvic acid like that of creatinephosphate has hitherto been considered to involve only a straightforward transfer of the phosphate to adenylic acid. This reaction has now been investigated further and it will be made clear that it is not by any means such a simple exchange as has been supposed.

In normal glycolysis where glycogen is the substrate it is easy to conceive of the adenylic acid being continuously esterified and reformed, the former during the dephosphorylation of phosphopyruvic acid, the latter by donating the phosphate to glycogen or hexosemonophosphate. This latter reaction will go on as long as any carbohydrate is left and will thus be regenerating the adenylic acid to act as acceptor for the phosphate from phosphopyruvic acid. The enzyme adenylypyrophosphatase would also help in the dephosphorylation but on the other hand the coupled esterification of adenylic acid recently demonstrated [Needham & Pillai, 1937; Meyerhof *et al.* 1937] would tend to remove some of the adenylic acid. It was supposed however that when hexosediphosphate is the substrate the adenylic acid is regenerated solely by the activity of the adenylypyrophosphatase since the hexose is already phosphorylated and cannot act in this way, and no other intermediate compounds are known which could serve as phosphate acceptor. An extract which does not contain adenylypyrophosphatase should therefore be incapable of producing lactic acid from hexosediphosphate when adenylypyrophosphate and not adenylic acid has been added as activator. This however is by no means the case and it therefore remained to be seen how phosphopyruvic acid could be dephosphorylated under these conditions. The possibility of coenzyme I acting in a capacity similar to that of adenylic acid, where it is alternately phosphorylated and dephosphorylated must be borne in mind but the mechanism of this reaction is still quite obscure even if it takes place at all [Ohlmeyer & Ochoa, 1937].

A fairly stable powder can be prepared from a fresh muscle extract by precipitation with acetone and drying: this gives an active extract with water and it is found that of the two pyrophosphatases present in the fresh extract, the adenylypyrophosphatase, which is comparatively weak even in the fresh extract, has almost completely disappeared while the inorganic pyrophosphatase is still quite active. The third phosphatase, the hexosediphosphatase has not suffered any serious inactivation. While this extract is therefore unable to decompose adenylypyrophosphate it was still found to be able to dephosphorylate

phosphoglyceric acid. That some hitherto unnoticed mechanism of phosphoglycerate dephosphorylation other than transfer through adenylic acid is here concerned is shown in a striking way by the fact that this extract cannot dephosphorylate creatinephosphate in the presence of adenylypyrophosphate. Further, this dephosphorylation of phosphoglycerate is increased several times by arsenate but with this difference: the activation by arsenate can be inhibited by iodoacetate while the original dephosphorylation is not.

The whole question of the mechanisms concerned in formation of inorganic phosphate in muscle is of interest, because in normal muscle contracting anaerobically increase in free phosphate, as well as in lactic acid and creatine, is always observed. Here again, the formation of the free phosphate has usually been explained by the action of adenylypyrophosphatase alone.

The present work includes some preliminary experiments on the pyrophosphatases and on hexosediphosphatase, particularly on separation of the enzymes and comparison of their activities. The dephosphorylation of phosphoglycerate was then investigated, especially the activation of this reaction by arsenate and the inhibition of the activation by iodoacetate. The experiments will be described in this order and the results examined and discussed at the end.

EXPERIMENTAL DETAILS

Enzyme preparation

The preliminary work on the pyrophosphatases was done on fresh muscle extract prepared by extracting fresh rat or rabbit muscle with ice-cold water or 0.5% KCl for $\frac{1}{2}$ –1 hr., centrifuging and dialysing the extract for 3–5 hr. For all remaining experiments, except when specified otherwise, aqueous extract of dried acetone powder from rabbit muscle prepared according to the method described in a previous paper [Needham & Pillai, 1937] was used. The extract was dialysed for 3–88 hr. according to the type of experiment and the details are given with each experiment. As will be evident from the experimental part of the work the acetone powder does not contain any adenylypyrophosphatase and it was not therefore necessary to keep the extracts for 5 days before use to inactivate this enzyme.

Chemical preparations used

Adenylypyrophosphate was made according to the method of Lohmann [1931]. For cozymase the method described by Green *et al.* [1937] was used. Phosphoglyceric acid was made by the method of Vercellone & Neuberg [1935], barium creatinephosphate according to Meyerhof *et al.* [1937]. Adenylic acid was obtained from the Laakoon Factory, Lwow, Poland.

Methods of estimation

Inorganic phosphate was estimated by the method of Fiske & Subbarow [1925]; when arsenate was present the Pett [1933] modification was used. The adenylypyrophosphate was estimated by means of its easily hydrolysable phosphate formed by 7 min. hydrolysis in *N* HCl at 100° [Lohmann, 1928, 1] after being separated from other interfering substances when present by barium precipitation. Creatinephosphate P was separated by barium precipitation of other phosphates and estimated according to Fiske & Subbarow [1929] when necessary; phosphopyruvic P was found by estimating P decomposed in 1 hr. hydrolysis in *N* HCl at 100°, with proper controls for small amounts of

other easily hydrolysable phosphorus compounds which might be present; the difficultly hydrolysable phosphate was found by subtracting the inorganic P present after 180 min. hydrolysis in *N* HCl at 100° from the total P. Pyruvate was determined by bisulphite treatment and subsequent iodine titration [Clift & Cook, 1932], lactic acid by the method of Friedemann & Graesser [1933].

Experimental procedure

For most of the preliminary experiments with pyrophosphatases 1 ml. of the fresh extract made up to 3–6 ml. with additions was used. 1 ml. of adenylypyrophosphate containing 0.55 mg. pyro-P, 1 ml. of 2.5 % $\text{Na}_4\text{P}_2\text{O}_7$, $10\text{H}_2\text{O}$ and 1 ml. of 1.5 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added to each when necessary, with sodium bicarbonate as buffer. The time of experiment was usually 1 hr. at 38°.

When the acetone powder extract was used for experiments with hexosediphosphate and phosphoglycerate 2 ml. extract were usually made up to 4–5 ml. with additions. The concentration of sodium hexosediphosphate was about 0.011–0.0125 *M*, of sodium phosphoglycerate 0.025 *M*, of A.T.P. 0.0006–0.0003 *M*, of cozymase 0.0003–0.0001 *M*. In the early experiments the arsenate concentration was 0.0025 *M*, later this was reduced to 0.001 *M*. The strength of iodoacetate used was in some cases 0.004 *M*, in other cases it was reduced to 0.0012 *M*. Sodium bicarbonate was used as buffer to give a final concentration of 0.45–0.22 %; the strengths of other additions are given in the proper places.

In all cases suitable controls were carried out simultaneously without addition of substrates but with all coenzymes and also with the substrates but without coenzymes.

Most of the experiments were done at 38° and the duration was 30 min.; those with arsenate were usually at room temperature (20°). Trichloroacetic acid was used to precipitate proteins and after filtration through paper P estimations were done on the filtrate. In the case of creatinephosphate P centrifuging was used in preference to filtration.

The results are expressed unless otherwise stated as mg. increase or decrease per ml. of extract.

Pyrophosphatases in fresh muscle extract and in acetone powder extract

Fresh muscle extract is known to contain a hexosediphosphatase, an inorganic pyrophosphatase and adenylypyrophosphatase [Lohmann, 1933]. Glycerophosphatase has generally been found to be absent. Of these the inorganic pyrophosphatase and the hexosediphosphatase are both quite stable and very active while the adenylypyrophosphatase is easily inactivated and is in any case not so active even in a freshly prepared extract as the inorganic pyrophosphatase. There is conflicting evidence in the literature as to whether these two pyrophosphatases are distinct and separate enzymes. Jacobsen [1931, 1933] claimed to have separated from liver an enzyme which was active towards adenylypyrophosphate but not inorganic pyrophosphate by selective destruction with 10 % NH_4Cl at 55° but doubts on the validity of this have been thrown by other workers [Lohmann, 1933; Satoh, 1935; Haase, 1936]. It is a comparatively easy matter to get the inorganic pyrophosphatase free from the adenylypyrophosphatase as the latter is very easily inactivated. But it must be remarked in passing that the enzyme is not completely destroyed by keeping for 5 or 6 days in the ice-chest or by long dialysis. Certain extracts were found to have considerable adenylypyrophosphatase activity even after 2 weeks and dialysis for 16 hr. The

activity seems to vary a good deal with different extracts and a minimum of 3 weeks would seem to be necessary. Table I shows the adenylypyrophosphatase activity of an extract during the course of 2 weeks.

Table I

	mg. P produced
Extract 5 days old, dialysed $8\frac{1}{2}$ hr.	0.42
Extract 9 days old, dialysed $8\frac{1}{2}$ hr.	0.22
Extract 11 days old, dialysed 16 hr.	0.11
Extract 13 days old, dialysed $8\frac{1}{2}$ hr.	0.12

0.55 mg. P present as pyrophosphate fraction of A.T.P.; time 1 hr. at 38° .

The fact that adenylypyrophosphatase so readily decreases in activity on dialysis or standing suggests the possibility that a coenzyme may be necessary.

Experiments were tried to see whether a solution freed from adenylypyrophosphatase by long standing could be reactivated by adding a fresh boiled extract which contained active adenylypyrophosphatase but no reactivation could be obtained. Table II shows the result of an experiment in which a fresh extract containing adenylypyrophosphatase acted upon inorganic and organic pyrophosphate separately and together and would seem to show that the enzymes were separate as the amounts of inorganic P produced are additive.

Table II

	Inorganic P		
	I	II	III
A.T.P. only	0.35	0.38	0.28
Na pyrophosphate only	1.97	2.09	2.10
A.T.P. + $\text{Na}_4\text{P}_2\text{O}_7$	2.58	2.87	2.57

The reason for the total being rather more than the sum of the individual values is not clear but the fact that the two dephosphorylations do not inhibit each other is evident.

Attempts were now made to separate the two enzymes by adsorption and precipitation methods. Three different aluminium hydroxides, kaolin and magnesium carbonate at different ranges of acidity and alkalinity were tried without success.

Finally it was found that the acetone powder muscle extract prepared by precipitating a fresh muscle extract with acetone and extracting the dried powder was quite free from adenylypyrophosphatase even immediately after preparation while it still contained both inorganic pyrophosphatase and hexosediphosphatase in large amounts. Most of the experiments with hexosediphosphate and phosphoglycerate described in the succeeding sections were done with such an extract.

The activity of such an extract towards both pyrophosphates will be seen from the following figures.

	mg. P
Adenylypyrophosphate	0.02
Sodium pyrophosphate	0.72

30 min. at 38°

If inorganic pyrophosphatase is really a separate enzyme (as the evidence suggests) its function in the muscle is a matter for conjecture, for, so far as is known, inorganic pyrophosphate does not occur there.

As regards the glycolytic power of these extracts it may be noted that they are more active towards hexosediphosphate than glycogen but even with

hexosediphosphate their activity is rather low (1 mg. lactic acid per ml. extract from hexosediphosphate). This activity is not increased appreciably by arsenate; a fact to be contrasted with the great effect of arsenate on the dephosphorylation of phosphoglyceric acid to be discussed later.

Dephosphorylation of sodium hexosediphosphate

The dephosphorylation of hexosediphosphate in muscle extract has been investigated very fully by Lohmann [1933]. One might add that manganese, as might be anticipated, can replace magnesium as the activator as well or even perhaps better and in much smaller molecular concentration. Neither adenylypyrophosphate nor cozymase is necessary.

	Inorganic P produced
Manganese (0.09 mg.)	0.63
Magnesium (0.15 mg.)	0.57

It must also be noted that in an acetone extract some of the hexosediphosphate when present in the usual concentrations ($M/80$) might always, even in the presence of all the coenzymes (magnesium, A.T.P. and coenzyme I), break down into inorganic P and hexosemonophosphate although part is at the same time undergoing dismutation. A comparison of the dephosphorylations of both hexosediphosphate and phosphoglycerate when present in equivalent amounts as regards P (approx. $M/40$) will make this clear.

Table III

	Inorganic P		
	I	II	III
Sodium hexosediphosphate	0.70	0.75	0.77
Sodium phosphoglycerate	0.41	0.56	0.64

All coenzymes are present in both cases.

Here the hexosediphosphate gives more free inorganic P than an equivalent amount of phosphoglycerate. This can only mean that some other dephosphorylating mechanism is at work for hexosediphosphate, besides that acting after its breakdown to phosphoglycerate. The same would seem to be true of an ordinary muscle extract as the following experiment with an extract kept for several days to destroy adenylypyrophosphatase shows:

Substrate	Lactic acid	Inorganic P
Hexosediphosphate	0.63	0.42.

The inorganic P is almost double the amount corresponding to the lactic acid. In drawing conclusions from the appearance or disappearance of inorganic P in experiments where hexosediphosphate is undergoing oxido-reduction this possibility must always be kept in mind.

Dephosphorylation of sodium phosphoglycerate

As has been mentioned before, the acetone powder extracts used for these experiments do not contain any appreciable amounts of adenylypyrophosphatase but have a strong dephosphorylating action upon phosphoglyceric acid. The extracts were frequently tested during the course of this work but in no

circumstances was any dephosphorylating action upon adenylypyrophosphate found. The following figures show the results of a typical experiment:

	Inorganic P
Adenylytriphosphate (0.28 mg. pyro-P)	0.02
Sodium phosphoglycerate (1.5 mg. P)	0.56
Extract dialysed 3 hr.	

The extract itself produced no free inorganic P under these conditions.

The requirements of coenzymes for this dephosphorylation were now investigated. As, however, the enzyme catalysing this reaction fell off in activity by long dialysis the extract could not be completely freed from the coenzymes already present by this method.

Effect of dialysis on the dephosphorylation of phosphoglycerate

	Inorganic P		
Extract dialysed for ...	3 hr.	22 hr.	88 hr.
	0.56	0.39	0.19
	—	—	0.17

However, the results in the following table are fairly clear and show that both magnesium and adenylypyrophosphate are necessary. It is not possible to make any conclusions about coenzyme I as it is well known that it is difficult to dialyse away the last traces of this and the requirement for it is very small, but that it can replace A.T.P. to some extent as a phosphate transporter seems clear enough.

Table IV

Extract dialysed 22 hr.	Inorganic P	
No coenzymes	0.10	0.12
Mg	0.16	0.16
Mg + A.T.P.	0.38	0.34
Mg + A.T.P. + coenzyme I	0.39	0.33
Mg + coenzyme I	0.27	—
A.T.P. + coenzyme I	0.26	0.26

The optimum concentrations of coenzymes and substrate are similar to those in normal glycolysis. With 0.025 *M* sodium phosphoglycerate and 0.0006 *M* A.T.P. and 0.003 *M* Mg 50% of the maximal values are reached. The velocity increases with increasing concentrations of phosphoglycerate up to 0.07 *M* and at this high concentration of phosphoglycerate slightly with increased A.T.P. and magnesium.

For identifying the end product of the dephosphorylation the total bisulphite-binding substances formed were estimated according to the method of Clift & Cook [1932]. Reckoning these as pyruvic acid an amount roughly equivalent to the inorganic phosphate set free was found after making due correction for any phosphopyruvic acid which might be present.

Total pyruvic acid	Present as phosphopyruvic acid	Free pyruvic acid	Equivalent P that should be set free	P found
1.69	0.38	1.31	0.46	0.35

As under these conditions sodium glycerate added to muscle extract does not give any bisulphite-binding substance it may be concluded that the dephosphorylation takes place after conversion into phosphopyruvic acid. This is more-over confirmed by the fact that the dephosphorylation is inhibited by fluoride as usual. *M*/200 iodoacetate does not inhibit the reaction.

In trying to find out the mechanism of this dephosphorylation, in which apparently adenylypyrophosphate plays a prominent part, various possibilities were tested. Since hexosediphosphate is able to decompose in such an extract into hexosemonophosphate and inorganic phosphate and adenylypyrophosphate can rephosphorylate the hexosemonophosphate it is easy to conceive of a small trace of hexosediphosphate, acting catalytically, converting the phosphate from adenylypyrophosphate into inorganic form, the resulting adenylic acid being immediately rephosphorylated by the phosphopyruvic acid. The necessity for both A.T.P. and magnesium would be explained by this as also the dispensability of coenzyme I. It is practically impossible to make sure that a trace of hexosediphosphate or hexosemonophosphate does not occur in the phosphoglycerate especially in view of the nature of its preparation from hexosediphosphate. However, one would expect in such a case that a trace of hexosediphosphate added to an extract would catalyse the breakdown of adenylypyrophosphate. Experiments were done in which small amounts of hexosediphosphate (0.1 mg. P) were added to an extract acting upon fairly large amounts of adenylypyrophosphate (0.55 mg. pyro-P) but no activation of the dephosphorylation could be obtained over and above that due to the added hexosediphosphate itself. Similar amounts of hexosediphosphate also failed to produce any activation of the dephosphorylation of phosphoglycerate when added to an extract acting upon that substrate.

There is however some ground for believing that when large quantities of hexosediphosphate are present adenylypyrophosphate can be dephosphorylated in this way. Thus when an extract acts upon fairly equivalent amounts of hexosediphosphate and phosphoglycerate at the same time the amount of inorganic P produced is much less than the sum of the individual dephosphorylations when they are acted upon separately. While this may be due partly to some of the triosephosphate being removed by dismutation with the pyruvic acid formed as a result of the dephosphorylation of phosphoglycerate (this reaction being much faster than the oxido-reduction of triosephosphate and subsequent dephosphorylation), thus upsetting the equilibrium hexosediphosphate $\rightarrow$ triosephosphate, the difference as will be evident from the table is too great to be accounted for in this way. It is therefore legitimate to conclude that some of the phosphorus from phosphopyruvic acid is being conveyed by adenylic acid to rephosphorylate hexosemonophosphate. The effect of the coupled esterification of adenylic acid consequent upon the dismutation is unlikely to be prominent, due to the lack of free adenylic acid.

Table V

	Inorganic P	
	1.02	0.39
Hexosediphosphate only	0.57	0.36
Phosphoglycerate only	1.23	0.40
Hexosediphosphate + phosphoglycerate		

Another possibility that was considered [*v. Ohlmeyer & Ochoa, 1937*] is that reduced coenzyme I might act as a phosphate acceptor from adenylypyrophosphate, being then itself broken down by a cozymase pyrophosphatase. Traces of hexosediphosphate present might then be able to reduce the coenzyme, enabling it to act in this way. Addition of traces of hexosediphosphate to an extract acting upon adenylypyrophosphate in the presence of coenzyme I failed however to produce any extra dephosphorylation beyond that of the added hexosediphosphate. The dephosphorylation of phosphoglycerate was also not increased by the addition of both coenzyme I and traces of hexosediphosphate.

Moreover, the fact that iodoacetate does not inhibit the reaction makes this possibility even more unlikely.

The influence of other substances like sodium pyruvate, sodium glycerate and large amounts of coenzyme I on the dephosphorylation of adenylypyrophosphate was tested but none of them gave any increase of inorganic P. Finally, small amounts of phosphoglycerate (0.1 mg. P) were also found not to have any activating effect on the breakdown of adenylypyrophosphate.

Since phosphoglycerate is so readily dephosphorylated by these extracts it seemed to be of great interest to find out whether creatinephosphate could be dephosphorylated under these conditions. As creatinephosphate decomposes more readily at a lower pH than that at which most of the other experiments were carried out (about 8.6) the solutions were brought to about pH 7.4 by bubbling a gas mixture of 5% CO₂ and 95% nitrogen through them and keeping them tightly stoppered during the course of the experiment. Both inorganic P and creatinephosphate P were estimated. No breakdown of creatinephosphate was observed.

The effect of adding a phosphate acceptor such as creatine during the dephosphorylation of phosphoglycerate was tried and showed very large amounts of creatinephosphate accumulating. Very little inorganic P was formed.

	Inorganic P	Creatine-phosphate P
Phosphoglycerate	0.35	—
+ creatine (13 mg.)	0.14	0.95

Finally it remained to test how much inorganic P would be formed from phosphoglycerate by a fresh extract containing adenylypyrophosphatase.

	Inorganic P
(1) Adenylypyrophosphate (0.60 mg. pyro-P)	0.29
(2) A.T.P. (0.09 mg. pyro-P) + sodium phosphoglycerate (1.5 mg. P)	0.60

Here, even though the extract has produced only 0.29 mg. P from a large excess of adenylypyrophosphate, it has produced 0.60 mg. P from sodium phosphoglycerate with only a seventh of the amount of adenylypyrophosphate to act as carrier, showing that even in a fresh extract there must be mechanisms producing dephosphorylation of phosphoglycerate other than through adenylic acid formed by dephosphorylation of A.T.P. by adenylypyrophosphatase.

In conclusion one might refer to the velocity of the dephosphorylation of phosphoglycerate in an acetone powder extract. It is interesting because of the very steady rate at which it proceeds as will be evident from the following table:

Table VI

	Inorganic P produced
10 min. at 38°	0.19
20 " "	0.29
30 " "	0.36
60 " "	0.50

The action of arsenate upon this dephosphorylation will now be considered.

The action of arsenate

Arsenate increases the rate of dephosphorylation of phosphoglycerate enormously even in very small concentrations, a fact which has been observed before [Meyerhof & Kiessling, 1935].

Table VII

Inorganic P		
No arsenate	0.09	Extract dialysed 36 hr. A.T.P. and Mg added. Time of exp. 15 min. at room temperature
0.005 <i>M</i> arsenate	0.92	
0.0025 <i>M</i> arsenate	0.77	
0.002 <i>M</i> arsenate	0.69	
0.001 <i>M</i> arsenate	0.57	
0.0002 <i>M</i> arsenate	0.36	
0.0001 <i>M</i> arsenate	0.30	

With $M/10,000$ arsenate the rate is still at least three times the rate in the absence of arsenate while with $M/200$ arsenate it is as much as ten times. That the dephosphorylation proceeds normally with production of pyruvate was shown by estimation of the pyruvic acid formed according to the method of Clift & Cook. The next point is to find what coenzymes if any are required for this reaction with arsenate. This is easier than with the dephosphorylation in the absence of arsenate since it was found that the activation by arsenate was not damaged seriously by very long dialysis.

Inorganic P		
Extract dialysed 22 hr.	1.22	15 min. at room temp. Mg, A.T.P. and cozymase added
88 hr.	0.97	

As the following table shows, 79 hr. dialysis is sufficient to remove most of the coenzymes and the necessity of all the three coenzymes, A.T.P., cozymase and magnesium for this activation by arsenate is quite clear.

Table VIII

Inorganic P		
No addition	0.03	30 min. at room temp. Arsenate $M/1000$
Mg only	0.14	
Mg + A.T.P.	0.34	
Mg + adenylic acid (0.3 mg.)	0.16	
Mg + coenzyme I	0.11	
Mg + A.T.P. + coenzyme I	1.04	
Mg + adenylic acid + coenzyme I	1.25	
Coenzyme I + adenylic acid	0.04	

The adenylypyrophosphate was found to contain traces of cozymase; adenylic acid was therefore used as a check.

It now remains to elucidate the mechanism of this reaction. Experiments showed that adenylypyrophosphate is not broken down to adenylic acid and inorganic phosphate in the presence of arsenate, coenzyme I and magnesium. Reduced coenzyme I prepared by reducing coenzyme I with sodium hydrosulphite according to Euler & Hellström [1938], small amounts of hexosediphosphate and pyruvate (0.0003 and 0.0006 *M* respectively) separately and together were all found not to activate the dephosphorylation of adenylypyrophosphate in the presence of arsenate. A sample of phosphoglycerate prepared after treating it with *N* HCl at 100° for 4 hr. to remove any easily hydrolysable phosphate compounds including hexosediphosphate was found to produce inorganic phosphate in the presence of arsenate as rapidly as the original phosphoglycerate itself. One must of course remember that if any hexosemonophosphate was present it was not likely to decompose completely under these conditions.

The effects of the two poisons, fluoride and iodoacetate, were next tried and both were found to inhibit the activation by arsenate.

Table IX

	Inorganic P		30 min. at room temp.
	0.99	1.23	
Arsenate only <i>M</i> /400	0.08	—	
+ fluoride <i>M</i> /25	—	0.20	
+ iodoacetate <i>M</i> /840	—	—	

It is interesting to notice that, using a yeast enzyme preparation in the presence of arsenate, Meyerhof *et al.* [1937] found that phosphopyruvic acid is readily broken down to pyruvic acid and free phosphate, provided that cozymase, a trace of hexosediphosphate and an active oxido-reduction enzyme system are present. This mechanism of dephosphorylation has not so far been explained. It also is inhibited by iodoacetate.

The action of iodoacetate is dissimilar from its effect on the dephosphorylation of phosphoglycerate in the absence of arsenate where it does not inhibit the reaction. In order to make sure that the arsenate and iodoacetate were not for some reason neutralizing each other's effects the following experiment was tried, making use of the fact that iodoacetate inhibits the dismutation between triose-phosphate and pyruvic acid. If now the effects of arsenate and iodoacetate were to antagonize each other arsenate should remove the inhibition of this dismutation by iodoacetate and we should get the same amount of lactic acid formed.

Table X

	Lactic acid
Triosephosphate + pyruvate only	2.4
+ iodoacetate	0.3
+ arsenate	4.5
+ arsenate + iodoacetate	0.3

Time of exp. 30 min. at 38°

The inhibition by iodoacetate therefore seems to be quite specific and not influenced by arsenate.

It has been mentioned before that small amounts of pyruvate had no action upon adenylypyrophosphate in the presence of arsenate and the coenzymes. With much larger amounts of pyruvate (0.004–0.020 *M*) a small amount of dephosphorylation can be observed. This dephosphorylation is also inhibited by iodoacetate.

Table XI

	Inorganic P	
Extract dialysed 88 hr.		
A.T.P. (0.3 mg. pyro-P) only	0.01	30 min. at 38°
A.T.P. + 0.004 <i>M</i> pyruvate	0.05	
A.T.P. + 0.020 <i>M</i> pyruvate	0.10	Arsenate <i>M</i> /1000
A.T.P. + 0.020 <i>M</i> pyruvate + iodoacetate <i>M</i> /800	0.01	

The reason for this dephosphorylation is not known. It may be due to certain impurities in the pyruvate but the amount is perhaps too small to be of significance.

Finally the effect of arsenate on creatinephosphate breakdown in the presence and absence of coenzymes was studied.

The results (Table XII) show that creatinephosphate is to some extent broken down when arsenate is present although quite stable in its absence. There are, however, some great differences between the breakdown of creatinephosphate and that of phosphoglycerate. The former as will be evident from the table is hardly

Table XII

Arsenate $M/1250$ present in all	Inorganic P
Creatinephosphate only (0.98 mg. P)	0.15
+ Mg + A.T.P. + cozymase	0.17
+ Mg + A.T.P. + cozymase + pyruvate (0.02 M)	0.28
+ Mg + A.T.P. + cozymase + hexosediphosphate ($M/4000$)	0.29
+ Mg + A.T.P. + cozymase + iodoacetate $M/800$	0.04
Phosphoglycerate only	0.20
Time of exp. 30 min. at 38°	
Extract dialysed 88 hr.	

affected by the absence of the coenzymes, which produce no appreciable increase in inorganic phosphate. Pyruvate produces a small increase comparable with that which it produces with A.T.P. (see Table XI) and hexosediphosphate in like amount. Iodoacetate inhibits the reaction at a concentration of $M/800$ though at this concentration it hardly affects the transfer of phosphate from creatinephosphate to adenylic acid. It may also be noted that phosphoglycerate under the same conditions (in the absence of all coenzymes) gives a similar amount of inorganic phosphate to that given by creatinephosphate in this experiment but with all coenzymes it gives as much as 3.32 mg. P or about 16–20 times the amount.

DISCUSSION

It is abundantly clear from these experiments that dephosphorylation of phosphoglycerate in muscle extracts involves several different mechanisms all of which seem to require one thing in common, adenylic acid. When glycogen is present to act as a phosphate acceptor the adenylic acid is easily regenerated and then re-esterified. The action of adenylypyrophosphatase is also clear. It is when both these are lacking that the two mechanisms of dephosphorylation treated here become more prominent and clear.

That the phosphoglycerate passes through the stage of phosphopyruvate and then breaks down into pyruvate and phosphate is clear both from the estimations of pyruvate formed as well as from the fact that the dephosphorylation both in the absence as well as in the presence of arsenate is poisoned by fluoride. We shall now consider briefly in review the evidence for the nature of the dephosphorylation first in the absence of arsenate, and secondly when arsenate is present.

Dephosphorylation without arsenate. It is clear from the figures given before that this dephosphorylation requires both Mg and adenylypyrophosphate for full activation. Addition of cozymase does not seem to increase the rate of breakdown. This may be due to there being already a sufficiency of this coenzyme in the extract as it is known to be required only in minute quantities and it is difficult to dialyse the last traces away completely. However, it will be seen that it can replace adenylypyrophosphate to some extent but is not quite so active.

The absence of adenylypyrophosphatase from the extracts has been demonstrated. Large quantities of adenylypyrophosphate added to the extract do not give any measurable increase in inorganic phosphate even when cozymase and magnesium are added. It remains therefore to see how the adenylypyrophosphate can serve as phosphate acceptor for the phosphate of phosphoglycerate, assuming that it is the only way in which it can catalyse the dephosphorylation.

Two possibilities have been considered.

(1) Setting free of phosphate by the system adenylypyrophosphate, hexosemonophosphate, hexosediphosphate and hexosediphosphatase

(2) Setting free of phosphate by the system adenylypyrophosphate, reduced cozymase, reduced cozymase pyrophosphate and reduced cozymase pyrophosphatase with traces of hexosediphosphate serving to form an oxido-reduction system for reducing cozymase in the first instance.

Since traces of hexosediphosphate which might be supposed to reduce cozymase do not activate the dephosphorylation of either A.T.P. or phosphoglycerate with or without cozymase, and the dephosphorylation of phosphoglycerate, moreover, is not inhibited by iodoacetate, it must be considered that no evidence exists for either of these mechanisms.

Finally the idea that the dephosphorylation is the result of the action of a phosphopyruvic phosphatase might be considered but for the fact that it is hard to imagine a phosphatase so specific as to be activated by adenylypyrophosphatase or cozymase.

Dephosphorylation in the presence of arsenate. Here also the phosphoglycerate passes through the phosphopyruvic stage before dephosphorylation, as shown by the formation of pyruvate and by the inhibition of the reaction by fluoride. The reaction needs all the three coenzymes, adenylypyrophosphate, magnesium and cozymase for full activation. The poisoning of the activity by iodoacetate might lead one to think that here indeed the coenzyme I is taking part in a system of oxido-reduction with perhaps traces of hexosediphosphate. However, in this case also, as before, small amounts of added hexosediphosphate alone and along with pyruvate were not found to give any increase of dephosphorylation of either adenylypyrophosphate or phosphoglycerate or of creatinephosphate in the presence of all the coenzymes. Reduced cozymase added to A.T.P. also does not have any noticeable action. One must wait for further experimental evidence before any idea of the true role of cozymase in the mechanism can be formulated.

The stimulation of the breakdown of creatinephosphate in the presence of arsenate and its poisoning by iodoacetate would tend to show that it is perhaps of similar character. Against this, however, is the fact that addition of coenzyme does not activate this breakdown and that the activity itself is very slight compared with the dephosphorylation of phosphoglycerate under the same conditions. The slight activation of adenylypyrophosphate breakdown by pyruvate is perhaps also of similar origin.

A word might be added about arsenate activation in general in these extracts. It was found that while the arsenate did not activate the production of lactic acid from glycogen and hexosediphosphate both the dismutation between triose-

phosphate + pyruvate as well as the dephosphorylation of phosphoglycerate are considerably accelerated showing that arsenate perhaps acts only in the later stages of glycolysis in such an extract.

SUMMARY

1. Of the three phosphatases present in fresh muscle extract, inorganic pyrophosphatase, adenylypyrophosphatase and hexosediphosphatase which breaks down hexosediphosphate into hexosemonophosphate and inorganic P, the adenylypyrophosphatase is the least stable, but it is not completely destroyed even after 2 weeks at 0° and 16 hr. dialysis. Attempts to separate the two pyrophosphatases by adsorption did not meet with success though there was some evidence that they were distinct and separate enzymes.

2. An acetone powder extract of muscle is quite free from adenylypyrophosphatase even though the other two phosphatases are present in active form. Such an extract does not dephosphorylate adenylypyrophosphate but readily dephosphorylates phosphoglycerate when small amounts of magnesium and adenylypyrophosphate are added. Creatinephosphate is not broken down under these conditions. The dephosphorylation is inhibited by fluoride but not by iodoacetate.

3. The dephosphorylation of phosphoglycerate is activated greatly by arsenate. A.T.P., magnesium and cozymase are necessary for this activation. Fluoride and iodoacetate inhibit it. Creatinephosphate is slightly broken down in the presence of arsenate.

4. Two possibilities were considered to explain the mechanism of the reaction by which A.T.P. could serve as phosphate acceptor in the absence of any known mechanism for its breakdown into adenylic acid. One assumed the presence of traces of hexosemonophosphate formed by dephosphorylation of hexosediphosphate by the phosphatase present, whilst the other postulated the presence of reduced cozymase acting as phosphate carrier. So far no experimental evidence for either of these mechanisms has been obtained.

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