CCLV. ACTION OF ARSENATE IN GLYCOLYSIS

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The activating effect of arsenate on glycolysis in alcoholic fermentation as well as in muscle brei and extracts has been known for a long time [Harden & Young, 1906; 1911; Meyerhof, 1921], and it was also known that this activation is due specifically to increased hexosediphosphate breakdown [Harden & Young, 1911; Meyerhof, 1918]. The assumption that the effect of the arsenate was to activate a hexosediphosphatase splitting hexosediphosphate into hexose and inorganic phosphate was early abandoned [Meyerhof, 1927; Raymond, 1928; Macfarlane, 1930; Harden, 1932] as it was shown that arsenate activation of hexosediphosphate breakdown could not be obtained in the presence of the phosphatase only, independently of increased fermentation. Shortly after, it was found in yeast juice as well as in muscle extract that the production of alcohol or lactic acid from dihydroxyacetonephosphate, the breakdown product of hexosediphosphate, was increased by arsenate as much as that from hexosediphosphate, and further that the oxidoreduction of triosephosphate and acetaldehyde or pyruvate as well as the dephosphorylation of phosphoglycerate (phosphopyruvate) proceeded very much faster in the presence of arsenate [Meyerhof, 1934; Meyerhof & Kiessling, 1935]. Several attempts were made to explain the increased dephosphorylation of phosphoglycerate in the presence of arsenate. Since phosphoglycerate was known to be dephosphorylated after conversion into phosphopyruvate by transfer of its phosphate to adenylic acid forming adenylpyrophosphate and pyruvate, it was thought that arsenate, by activating the enzyme adenylpyrophosphatase, might increase the production of adenylic acid from the pyrophosphate. However, Pett & Wynne [1934] and Schäffner & Krumey [1936] showed conclusively that all the phosphatases investigated with the possible exception of adenylpyrophosphatase were not activated but even inhibited by arsenate; Needham & Pillai [1937] later showed that with adenylpyrophosphatase also the activation was only very slight and could not in any case account for such very greatly increased production of phosphate as is usually observed with arsenate. The dephosphorylation of phosphoglycerate in the presence of arsenate and catalytic amounts of adenylic acid in yeast juice or muscle extract occurs as fast as the transfer of phosphate from phosphoglycerate to equivalent amounts of adenylic acid, and it was therefore supposed that in such extracts the adenylic acid is somehow constantly being regenerated, but Meyerhof & Kiessling [1936] could not find any evidence of this and postulated the formation and breakdown of a cozymasepyrophosphate.

The coupled esterification of adenylic acid with inorganic phosphate consequent upon the oxidation of triosephosphate in muscle extract had by now been demonstrated [Meyerhof *et al.* 1937, 2; Needham & Pillai, 1937]. Needham & Pillai found that if arsenate was present during the oxidation no accumulation of adenylpyrophosphate resulted and they concluded that arsenate inhibited the coupling of esterification and oxidation. This interpretation was advanced

by other workers also [Meyerhof, 1937; Meyerhof *et al.* 1937, 1; 1938, 1] who supposed that the rapid oxidation of triosephosphate could take place only in conjunction with the esterification of inorganic phosphate with adenylic acid, and that arsenate, by inhibiting the coupling of oxidation and esterification, enabled the former to go on at the same rate even in the absence of large amounts of adenylic acid and inorganic phosphate. This assumption, besides explaining the observed activation by arsenate of the reaction triosephosphate + pyruvate (or acetaldehyde), would also show why hexosediphosphate breakdown itself can be influenced by arsenate in still another way. In normal glycolysis, where glycogen or glucose is the substrate, the adenylpyrophosphate formed during the dephosphorylation of phosphopyruvate is immediately dephosphorylated by donating its phosphate to glycogen or glucose, quite apart from the action of the adenylpyrophosphatase which is considered to be much slower. The adenylic acid is thus continuously regenerated to dephosphorylate more and more phosphopyruvate. When hexosediphosphate is the substrate it cannot however serve as phosphate acceptor for adenylpyrophosphate. The coupled esterification of adenylic acid during oxidation of triosephosphate goes on in both cases, but when arsenate is present the adenylic acid is not esterified and is free to dephosphorylate phosphopyruvate so that the breakdown of hexosediphosphate proceeds readily. In the case of glucose or glycogen this mechanism is of less importance.

Shortly afterwards Pillai [1938] showed that phosphoglycerate could be rapidly dephosphorylated in long-dialysed extracts of acetone muscle powder in the presence of arsenate if catalytic amounts of adenylic acid, Mg and cozymase are present, a result somewhat different from that of Meyerhof *et al.* [1937, 1] who found that in their A and B protein enzyme system a trace of hexosediphosphate too was necessary. They also stated that if an excess of adenylpyrophosphate is added to the arsenate-activated system, the phosphopyruvate breaks down but not the adenylpyrophosphate.

Since the arsenate activates not only the breakdown of hexosediphosphate as a whole but also all the known intermediate reactions individually, it seemed possible that the inhibition of the coupled esterification observed might be more apparent than real and that the action of the arsenate on this reaction should be investigated more fully. The muscle extracts used for the dephosphorylation experiments with phosphoglycerate contained no adenylpyrophosphatase [Pillai, 1938], and since, besides Mg and cozymase, adenylic acid was indispensable, it seemed, assuming as usual that the phosphopyruvate was dephosphorylated by transferring phosphate to adenylic acid, obvious to conclude that the adenylpyrophosphate must constantly be breaking down and reforming in the presence of arsenate to enable this reaction to take place. The effect of the arsenate in inhibiting the coupling might therefore very well be due to the accelerated breakdown by some mechanism, other than adenylpyrophosphatase, of the adenylpyrophosphate that is formed. The experiments described below show that in fact this is quite probable and that in the presence of arsenate the adenylpyrophosphate readily breaks down if a trace of phosphoglycerate, fluoride and cozymase are added.

Experimental

Enzyme preparation

An aqueous extract of a muscle powder prepared by precipitating fresh rabbit muscle extract with acetone was used [Needham & Pillai, 1937]. The extract was dialysed in cellophane tubes for 1–4 days according to the needs of the experiment. The enzyme solution does not contain any adenylpyrophosphatase.

Chemical preparations. Besides the preparations described by Pillai [1938], synthetic phosphopyruvate was made by the method of Kiessling [1936].

Methods of estimation. The usual methods adopted were the same as those given before [Pillai, 1938]. Adenylic acid and adenylpyrophosphate were determined when necessary by the deaminase method of Parnas & Lutwak-Mann [1935]. Details of the procedure will be found elsewhere [Needham & Pillai, 1937].

Experimental procedure. For most of the experiments 2 ml. enzyme solution were diluted to 4–6 ml. with the several additions of substrates, coenzymes, fluoride, arsenate etc. The amounts of these additions are given along with the experimental results. 0·3 ml. 6% $NaHCO_3$ was used as buffer in most cases and the experiments were carried out in ordinary 25 ml. centrifuge tubes. Suitable controls were always done simultaneously. The time of experiment was 15–30 min. at 38° or at room temp. (20°). Trichloroacetic acid was used to precipitate the proteins and the P estimations were carried out after filtration through dry filter paper. In the creatinephosphate estimations centrifuging was found quicker and more satisfactory than filtration.

The results are expressed, unless otherwise stated, as mg. increase or decrease per 2 ml. extract.

Details of experiments

It has been suggested that arsenate increases the rate of oxidoreduction between triosephosphate and pyruvate because this reaction goes on rapidly only when accompanied by simultaneous esterification of adenylic acid, and arsenate, by breaking this coupling, allows the reaction to proceed independently of the esterification [Meyerhof *et al.* 1937, 1; 1938, 1]. The following table shows the yields of lactic acid obtained during the dismutation of triosephosphate + pyruvate in the presence and absence of adenylic acid and inorganic P. The enzyme solution was an extract dialysed 73 hr. and containing practically no inorganic P.

Exp. I. All the tubes, including the control, received 2 ml. enzyme solution, 0·6 ml. *M*/6 Na hexosediphosphate, 0·8 ml. *M*/5 Na pyruvate, 0·6 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2, 6H_2O$, 0·15 ml. 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$.

	mg. lactic acid
Control	+2·2
+ phosphate (1 ml. 0·16 *M*)	+4·5
+ phosphate + adenylic acid (12 mg.)	+6·6

The adenylic acid was neutralized before addition and all tubes made up to 6 ml. with water. Time of exp. 15 min. at 38°.

Both inorganic P and adenylic acid give increased dismutation but even in their absence there is a fairly active reaction. The effect of adding P alone seemed peculiar, but the following exp. shows that even in the absence of adenylic acid esterification is taking place, though of a different nature.

Exp. II. All preliminary additions as in Exp. I, except that an extract dialysed only 25 hr. and therefore probably containing traces of adenylic acid was used. 30 min. at 38° C.

	mg. inorganic P
Control + phosphate (1 ml. 0·16 *M*)	−1·01
+ phosphate + arsenate (0·15 ml. *M*/10)	+0·22

There is a considerable disappearance of inorganic phosphate when it is present during dismutation even in the absence of adenylic acid; this is inhibited by arsenate. The ester formed cannot be adenylpyrophosphate or cozymase-pyrophosphate as the amount of adenylic acid present in the 25 hr.-dialysed

extract must be very small, also only 0·15 mg. cozymase is added (0·5 ml. 0·15 % solution of 20% pure cozymase). The ester was not identified but it might be formed by esterification of some of the pyruvate, the resulting phosphopyruvate being dephosphorylated in the presence of arsenate and the traces of adenylic acid still present. In some later experiments where an extract dialysed for much longer was used the esterification was found to be less but still noticeable.

The above experiment seems to show that the effect of the arsenate might be to increase the breakdown of any ester formed rather than to inhibit the esterification itself since it is known that arsenate activates the breakdown of phosphopyruvate. The next experiment shows that this is very probably the case for the oxidoreduction of triosephosphate + pyruvate and the corresponding esterification of adenylic acid.

Exp. III. Besides the preliminary additions given in Exp. I all tubes received 12 mg. adenylic acid and 1 ml. 0·16 *M* phosphate. In no. 3 the arsenate was added at the beginning of the experiment, but in 4 only after 15 min. at 38° when it was kept at 38° for a further 15 min.

	mg. inorganic P		min. at 38°
1. Control	−2·08	−2·16	15
2. Control	—	−2·37	30
3. + arsenate (0·15 ml. *M*/10)	−0·07	+0·13	15
4. + arsenate	−0·29	−0·31	15 + 15

The effect of the arsenate added before or after esterification is the same. In no. 4 the esterification has taken place but arsenate added later breaks down all the adenylpyrophosphate formed so that there is no difference between nos. 3 and 4. Whether in the presence of arsenate the esterification takes place at all is impossible of direct proof.

In order to see how the activation of phosphate breakdown actually happens it was necessary to exclude once for all the possibility of arsenate activation of hexosediphosphatase, to make sure that the extra inorganic P in the presence of arsenate did not come from this source. Exp. IV shows that in the presence of NaF only, the arsenate considerably inhibits the production of inorganic P from hexosediphosphate. This however is due to the fact that the arsenate greatly activates the dismutation of triosephosphate and thus reduces the effective concentration of hexosediphosphate itself. With both NaF and iodoacetate the inhibition is very much less but still noticeable.

Exp. IV. All tubes received 2 ml. extract, 0·6 ml. *M*/6 hexosediphosphate 0·1 ml. 2·5% $MgCl_2$, 0·1 ml. of 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$. Made up to 4 ml. with additions. All 30 min. at 38°.

	mg. inorganic P		
Control	0·91	—	0·69
+ arsenate (0·1 ml. *M*/10)	0·37	—	0·45
+ iodoacetate (0·1 ml. *M*/10)	—	0·79	0·72
+ iodoacetate + arsenate	—	0·66	0·63

The next point was to test whether added adenylpyrophosphate would be broken down during the dismutation of hexosediphosphate + pyruvate in the presence of arsenate and the following experiment shows that this is indeed the case.

Exp. V. All preliminary additions as in Exp. I. 15 min. at 38°.

	mg. inorganic P
Control	−0·04
+ A.T.P. (0·8 mg. pyro-P)	+0·01
+ arsenate	+0·33
+ arsenate + A.T.P.	+1·19

In the absence of arsenate the adenylpyrophosphate does not break down.

It seemed therefore as if the oxidoreduction itself or one of the products formed could, in the presence of arsenate, cause adenylpyrophosphate to split off inorganic P. In accordance with this assumption the dismutation of two triosephosphate molecules alone without pyruvate could bring about decomposition of A.T.P., as is shown in the following experiment which was made with all additions as in Exp. V except for pyruvate which was omitted:

	mg. inorganic P
Control + arsenate	0·28
+ arsenate + A.T.P.	0·72

Since for the dephosphorylation of phosphopyruvate in muscle extract in the presence of arsenate and no adenylpyrophosphatase a small amount of adenylic acid acts catalytically and is indispensable, there is an obvious possibility that adenylpyrophosphate is continually being broken down and reformed. One would therefore expect that large amounts of adenylpyrophosphate should break down in extracts in which phosphopyruvate is at the same time experiencing decomposition. Addition of considerable amounts of adenylpyrophosphate to muscle extracts in which phosphoglycerate was being dephosphorylated in the presence of arsenate, however, failed to produce appreciable extra dephosphorylation.

Exp. VI. 0·5 ml. 0·1 *M* phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. $MgCl_2$, 0·2 ml. A.T.P. (0·1 mg. pyro-P) and 0·25 *M*/50 arsenate were added to 2 ml. muscle extract (dialysed 23 hr.) in the control and the other tubes. Made up to 5 ml. with 0·3 ml. $NaHCO_3$, water and other additions if any.

	Inorganic P	min. at 38°
	1·54	30
Control	1·60	60
+ A.T.P. (0·6 mg. pyro-P)	1·68	30

The negative result in this case, however, might be due to the fact that we are here dealing with the same enzyme reaction in both cases (the dephosphorylation of A.T.P. and phosphoglycerate requiring the same enzyme), or because at the moment when all phosphoglycerate has been dephosphorylated the A.T.P. has still remained intact. The former is unlikely because practically all the phosphoglycerate has broken down in 30 min. The latter would follow from the fact that so long as any phosphoglycerate (phosphopyruvate) is present the adenylic acid formed by breakdown of A.T.P. would be immediately rephosphorylated. Addition of NaF would of course prevent the dephosphorylation of phosphoglycerate and the consequent regeneration of A.T.P. The following experiment in which this was done shows that under these conditions the adenylpyrophosphate is completely broken down [compare Meyerhof *et al.* 1937, 1].

Exp. VII. All additions as in Exp. VI except that 0·5 ml. 0·4 *M* NaF was added (conc. of NaF *M*/28).

	Inorganic P
Control	0·30
+ A.T.P. (0·6 mg. pyro-P)	0·86

That the adenylpyrophosphate has actually broken down and has not merely removed the inhibition of phosphoglycerate dephosphorylation by NaF [Runnström & Hemberg, 1937] was shown by precipitating the residual adenylpyrophosphate with Ba acetate and estimating NH_2-N by the method of Parnas & Lutwak-Mann [1935]. There was no A.T.P. left. Further proof of this is given

below in experiments with only a trace of phosphoglycerate, which could not provide more than a fraction of the inorganic P by its own decomposition.

It seemed therefore that in the mere presence of phosphoglycerate and the complete oxidoreduction enzymes with arsenate adenylpyrophosphate could break down. Obviously this reaction is at the basis of the dephosphorylation of phosphoglycerate observed [Pillai, 1938] in the same system. There a small amount of adenylpyrophosphate is able to act catalytically, for immediately on decomposition it is rephosphorylated by some of the phosphopyruvate and this would go on continuously till all the phosphoglycerate has been decomposed. In view of the fact that the mechanism of the coupled esterification of adenylic acid

triosephosphate + cozymase + adenylic acid + inorganic phosphate
→ phosphoglycerate + reduced cozymase + A.T.P., ...(A)

has been shown to be reversible [Meyerhof *et al.* 1938, 2]

A.T.P. + phosphoglycerate + reduced cozymase
→ triosephosphate + cozymase + adenylic acid + P, ...(B)

it was natural to suppose that the dephosphorylation of phosphoglycerate in the presence of traces of cozymase and A.T.P. was related to reaction B. This reaction is stoichiometric, but in the presence of alcohol or lactic acid to reduce the cozymase as soon as it is formed a small amount of cozymase can act catalytically. Leaving aside for a moment the mechanism of cozymase function one would suppose that part of the phosphoglycerate would undergo conversion into triosephosphate during its dephosphorylation. No accumulation of triosephosphate can however be expected, since triosephosphate in the presence of traces of cozymase and of the pyruvate formed by the dephosphorylation of phosphopyruvate would be reoxidized completely to phosphoglycerate when arsenate is present in the system [Adler & Günther, 1938]. As a matter of fact neither triosephosphate nor lactic acid could be detected among the products formed. However, if conversion into triosephosphate is concerned at all in the reaction one should get an inhibition of the dephosphorylation of phosphoglycerate if this change is prevented. By using synthetic phosphopyruvate instead of phosphoglycerate and employing NaF to prevent any conversion of the former into phosphoglycerate and then into triosephosphate, the dephosphorylation of phosphopyruvate should be considerably inhibited if the supposition is true. The following experiment in which this was done shows that the dephosphorylation of phosphopyruvate in the presence of traces of adenylic acid and cozymase is inhibited by NaF only slightly, both in presence as well as absence of arsenate.

Exp. VIII. 1·2 ml. Na phosphopyruvate containing 2·7 mg. phosphopyruvic P, 0·2 ml. A.T.P. containing 0·1 mg. pyro-P, 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$ and 0·3 ml. 6% $NaHCO_3$ were added to all tubes together with 2 ml. extract dialysed 23 hr. Made up to 5 ml. with further additions or water.

	Inorganic P	
Control	0·55	30 min. at 38°
+ NaF (0·3 ml. 0·4 *M*)	0·39	
+ arsenate (0·25 ml. *M*/50)	2·01	15 min. at 20°
+ arsenate + NaF	1·58	

The slight inhibition observed may be due to injury to the enzymes by the high concentration of NaF (*M*/40), or because, since only a trace of phospho-

glycerate is required for the reaction and the NaF poisoning is not 100% effective, especially with large amounts of added phosphopyruvate, a small but sufficient amount of phosphoglycerate has formed in a short time. The following experiment in which decreasing amounts of phosphoglycerate are added in the presence of NaF shows that even with a trace of phosphoglycerate (0·12 mg. P) the adenylpyrophosphate is completely dephosphorylated.

Exp. IX. 0·5 ml. 0·2% cozymase, 0·1 ml. $MgCl_2$, 0·3 ml. *M*/50 arsenate, 0·6 ml. 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$ were added to 2 ml. extract (dialysed 23 hr.) in all tubes including controls. Made up to 6 ml. with water or further additions. In each case a control containing the same amount of phosphoglycerate and a trace of A.T.P. was run to compensate for any dephosphorylation of the phosphoglycerate itself. 30 min. at 38°.

	mg. inorganic P
Control + 0·5 ml. *M*/5 phosphoglycerate + A.T.P. (0·75 mg. pyro-P)	−0·66
+ 0·1 ml. *M*/5 ,, + A.T.P. (0·75 mg. pyro-P)	0·60
+ 0·5 ml. *M*/50 ,, + A.T.P. (0·83 mg. pyro-P)	0·87
+ 0·2 ml. *M*/50 ,, + A.T.P. (0·83 mg. pyro-P)	0·79

Practically all the pyrophosphate is broken down under these conditions and even at room temp. the reaction is quite rapid.

Exp. X. All additions were the same as in previous experiment except that 0·2 ml. *M*/50 phosphoglycerate and A.T.P. (1·1 mg. pyro-P) were added to all tubes. Reaction at room temp. (20°).

	Inorganic P
15 min.	0·69
30 ,,	0·84
60 ,,	0·99

It was observed that Mg is not necessary, but cozymase is indispensable and that iodoacetate completely inhibits the reaction. In the absence of NaF the dephosphorylation is very small, evidently owing to the disappearance of the phosphoglycerate by dephosphorylation after conversion into phosphopyruvate.

Exp. XI. All tubes received 2 ml. extract dialysed 77 hr., 0·2 ml. *M*/50 phosphoglycerate, 2 ml. A.T.P. containing 1·1 mg. pyro-P, 3 ml. *M*/50 arsenate and 0·3 ml. 6% $NaHCO_3$. Made up to 6 ml. with further additions or water. 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$, 0·6 ml. 0·4 *M* NaF and 0·1 ml. *M*/10 iodoacetate added as indicated. 15 min. at room temp.

	Inorganic P
Control + NaF + Mg + cozymase	0·69
+ NaF + Mg only	0·46
+ NaF + cozymase only	0·68
+ cozymase + Mg (no NaF)	0·22
+ cozymase + Mg + NaF + iodoacetate	0·08

The dephosphorylation is quite high without cozymase but this is due to the fact that the adenylpyrophosphate used contains small amounts of cozymase as impurity. With adenylpyrophosphate freed from cozymase by leaving the solution overnight at *p*H 9·0, at which alkalinity the cozymase is inactivated, the indispensability of cozymase is quite clear. 15 min. at room temp.

	Inorganic P
No cozymase	0·07
+ cozymase	0·21

That no impurity in the sodium phosphoglycerate itself is responsible for the activation of adenylpyrophosphate breakdown can be shown by adding a small amount of synthetic phosphopyruvate to the extract, allowing it to remain

a few min. so that some of it is converted into phosphoglycerate and then adding the NaF, arsenate, coenzymes and A.T.P. This works as satisfactorily as the phosphoglycerate itself.

Sodium glycerate substituted for phosphoglycerate is completely inactive so that the possibility of decomposition of phosphoglycerate in such manner in the dephosphorylation system may be set aside. α-glycerophosphate and pyruvate have little effect. Even glyceraldehyde and pyruvate together did not influence the dephosphorylation of A.T.P. to any considerable extent beyond that by pyruvate itself.

Exp. XII. 2 ml. extract, 0·5 ml. cozymase, 0·1 ml. $MgCl_2$, 0·3 ml. *M*/50 arsenate, 1·7 ml. A.T.P. containing 1 mg. pyro-P and 0·3 ml. $NaHCO_3$ were mixed and made up to 6 ml. with water or further additions as follows: 0·4 ml. *M*/5 Na glycerate, 0·5 ml. *M*/5·5 Na glyeerophosphate, 0·5 ml. *M*/5 pyruvate, 0·5 *M*/5 glyceraldehyde and 0·6 ml. 0·4 *M* NaF. Time 30 min.

	Inorganic P	Temp.
Control + Na glycerate + NaF	+0·01	20°
+ glycerophosphate	+0·07	38°
+ glycerophosphate + NaF	+0·02	38°
+ pyruvate + NaF	+0·18	38°
+ pyruvate + glyceraldehyde	+0·31	38°

The reaction would therefore seem to be specific for phosphoglycerate.

In the absence of arsenate, A.T.P. is not decomposed in this enzyme system even in the presence of large amounts of phosphoglycerate and NaF, notwithstanding the fact that if no NaF is added phosphoglycerate itself will be dephosphorylated in such an extract with small amounts of A.T.P. added as coenzyme.

It is of interest to see whether creatinephosphate will be decomposed under the same conditions as adenylpyrophosphate in this system. The following experiment shows that in the presence of traces of phosphoglycerate and arsenate creatinephosphate will split off inorganic P even in the absence of adenylic acid. Mg is necessary for this, unlike the decomposition of A.T.P., and iodoacetate inhibits the reaction. Cozymase is indispensable.

Exp. XIII. 2 ml. extract dialysed 94 hr., 2 ml. creatinephosphate containing 1·2 mg. P, 0·2 ml. *M*/50 phosphoglycerate, 0·6 ml. 0·4 *M* NaF, 0·3 ml. *M*/50 arsenate, and 0·3 ml. 6% $NaHCO_3$ were mixed. Made up to about 6 ml. with further additions as indicated. 0·5 ml. 0·15% cozymase, 0·2 ml. 4% adenylic acid and 0·1 ml. 2·5% $MgCl_2$, $6H_2O$. 30 min. at 20°.

	Inorganic P		
Control + Mg	0·22	—	—
+ Mg + cozymase	0·63	0·76	0·88
+ Mg + cozymase + adenylic acid	0·68	—	—
+ cozymase	—	—	0·49
+ cozymase + Mg + iodoacetate	—	—	0·10

It is curious that adenylic acid is unnecessary for the dephosphorylation of creatinephosphate. That the creatinephosphate itself contains no trace of adenylic acid as impurity can be shown by estimating the adenylic acid by the deaminase method of Parnas and also indirectly. It is known that adenylic acid is obligatory for the dephosphorylation of phosphoglycerate in the presence of arsenate and of the complete oxidoreduction enzymes [Pillai, 1938]. Creatinephosphate is also broken down in such an extract in the presence of phosphoglycerate. Even supposing that the mechanism of breakdown is the same in

both cases, if a small amount of creatinephosphate, which will be completely decomposed, is added to phosphoglycerate in the same extract with all additions except adenylic acid, one would expect some of the phosphoglycerate also to be dephosphorylated provided that the creatinephosphate contains some adenylic acid as impurity, but not otherwise. The following experiment shows that no extra phosphoglycerate is broken down if creatinephosphate alone is added, but the further addition of a trace of adenylic acid (0·1 mg.) immediately increases the production of inorganic P.

Exp. XIV. To 2 ml. extract dialysed 94 hr., 0·5 ml. *M*/5 phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$, 0·25 ml. *M*/50 arsenate and 0·3 ml. 6% $NaHCO_3$ were added and made up to 5 ml. with water or further additions as follows: creatinephosphate (containing 0·6 mg. P) and 0·1 mg. adenylic acid when indicated. 30 min. at room temp.

	Inorganic P
Control	0·38
+ NaF	0·17
+ NaF + creatinephosphate	0·77
+ creatinephosphate	0·90
+ creatinephosphate + adenylic acid (0·1 mg.)	1·37

Further, adenylic acid does not activate the production of phosphate from creatinephosphate alone.

	Inorganic P
Creatinephosphate only (1·2 mg. P)	0·63
+ adenylic acid (0·8 mg.)	0·68

Creatinephosphate therefore seems to be able to split off inorganic phosphate in the presence of arsenate without the agency of adenylic acid or at most in the presence of extremely minute amounts.

It has already been mentioned that the mechanism of breakdown of A.T.P. in the presence of phosphoglycerate might possibly be related to the reaction phosphoglycerate + reduced cozymase + A.T.P. → triosephosphate + oxidized cozymase + adenylic acid + P which has been shown to occur in muscle extracts. This requires reduced cozymase to start the reaction and since neither reduced cozymase nor any reducing agent such as lactic acid or acetaldehyde was added, it was thought possible that some material present in the extract itself might bring about this reduction.

Experiments in which considerable amounts of cozymase were added to the extract, either alone or in the presence of arsenate or with all further additions, failed to show the presence of any reduced cozymase when examined spectrophotometrically. Reduced coenzyme if present at all must be in very small amounts as the method detects amounts as low as 0·05 mg. reduced cozymase [Warburg *et al.* 1935].

Discussion

The breakdown of adenylpyrophosphate by muscle extract containing no adenylpyrophosphatase, in the presence of arsenate, cozymase and small traces of phosphoglycerate (prevented from decomposition by NaF), has been demonstrated. This reaction is the basis of the dephosphorylation of phosphoglycerate in the presence of catalytic amounts of adenylic acid, observed under the same conditions in muscle extract [Pillai, 1938]. There the adenylic acid is esterified by phosphopyruvate and broken down continuously by the excess phosphoglycerate, and the reaction requires the presence of Mg, though adenylpyrophosphate itself seems to break down in its absence. Since Mg is obligatory for

the transfer of phosphate from phosphopyruvate to adenylic acid, it is easy to see that the dephosphorylation of phosphopyruvate proceeds via adenylic acid.

In the absence of arsenate, adenylpyrophosphate does not decompose even in the presence of very large amounts of phosphoglycerate and NaF, though phosphoglycerate itself under these conditions undergoes considerable dephosphorylation in the absence of NaF if a trace of adenylpyrophosphate is added. This shows that the mechanism of the reaction in which, in the absence of arsenate, phosphoglycerate is dephosphorylated in muscle extract containing no adenylpyrophosphatase and only traces of A.T.P. must be something entirely different from the reaction in the presence of arsenate as has been noticed before [Pillai, 1938].

It is difficult to understand the mechanism of the reaction by which adenylpyrophosphate is decomposed by muscle extract in the presence of traces of phosphoglycerate, cozymase and arsenate. It has been shown that the reaction

$$\text{triosephosphate} + \text{cozymase} + \text{adenylic acid} + \text{phosphate} \rightleftharpoons \text{phosphoglycerate} + \text{reduced cozymase} + \text{A.T.P.}$$

is reversible and can take place both in yeast and muscle extracts. The reaction is stoichiometric and the function of the A.T.P. in the reaction from right to left is to provide the energy by its breakdown to adenylic acid and inorganic phosphate for the conversion of phosphoglycerate into triosephosphate, an endothermic reaction. If lactic acid or alcohol is added to reduce the cozymase as soon as it is oxidized by the phosphoglycerate a small amount of cozymase can act catalytically. A small amount of A.T.P. would also suffice if some phosphate donator like creatinephosphate were added or the NaF omitted, so that some of the phosphopyruvate might itself rephosphorylate adenylic acid.

In the presence of arsenate not only does a trace of cozymase suffice but even a small amount of phosphoglycerate can act catalytically provided that NaF is added to prevent its decomposition to phosphate and pyruvic acid. It has been shown that the phosphoglycerate cannot act by directly splitting off phosphate since Na glycerate is inactive; also the inactivation by iodoacetate shows that the reaction involves some kind of oxidoreduction. Arguing from analogy with phosphoglycerate, glycerophosphate should be capable of reducing cozymase and being oxidized to triosephosphate in the same enzyme system but it has very little action upon the dephosphorylation of A.T.P. Similarly pyruvate, as well as pyruvate + glyceraldehyde, which can form a powerful oxidoreduction system with the formation of lactic acid, has comparatively little action. This breakdown of A.T.P. in the presence of arsenate, like the coupled esterification of adenylic acid, therefore seems to be more or less specific to phosphoglycerate $\rightleftharpoons$ triosephosphate.

It will be observed that no reduced coenzyme could be found spectrophotometrically in the enzyme system producing dephosphorylation of A.T.P. even when large amounts of cozymase were added. The possibility of small amounts of reduced cozymase which cannot be detected by the analytical method employed being present must however be borne in mind.

That cozymase can act under certain circumstances as phosphate carrier must now be considered to have been proved beyond doubt [see Ohlmeyer & Ochoa, 1937] and it is likely that in all the reactions considered below a cozymasepyrophosphate is an intermediate compound, both in esterification and in liberation of inorganic phosphate from A.T.P. or creatinephosphate. The function of the adenylic acid, arguing from analogy with creatine, would then be to act as an acceptor of phosphate from the cozymasepyrophosphate, and of the A.T.P., like creatinephosphate, to be a donator of phosphate to cozymase.

Suppose now that a small amount of reduced cozymase is initially present to start the reaction from right to left in the reversible coupled reaction given above. It will be seen that very soon an equilibrium will be established, when as much triosephosphate will be oxidized by the cozymase to phosphoglycerate as phosphoglycerate is being reduced by the reduced coenzyme. No adenylpyrophosphate will however disappear in the sequel because an equivalent amount of inorganic phosphate will be esterified during the triosephosphate oxidation to replace the A.T.P. being broken down in the reverse reaction. To explain the actual breakdown of A.T.P. observed in the presence of the arsenate one would therefore have to assume that arsenate prevents the esterification of inorganic phosphate accompanying the oxidation of triosephosphate, without affecting the reverse reaction in any way, so that the equilibrium will be upset and the A.T.P. continually decomposed but not reformed. A small amount of phosphoglycerate and cozymase would then act catalytically, being alternately reduced and then oxidized. Of course the formation of an intermediate phosphorylated cozymase both in esterification as well as in dephosphorylation would not affect the final balance of the reaction.

However, this assumption of the inhibition by arsenate of the esterification of inorganic phosphate coupled with the oxidation of triosephosphate [Needham & Pillai, 1937; Meyerhof *et al.* 1938, 1] is difficult of experimental proof because the product of the oxidation of triosephosphate itself (phosphoglycerate) would cause any adenylpyrophosphate, even if it were formed, to break down immediately in the presence of the arsenate. No other similar reactions are known, so that this action of arsenate cannot be tested elsewhere. Arsenate as far as is known only slightly inhibits the phosphatases and does not prevent the transfer of phosphate from phosphopyruvate to adenylic acid, but these are rather different reactions, not comparable with esterification of inorganic phosphate. However, it seems unwarranted to assume in the absence of clear experimental evidence that arsenate inhibits the coupled esterification of inorganic phosphate, and the explanation of the breakdown of adenylpyrophosphate in the presence of arsenate has to await further knowledge.

It is known that arsenate activates the reaction triosephosphate + pyruvate (or acetaldehyde) → phosphoglycerate + lactic acid (or alcohol) to the same rate in the absence of stoichiometric amounts of adenylic acid and inorganic phosphate as when these are present in such amount. The supposition has been advanced that this is due to the fact that the reaction goes on rapidly only when accompanied by esterification of inorganic phosphate, and that arsenate, by breaking the coupling in some manner, allows it to proceed as rapidly even without simultaneous esterification. Why the reaction should go on rapidly, in the absence of large amounts of adenylic acid or inorganic phosphate, merely because the esterification is prevented by arsenate is difficult to understand. Without any assumption of inhibition by arsenate this activation can be explained by the fact that any ester formed (A.T.P. or cozymasepyrophosphate) is decomposed rapidly in the presence of phosphoglycerate and arsenate. Since there are always traces of coenzymes as well as inorganic phosphate present, the function of the arsenate would be to regenerate the coenzyme and inorganic phosphate by rapid breakdown of the coenzymepyrophosphate formed during the oxidation of triosephosphate, so that continuous esterification may occur. The arsenate thus assures a constant supply of coenzyme and inorganic phosphate for esterification and the reaction can go on at the same rate as when large amounts of adenylic acid and phosphate are present.

The behaviour of creatinephosphate is interesting. Since it can break down

in the absence of adenylic acid it is able to transfer phosphate directly to cozymase in the presence of arsenate, Mg and the complete oxidoreduction enzymes. Phosphopyruvate however is not dephosphorylated in the absence of adenylic acid, showing that it cannot transfer phosphate directly on to cozymase but only through adenylic acid, a result in keeping with other observed facts. Further, the necessity for the presence of Mg for the dephosphorylation of creatinephosphate but not for that of A.T.P. would indicate that the function of the Mg is to facilitate the transfer of phosphate between molecules of two dissimilar substances like creatinephosphate and cozymase and that its aid is not necessary for the exchange between two closely related compounds like adenylpyrophosphate and cozymase.

Finally, it has been observed that arsenate instead of activating sometimes inhibits glycolysis [Meyerhof, 1927], and also that it inhibits esterification of glucose [see Schäffner & Krumey, 1936]. While with the higher concentrations of arsenate the inhibition might to some extent be due to injury to the enzymes, it seems likely that the arsenate, by accelerating the breakdown of cozymase-pyrophosphate and adenylpyrophosphate, may prevent under certain conditions the esterification of carbohydrate by these sources and thus retard glycolysis as a whole.

Summary

1. Adenylpyrophosphate breaks down rapidly into adenylic acid and inorganic P in the presence of arsenate in muscle extract containing no adenylpyrophosphatase if cozymase, a trace of phosphoglycerate and NaF (to prevent the decomposition of the phosphoglycerate) are added.

2. This reaction is the basis of the activation of hexosediphosphate breakdown by arsenate. The activation is prominent at two stages, (1) the oxidoreduction between triosephosphate and pyruvate (or acetaldehyde), and (2) the dephosphorylation of phosphoglycerate. The first reaction is considerably activated by the presence of stoichiometric amounts of adenylic acid and inorganic P which are esterified simultaneously, and since, in the presence of arsenate, the phosphoglycerate formed by oxidation of the triosephosphate causes a rapid breakdown of any A.T.P. formed, thus constantly regenerating adenylic acid and inorganic P for continuous esterification, the reaction goes on as quickly as before even when only traces of adenylic acid and inorganic P are available. The dephosphorylation of phosphoglycerate similarly proceeds as rapidly as when an equivalent amount of adenylic acid is present.

3. The mechanism of the reaction is not known but it is possible that it involves reduction of the phosphoglycerate to triosephosphate and formation of a cozymasepyrophosphate as an intermediate phosphorylated compound.

4. Creatinephosphate can break down similarly in the presence of arsenate, cozymase, Mg and a trace of phosphoglycerate and NaF, apparently without adenylic acid as intermediary phosphate transporter.

5. This activated breakdown in the presence of arsenate of adenylpyrophosphate, which is the most important phosphorylating intermediary, might by preventing esterification of carbohydrate account for the inhibition of glycolysis by arsenate sometimes observed.

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