CCXXVIII. THE COUPLING OF OXIDO-REDUCTIONS AND DISMUTATIONS WITH ESTERIFICATION OF PHOSPHATE IN MUSCLE

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THE disappearance of free phosphate during glycogenolysis in muscle extract or brei is a striking phenomenon, which has been well known for many years. It is only recently, however, that it has become possible to analyse this phenomenon, and it is now clear that more than one mechanism can be involved.

Parnas and his colleagues have lately elucidated one of these mechanisms, showing that in a dialysed muscle extract glycogen can react with inorganic phosphate giving hexosemonophosphate [Parnas & Baranowski, 1935; Parnas *et al.* 1936; Ostern *et al.* 1936]. In the absence of adenylpyrophosphate no further reaction takes place and hexosemonophosphate accumulates, until the whole of the glycogen or the whole of the phosphate is used up. If adenylpyrophosphate is now added, hexosediphosphate is formed.

We have been studying another method of esterification of free phosphate—a phosphorylation of adenylic acid which is coupled with dismutation or oxido-reduction.

That disappearance of free phosphate and phosphorylation of carbohydrate might depend upon oxido-reduction has from time to time been observed, e.g. by Nilsson [1933] in yeast extract, by Meyerhof & Kiessling [1935, 1, 2] in yeast and muscle extract, by Schäffner & Berl [1936] in yeast extract and by Dische [1934; 1936] in red blood cells and haemolysed blood. It is to be noticed that the oxido-reduction, e.g. of triosephosphate with pyruvic acid, is an exothermic reaction; this fact suggested that the esterification dependent on it might be an endothermic reaction requiring provision of free energy. Now, little energy is given out on the hydrolysis of hexosemonophosphate and little energy is needed for its synthesis; on the other hand, the hydrolysis of adenylpyrophosphate is strongly exothermic and its synthesis would require provision of free energy. These considerations led us to enquire into the possibility of the coupling of oxido-reduction with synthesis of adenylpyrophosphate from adenylic acid and free phosphate. Dische, in his work on oxido-reduction and carbohydrate esterification in erythrocytes, had already mentioned an increase in easily hydrolysable phosphate in some experiments and had suggested that formation of adenylpyrophosphate was an intermediate stage in the phosphorylation of carbohydrate by free phosphate.

Our results [1937] showed that synthesis of adenylpyrophosphate from adenylic acid and free phosphate can take place in muscle extract, and that this synthesis is coupled with or is dependent upon simultaneous oxido-reduction; in most cases we used the reaction between triosephosphate and pyruvic acid giving phosphoglyceric and lactic acids. While these results were in the press, a

note by Meyerhof [1937] appeared, in which it was shown that synthesis of creatinephosphate (via adenylic acid) can take place in muscle extract from creatine and free phosphate in proportion to accompanying oxido-reduction.

The experimental results on muscle extract will first be described, and their importance in the chemistry of contraction of the intact muscle will be discussed later.

Experimental details

(1) *Enzyme preparation*

An aqueous extract of dried acetone powder from rabbit muscle was used. This was prepared by a modification of the technique described by Meyerhof & Kiessling [1935, 2]. The rabbit was killed and bled, and its muscle was rapidly removed and cooled. This was passed twice through a cooled mincer, well mixed with one and a half times its weight of ice-cold distilled water and allowed to stand, with occasional stirring, for half an hour. The suspension was then strained through muslin, and the extract allowed to stand at room temperature 2–3 hr. At the end of this time, three volumes of acetone were added, and the precipitate was spun off. It was washed once with acetone, once with ether, dried *in vacuo* and powdered finely. The aqueous extract from this powder was made by grinding 2 g. with 25 ml. water. It was necessary for our experiments that the extracts should be free from adenylpyrophosphatase, so the suspension was allowed to stand 5 days at 0° before use [see Lehmann, 1935].[1] It was also dialysed $3\frac{1}{2}$–$4\frac{1}{2}$ hr. to remove any remaining substrates. Finally it was spun, and the almost clear slightly yellow solution was used.

(2) *Chemical preparations used*

The adenylpyrophosphate used was made by the method of Lohmann [1931].

We are indebted to Dr P. Ostern for some of the adenylic acid used.

For the preparation of cozymase, the procedure described by Green *et al.* [1937] was used. This follows very closely the methods of Myrbäck [1933] and Euler *et al.* [1936]. The phosphopyruvic acid was made in this laboratory according to Kiessling's method [1935] by Mr S. Williamson and Dr H. Lehmann, to whom we express our thanks. Triosephosphate was made by the method of Meyerhof & Lohmann [1934, 2].

(3) *Methods*

Lactic acid was estimated by the method of Friedemann & Graeser [1933]. For inorganic phosphate the method of Fiske & Subbarow [1925] was used; when arsenate was present the preliminary treatment described by Pett [1933] was introduced. Triosephosphate was estimated as inorganic phosphate after alkaline hydrolysis [Meyerhof & Lohmann, 1934, 1]; creatinephosphate was separated from true inorganic phosphate by barium precipitation, then estimated by Fiske & Subbarow's method [1929]; difficultly hydrolysable phosphate (phosphoglyceric acid and glycerolphosphate) was found by subtracting the inorganic P present after 180 min. hydrolysis in *N* HCl at 100° from the total P. A correction must be made for the adenylic P and cozymase P remaining unhydrolysed after 180 min.; this amounts to 40 % of the adenylic P and about the same fraction of the cozymase P.

[1] Later experiments showed that even after only 1 day at 0°, no interference by adenylpyrophosphatase took place.

The adenylpyrophosphate was estimated both by means of its easily hydrolysable phosphate (7 min. in *N* HCl at 100° [Lohmann, 1928]) and by the deaminase method of Parnas & Lutwak-Mann [1935].

In the latter method, adenylpyrophosphate is separated from free adenylic acid by addition to the trichloroacetic extract of 25% barium acetate followed by neutralization to phenolphthalein with NaOH. The precipitate is spun off and washed with 1% barium acetate (made just alkaline to phenolphthalein). The precipitate is dissolved in a little 0·1*N* HCl, and saturated sodium sulphate solution is added to remove excess of barium. The final adjustment can be made with more dilute sodium sulphate, as excess of sodium sulphate is to be avoided, although this salt is not so poisonous to the deaminase as barium ions. The barium sulphate precipitate must be washed very carefully, using dilute (0·1*N*) HCl; if water only was used, very great losses were observed, probably due to adsorption. Four washings were carried out and these were added to the main solution, which was then neutralized and made up to 15 ml. This solution was then divided into two parts, the preformed ammonia being estimated in one part, the ammonia present after action of frog muscle enzymes in the other. The enzyme preparation was made by grinding 1 g. frog (*R. temporaria*) muscle with 10 ml. phosphate buffer (*p*H 7·2; 0·1–0·5*M*) and 1 g. quartz sand. 1 ml. of this brei was used; it is capable of setting free up to 0·15 mg. ammonia N from adenylic acid or adenylpyrophosphate in 2 hr. at 37°. After this incubation period, ammonia was estimated by the method of Parnas & Heller [1924]. The free ammonia in the brei itself was also estimated. If the ammonia distillation is not performed at once after the action of the deaminase, the samples are precipitated with trichloroacetic acid; it is unnecessary to remove the protein before distilling. We are indebted to Dr C. Lutwak-Mann for advice on this method.

(4) *Experimental procedure*

1 ml. of extract was made up to 2·5–3 ml. with other solutions. The final concentration of hexosediphosphate in the mixture was 0·013*M*, of pyruvic acid 0·026*M*, of adenylic acid 0·006*M*, of phosphate about 0·03*M*, unless otherwise stated. The phosphate solution had *p*H 7·2. Fluoride, 0·02*M*, was always used to prevent breakdown of the phosphoglyceric acid formed. Cozymase was always added, as the extract contains less than the optimum amount; the solution used contained 150 mg. of the dry preparation in 100 ml., and 0·3 ml. was added per ml. extract or per 3 ml. final volume. In some cases the experiments were done at *p*H 7·2; in others bicarbonate was added (to make the mixture about 0·3%) and the *p*H was about 8·6.

In order to obtain values for the initial content of inorganic P, lactic acid etc., appropriate amounts were pipetted out of the mixture immediately after addition of the extract and were run into an equal volume of 8% trichloroacetic acid. After incubation of the rest of the mixture for the required time (usually 30 min. at 37°) this also was precipitated with an equal volume of 8% trichloroacetic acid. The trichloroacetic acid extracts were filtered through paper, and an aliquot part was neutralized and made up to a known volume. For 1 ml. of acetone powder extract (or about 3 ml. of reaction mixture) a convenient volume is 30 ml. 1 ml. can be used for each P fraction required and 15–20 ml. for lactic acid estimation.

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

THE PHOSPHORYLATION OF ADENYLIC ACID

In Table I are shown the results of some experiments in which oxido-reduction between triosephosphate and pyruvic acid was accompanied by formation of adenylpyrophosphate and disappearance of free phosphate.

Table I

Exp.	Lactic acid	Phospho-glyceric acid P	Inorganic P	Pyrophos-phate P	Remarks
4	+0·85	+0·31	−0·37	+0·35	5 min. at 37°, *p*H 7·2
	+1·58	+0·57	−0·62	+0·66	10 min. at 37°, *p*H 7·2
5	+1·80	+0·70	−0·78	+0·72	30 min. at 37°, *p*H 7·2
7	+2·68	+0·66	−0·74	+0·66	30 min. at 37°, *p*H 8·6

The extent of the oxido-reduction going on was determined by estimating the lactic acid and phosphoglyceric acid formed in each case. The fact that these two values are approximately equivalent shows that the fluoride poisoning is complete; the breakdown of phosphoglyceric acid has been completely inhibited, and therefore no adenylpyrophosphate formation can be accounted for by phosphate from this source via phosphopyruvic acid.

The adenylpyrophosphate formation was estimated by hydrolysis rate in Exps. 4 and 5, by deamination in Exp. 7. In all cases the inorganic P disappearing was equivalent to the adenylpyrophosphate P formed.

That the formation of adenylpyrophosphate and the disappearance of inorganic phosphate are dependent on the oxido-reduction is shown in Table II. The presence of 0·0025*M* iodoacetate prevents the oxido-reduction and at the same time completely inhibits esterification of inorganic phosphate and phosphorylation of adenylic acid. Also, deficiency in cozymase, which greatly slows the oxido-reduction, greatly decreases esterification.

Table II

Exp.		Lactic acid	Inorganic P	Pyrophos-phate P	Triosephos-phate P
5	No iodoacetate	+1·80	−0·78	+0·72	—
	+Iodoacetate	+0·32	0	0	0·28
7	No iodoacetate	+2·68	−0·74	+0·66	—
	+Iodoacetate	—	0	0	—
15	No added cozymase	+1·58	−0·15	—	—
	+Cozymase	+5·28	−0·93	—	—

It is interesting to remember that the formation of hexosemonophosphate from glycogen by reaction with inorganic phosphate is not inhibited by iodoacetate. It was, however, found by Parnas and his colleagues to be inhibited by phloridzin, and another clear distinction between the Parnas esterification and the esterification studied here, is that the latter is unaffected by phloridzin (see Table III).

Table III

Exp.		Lactic acid	Inorganic P	Pyrophos-phate P	Remarks
14	No phloridzin	—	−0·81	+0·72	—
	+Phloridzin	—	−0·81	+0·72	—
18	No phloridzin	+4·56	−0·51	+0·57	—
	+Phloridzin	+3·87	−0·45	+0·66	—
	No phloridzin	0	−1·71	—	Glycogen (1%) instead of hexosediphosphate + pyruvic + adenylic
	+Phloridzin	0	−0·36	—	

In Exp. 18 the lactic acid formation is 15% inhibited, the esterification 12% inhibited by 0·01 *M* phloridzin. A parallel test on the same extract in which glycogen was added (in place of hexosediphosphate, pyruvate and adenylic acid) showed that esterification of glycogen was 80% inhibited by the same phloridzin concentration.

The next consideration was the stoichiometric relation between dismutation and esterification. In passing it may be mentioned that the extract as used showed little or no deaminating activity, so that adenylic acid is not being destroyed in this way in the course of the experiments. In Table IV the ratios

Table IV

Exp.	Lactic acid produced / P esterified	mg. adenylic acid added per ml. extract	% adenylic acid esterified	Remarks
4	2·5	6	22	*p*H 7·2, 15 min. at 20°
	2·7	6	42	,, 30 min. at 20°
	2·3	6	34	,, 5 min. at 37°
	2·6	6	56	,, 10 min. at 37°
5	2·9	6	61	,, 30 min. at 37°
	2·3	6	76	,, 30 min. at 37°
7	3·6	6	68	*p*H 8·6, 30 min. at 37°
15	5·0	6	87	,, ,,
18	7·6	6	48	,, ,,
	7·0	6	42	,, ,,
25	4·1	6	78	,, ,,
	2·8	12	80	,, ,,
26	4·5	6	79	,, ,,
	3·0	12	74	,, ,,
	2·7	18	58	,, ,,
27	4·8	6	76	,, ,,
32	2·0	36	24	,, 30 min. at 37° 0·07 *M* phosphate
	2·0	54	16	,, 30 min. at 37° 0·07 *M* phosphate
42	1·9	18	24	,, 5 min. at 37° 0·03 *M* phosphate
	2·0	18	27	,, 15 min. at 37° 0·03 *M* phosphate
	2·1	18	28	,, 30 min. at 37° 0·03 *M* phosphate

of lactic acid formed to P esterified are collected together. If the reaction of one molecule of triosephosphate with one molecule of pyruvate is coupled stoichiometrically with the esterification of one atom of P, then the ratio would be 2·9. It will be seen that the ratio does reach and exceed this value, but is usually not significantly less. The oxido-reduction can, of course, go on in the absence of adenylic acid, and the ratio would then be infinity. These results, then, seem to show that, according to the conditions, a greater or less proportion of the oxido-reduction can be coupled with phosphorylation, but that, however favourable the conditions, not more than one atom of P is esterified per molecule of lactic acid formed.

The variability of the ratio is probably to be explained by the following considerations. In Exp. 4 no bicarbonate was added and the *p*H was 7·2; the time of experimentation was short. These conditions mean that the adenylic acid was present in excess, and in fact only 22–56% of the amount added was phosphorylated. In this experiment the ratio is near that required for one molecule of lactic acid to one atom of P esterified. In Exp. 5 also the *p*H was 7·2,

but the time allowed was longer, and nearly all the adenylic acid was phosphorylated. In the remaining experiments the *p*H was higher, owing to the addition of bicarbonate and the time of incubation was 30 min. Under these conditions practically the whole of the adenylic acid was phosphorylated (75–90 %) when only *M*/150 was present and this phosphorylation was probably complete some time before the oxido-reduction ceased. When larger quantities of adenylic acid were added (Exps. 25, 26, 32 and 42) phosphorylation was less complete and the ratio fell.

More work is, however, needed on these stoichiometric relations, as cases were encountered, for instance Exp. 42, where the ratio was as low as 2. It is possible that under certain conditions so far not ascertained, the dismutation of triosephosphate with triosephosphate is playing a part, even when pyruvic acid is present.

(5) *The effect of arsenate*

A third substance whose effect on phosphorylation is of great interest is arsenate. It has been known for many years that addition of arsenate prevents accumulation of phosphoric esters of carbohydrate during glucose fermentation by yeast extract or glycogenolysis by muscle extract; and further that, in presence of arsenate, the rate of alcohol or lactic acid formation from hexosediphosphate is as high as that from glucose or glycogen. Without arsenate, hexosediphosphate breakdown is very slow. Harden & Young originally suggested that arsenate activates a hexosediphosphate phosphatase, so that glucose and free phosphate were continually being reformed. But subsequent work on phosphatases has shown inhibition rather than activation by arsenate [see e.g. Pett & Wynne, 1934; Schäffner & Krumey, 1936]. One exception has recently been claimed—that of adenylpyrophosphatase [Schäffner & Krumey, 1936]. The work of Meyerhof & Kiessling [1936] seemed also to show that arsenate had a similar activating effect upon an enzyme breaking down the pyrophosphate compound of cozymase. If arsenate increases the rate of dephosphorylation of adenylpyrophosphate, then an explanation of the increased rate of lactic acid formation from hexosediphosphate would be at hand. One step in lactic acid formation is the dephosphorylation of phosphopyruvic acid, the phosphate being transferred to adenylic acid. In the presence of glycogen the phosphate is very rapidly again transferred with the formation of hexosemonophosphate or hexosediphosphate; if no substance capable of receiving the phosphate from adenylic acid is present, then the rate of phosphopyruvic acid breakdown will depend on adenylpyrophosphatase activity. In fresh muscle extract, the rate of formation of free phosphate from adenylpyrophosphate is much less than its rate of transfer to carbohydrate or creatine; and the adenylpyrophosphatase is far more readily inactivated by standing than the enzymes concerned with phosphate transport from one organic molecule to another. Thus any circumstance increasing the rate of adenylic acid reformation will increase the rate of breakdown of hexosediphosphate and phosphopyruvic acid.

We decided to try what effect, if any, arsenate has on oxido-reduction and the phosphorylation of adenylic acid. As Table V shows, the esterification is completely inhibited, although the oxido-reduction goes on as usual. A further question then arises. Is this effect due to an inhibition of the coupling? Or is adenylpyrophosphatase present and activated by the arsenate to such an extent that any adenylpyrophosphate synthesized by the coupling mechanism is again broken down? We had several times shown that the adenylpyrophosphatase activity of our extracts was nil, but it remained possible that arsenate might

be having a great activating effect upon otherwise undetectable traces of the enzyme. We therefore tested the adenylpyrophosphatase activity of this same extract, with and without arsenate (Exp. 19). In neither case was there any significant breakdown. It seems, therefore, that arsenate inhibits the coupling mechanism, without inhibiting oxido-reduction.

Table V

Exp.		Lactic acid	Inorganic P
17	No arsenate	—	−1·14
	+Arsenate *M*/75	—	0
19	No arsenate	4·28	−0·69
	+Arsenate	5·0	+0·18
	No adenylic acid; no arsenate but adenylpyrophosphate (0·8 mg. P)	—	−0·09
	Do. +arsenate *M*/100	—	+0·06
27	No arsenate	1·89	−0·78
	+Arsenate *M*/80	2·45	0
	Do. *M*/320	2·51	0

It is to be noticed that this inhibition of the coupling in itself leads to increased adenylic acid concentration, and thus would lead to increased rate of hexosediphosphate and phosphopyruvic acid breakdown. Further, in view of these results upon the effect of arsenate on the coupled mechanism the effect of arsenate upon adenylpyrophosphatase can only be studied with certainty when the conditions are such that no coupled resynthesis can come into operation in the unpoisoned control.

In order to study this question, we used fresh aqueous extract prepared in the usual way from rabbit muscle, not more than 2 days old and dialysed 3 hr. Such extracts contain active adenylpyrophosphatase, and their action on adenylpyrophosphate was tested with and without arsenate. In order to make sure that no coupled adenylpyrophosphate resynthesis obscuring breakdown was going on, controls were performed in which adenylic acid and inorganic phosphate were added instead of adenylpyrophosphate. No esterification of free phosphate took place in these controls (Exp. 20).

Table VI

Exp.		Inorganic P	Incubation time
20	Adenylpyrophosphate (1·2 mg. pyro P) but no adenylic acid or arsenate	+0·33	—
	Do. +arsenate	+0·31	
	Adenylic acid only without A.T.P. or arsenate	−0·01	—
31	Adenylpyrophosphate (1·45 mg. pyro P), no arsenate	+0·090	15 min.
	Do.	+0·171	30 min.
	+Arsenate *M*/240	+0·114	15 min.
	Do.	+0·204	30 min.

It will be observed that, especially during the first 15 min., arsenate has some effect in increasing adenylpyrophosphate breakdown. But even in the early period the breakdown without arsenate is about 80% of that with arsenate; it seems clear that this effect alone cannot account for the increased rate of hexosediphosphate breakdown, for during the first 15 min. this is usually at least twice as high with arsenate as without.

We also tested whether arsenate affects the rate of transfer of phosphate from phosphopyruvic acid to adenylic acid, and the result was negative.

Table VII

0·02 *M* phosphopyruvic acid instead of hexosediphosphate + pyruvic as usual. Arsenate *M*/250. Rest as in other experiments.

Exp		Amino-N in the adenylpyrophosphate formed by 1 ml. enzyme solution	sec.
35	No arsenate	0·068	60
	+ Arsenate	0·055	60
	No arsenate	0·095	120
	+ Arsenate	0·103	120

THE SPECIFICITY OF THE REACTANTS

In all the experiments so far described the oxido-reduction studied has been between triosephosphate and pyruvic acid. We have also tried the effect of other oxido-reductions upon esterification of adenylic acid (Table VIII).

The reaction of α-glycerophosphate with pyruvic acid led to esterification of free phosphate (Exps. 15 and 44). There are probably two oxido-reductions occurring here—that of α-glycerophosphate with pyruvic acid, producing triosephosphate and lactic acid, followed by that of the triosephosphate with pyruvic acid.

No lactic acid formation and no disappearance of free phosphate took place with β-glycerophosphate and pyruvic acid.

When pyruvic acid is present together with triosephosphate, the phosphoglyceric acid formed is equivalent to the lactic acid formed, and to the phosphate esterified. This seems to mean that the only oxido-reduction occurring is that between these two substances. But if pyruvic acid is absent, dismutation occurs between one molecule of triosephosphate and another. This dismutation, as was previously known [Meyerhof & Kiessling, 1935, 2], is slower than the reaction with pyruvate, but it is also coupled with adenylpyrophosphate synthesis [see also Meyerhof, 1937]. In this case, the difficultly hydrolysable phosphate formed is equivalent to twice the phosphate esterified (Exps. 15 and 39).

We are indebted to Dr D. E. Green for the information that, in muscle extract, triosephosphate can react with oxaloacetic acid instead of pyruvic acid; and further that glyceraldehyde can react with both pyruvic and oxaloacetic acids. All these oxido-reductions are inhibited by iodoacetate. The effect of each of these oxido-reductions upon phosphate esterification in presence of adenylic acid was tested. 0·026 *M* oxaloacetate and glyceraldehyde were used.

Table VIII

Exp.		Lactic acid	Inorganic P	Difficultly hydrolysable P	Triosephosphate P
15	Hexosediphosphate + pyruvic acid	5·28	−0·93	—	—
	α-Glycerophosphate + pyruvic acid	2·16	−0·51	—	—
	β-Glycerophosphate + pyruvic acid	0·57	−0	—	—
	Hexosediphosphate	1·22	−0·93	+2·36	—
44	α-Glycerophosphate + pyruvic acid	1·09	−0·25	—	—
40	Hexosediphosphate + oxaloacetic acid	—	−1·02	+0·96	—
39	Do.	—	−0·85	+0·82	—
	Hexosediphosphate	0·24	−0·47	+0·79	—
38	Hexosediphosphate + pyruvic acid	1·89	−0·75	—	—
	Do. + oxaloacetic acid	—	−0·87	—	—
	Glyceraldehyde + pyruvic acid	1·44	−0·09	—	—
	Do. + oxaloacetic acid	—	0	—	—
16	Triosephosphate + iodoacetic acid	—	−0·12	—	−3·9

In the presence of triosephosphate and oxaloacetic acid, the disappearance of free phosphate was as great as with pyruvic acid. These experiments were performed with the solution in equilibrium with an atmosphere of 95% N_2+5% CO_2, and in $M/40$ $NaHCO_3$. Control experiments in Barcroft manometers showed that under these conditions the decarboxylation of oxaloacetic to pyruvic acid is very slight. This is borne out by the "lactic acid" estimations, where the volatile bisulphite-binding substance was hardly greater in amount than that arising in a control from an amount of malic acid equivalent to the phosphoglyceric acid formed. The phosphoglyceric acid formed was equivalent to the phosphate esterified, showing that dismutation of triosephosphate with triosephosphate was playing no part.

With glyceraldehyde and pyruvic acid the oxido-reduction led to lactic acid formation not much less than with triosephosphate and pyruvic acid. But no esterification occurred. With glyceraldehyde and oxaloacetic acid (where the acid production has so far only been observed manometrically) there was also no esterification.[1]

It may be mentioned that the reversible reaction hexosediphosphate $\rightleftharpoons$ 2 triosephosphate cannot, in either direction, be coupled with adenylpyrophosphate synthesis. That the breakdown of hexosediphosphate is not coupled is shown by the lack of adenylpyrophosphate synthesis in presence of iodoacetic acid; that the reverse exothermic reaction is not coupled was shown by Exp. 16. Here $0{\cdot}06M$ triosephosphate was used, the other additions to the extract being phosphate, adenylic acid and iodoacetate in the usual concentrations. The mixture was kept 5 min. at room temperature. The decrease in alkali-labile phosphate shows that about 90% of the triosephosphate has been converted into hexosediphosphate.

We finally wished to know whether hexosediphosphate could replace free phosphate in providing phosphate groups for the esterification of adenylic acid. Lutwak-Mann & Mann [1935], and Ohlmeyer [1935] have found in Lebedev extract from yeast evidence of transfer of phosphate from hexosediphosphate to adenylic acid. This, like the reaction between adenylic acid and free phosphate, must be an endothermic reaction, and it was observed that it could only take place while some of the hexosediphosphate was breaking down; further, that it was impossible in the presence of iodoacetate. Ohlmeyer claimed that the product of dephosphorylation of the hexosediphosphate was hexosemonophosphate, but the evidence for this is not clearly given.

In muscle extract, we could find no evidence for this reaction. The extracts used were dialysed 6–7 hr., to ensure a very low phosphate content. Hexosediphosphate, pyruvate, adenylic acid and iodoacetate were added as usual but no phosphate (Table IX).

Table IX

Exp.		Inorganic P	Pyro P	Creatine-phosphate P
14	No inorganic P added	−0·06	+0·21	—
	+Inorganic P added	−0·81	+0·72	—
29	No inorganic P added, 5 min.	−0·26	+0·16	—
	No inorganic P added, 15 min.	−0·26	+0·20	—
37	No inorganic P added + creatine (15 min. at 37°)	−0·094	—	+0·097

[1] In a recent paper, Braunstein & Vyshepan [1937] have shown that, in muscle extract, triosephosphate can react with certain homologues of pyruvic acid, α-ketobutyric acid and α-ketovaleric acid. These oxido-reductions were accompanied by disappearance of inorganic phosphate.

The adenylpyrophosphate formed was never greater than could be accounted for by the inorganic phosphate disappearing. It seemed, however, possible that phosphate might at first be rapidly transferred to adenylic acid with formation of adenylpyrophosphate and hexosemonophosphate (by means of the coupled mechanism) and that this reaction might be later followed by rephosphorylation of the hexosemonophosphate at the expense of adenylpyrophosphate:

2 Hexosediphosphate + adenylic acid → 2 hexosemonophosphate + adenylpyrophosphate coupled with oxido-reduction,

2 Hexosemonophosphate + adenylpyrophosphate → 2 hexosediphosphate + adenylic acid.

We therefore tried stopping the incubation after shorter intervals of time.

As these attempts met with no success, we tried the addition of creatine; assuming that, if adenylpyrophosphate and hexosemonophosphate were formed, then later dephosphorylation of the adenylpyrophosphate would go on, at any rate partly, by reaction with creatine with formation of creatinephosphate. The results show that the creatinephosphate formed is equivalent only to the inorganic phosphate disappearing, and there is no evidence that any dephosphorylation of hexosediphosphate has occurred.

Discussion

(1) *The mechanism of the coupled reaction*

Little can be said about the mechanism of this coupling until the mechanism of the oxido-reduction processes concerned is itself understood. The oxido-reduction between, for example, pyruvic acid and triosephosphate may be written in two stages:

Triosephosphate + cozymase → phosphoglyceric acid + reduced cozymase,

Pyruvic acid + reduced cozymase → lactic acid + cozymase.

Here we have two separate reactions, each yielding heat and free energy; either or both may be coupled with the endothermic synthesis of adenylpyrophosphate. The analytical results of the present paper, as well as those of Meyerhof [1937] and Meyerhof & Kiessling [1935, 1, 2] suggest that, whatever the exact mechanism of the coupling, the relations are stoichiometric. Further work must elucidate the nature of the intermediate compounds and reactions playing a part for example in the balanced reaction which may be written:

2 Triosephosphate + cozymase + adenylic acid + 2 H_3PO_4 → 2 phosphoglyceric acid + reduced cozymase + adenylpyrophosphate.

The chemical relationship between cozymase and adenylic acid suggests possible mechanisms—e.g. the formation of a reduced phosphorylated cozymase, followed by the splitting off of adenylpyrophosphate, followed by combination of the reduced cozymase residue with free adenylic acid. This is only a hypothesis, but it makes possible the visualization of the production of free energy (in the reduction of the cozymase) and its utilization (in the phosphorylation of cozymase) in the same molecule.

The fact that all these experiments were done in extracts free from adenylpyrophosphatase shows clearly that the coupled mechanism of synthesis cannot depend in any way on a reversal of the action of this enzyme.

Dixon & Lutwak-Mann [1937, 1] have shown that the aldehyde mutase of liver is an enzyme distinct from acetaldehyde dehydrogenase. They suggest [1937, 2] that this dismutation is brought about by a single enzyme with two

active centres; at one, cozymase is reduced by aldehyde, at the other, reduced cozymase is oxidized by aldehyde. This enzyme, unlike the dehydrogenases, is poisoned by iodoacetate. All oxido-reductions which have been, in the present work, coupled with phosphate esterification, are poisoned by iodoacetate; that the enzymes concerned also resemble mutase in not consisting of linked dehydrogenase systems is made likely by the results of Dewan & Green [1937] and of Innes [1937]. It is as yet uncertain whether dehydrogenase systems can, in muscle extract, be coupled with phosphorylation of adenylic acid. For a discussion of lactic and triosephosphate dehydrogenases in this connexion, the reader is referred to the paper of Innes [1937].

(2) *The Harden and Young equation*

The knowledge of this coupled mechanism for the esterification of free phosphate and phosphorylation of adenylic acid clears up certain difficulties; for instance, it is well known that the Harden & Young equation (according to which for every molecule of carbohydrate breaking down to alcohol, a second molecule is esterified to hexosediphosphate) under certain experimental conditions describes the events in muscle extract, lactic acid being substituted for alcohol:

$$2\ \text{Hexose} + 2H_3PO_4 \rightarrow 2\ \text{lactic acid} + \text{hexosediphosphate}.$$

The mechanism underlying these stoichiometric relations has often been debated, but until the present time has remained obscure.

Consider now carbohydrate breaking down in muscle extract in presence of adenylpyrophosphate. The reaction between carbohydrate and adenylpyrophosphate is extremely rapid, and the whole of the pyrophosphate P is at once transferred to the carbohydrate. The amount of adenylpyrophosphate originally present is, of course, very small compared with the amount of carbohydrate, but let us suppose that it is enough to provide more triosephosphate than that required to saturate the mutase:

$$n\ \text{Glycogen residues} + n\ \text{A.T.P.} \rightarrow n\ \text{hexosediphosphate} + n\ \text{adenylic acid}$$

$$(n-m)\ \text{hexosediphosphate} + 2\ (n-m)\ \text{pyruvic acid} \rightarrow 2\ (n-m)\ \text{phosphoglyceric acid} + 2\ (n-m)\ \text{lactic acid}$$

$$\downarrow$$

$$2\ (n-m)\ \text{phosphopyruvic acid}$$

$$2\ (n-m)\ \text{phosphopyruvic acid} + (n-m)\ \text{adenylic acid} \rightarrow 2\ (n-m)\ \text{pyruvic acid} + (n-m)\ \text{adenylpyrophosphate}.$$

If we consider first the case where coupled resynthesis of adenylpyrophosphate is not possible, then we see that the above reactions result in a steady state of lactic acid production in which $(n-m)$ molecules of adenylpyrophosphate are continually built up and broken down; m molecules of hexosediphosphate (a very small, perhaps undetectable concentration) and m molecules of adenylic acid are continually present. But when we take into consideration the coupled resynthesis, then we see that in each cycle twice as much adenylpyrophosphate is resynthesized. This must mean that twice as much hexosediphosphate is formed each time as can be broken down by the mutase, and that for every $2\ (n-m)$ molecules of lactic acid formed, $(n-m)$ molecules of hexosediphosphate accumulate—in fact the relations are those expressed in the Harden & Young equation. That hexosephosphates do not accumulate to any marked extent in normal contracting muscle is due to the utilization of the excess adenylpyrophosphate to phosphorylate creatine, as described later in this discussion.

(3) *Inhibition of ammonia formation by pyruvate + phosphate*

Parnas and his colleagues have observed that ammonia formation in muscle brei poisoned with fluoride cannot be suppressed by addition of hexosediphosphate, but it can be suppressed if pyruvic acid together with free phosphate is also added [see Parnas & Lutwak-Mann, 1935]. It was at first supposed that phosphopyruvic acid could be synthesized from pyruvate and phosphate, but there is no evidence for this. It was then suggested by Needham & van Heyningen [1935] that the effect depended upon reaction between the added pyruvic acid and glycerophosphate (formed from the hexosediphosphate). The result of such oxido-reduction would be the formation of phosphoglyceric acid, and in the presence of this increased concentration of phosphoglyceric acid, the fluoride poisoning might be to some extent overcome [see Mann, 1935]. Parnas accepted this interpretation, with the important modification that the oxido-reduction leading to phosphoglyceric acid formation is rather between triosephosphate and pyruvic acid [Parnas *et al.* 1936]. It seems now, however, much more likely that the suppression of ammonia formation depends upon adenylpyrophosphate synthesis from free phosphate coupled with the oxido-reduction. The fact that addition of inorganic phosphate is necessary supports this view, which would also explain why suppression of ammonia formation by pyruvate + phosphate does not occur in iodoacetate-poisoned brei.

(4) *The role of the coupled esterification in the contracting muscle*

In the muscle contracting anaerobically, the most striking chemical changes are disappearance of glycogen and creatinephosphate and increase in lactic acid, free creatine and free phosphate. The creatine and the phosphate formed are equivalent to the creatinephosphate disappearing. After the anaerobic contraction there follows anaerobic "recovery". During this time (30–60 sec.) more lactic acid is formed (about as much again as during the contraction period proper, after 1–10 sec. tetanus) and about one-third of the lost creatinephosphate is resynthesized, equivalent amounts of creatine and phosphate disappearing. It seems that this synthesis of creatinephosphate must take place via adenylic acid, for creatine cannot react directly with inorganic phosphate. The problem of anaerobic recovery is therefore to provide an excess of adenylpyrophosphate above the amount needed for phosphorylating glycogen, so that this excess may react with creatine.

In the following scheme for the happenings during anaerobic recovery, only the well-established way of phosphorylating adenylic acid by means of phosphopyruvic acid is used; but the adenylpyrophosphate is economized by calling also upon the Parnas mechanism for formation of hexosemonophosphate from glycogen and free phosphate. The heats of reaction are taken from the work of Lohmann [1934] and Meyerhof & Schulz [1935; 1936]:

I. Glycogen + $2H_3PO_4$ → 2 hexosemonophosphate.

II. 2 Hexosemonophosphate + adenylpyrophosphate → 2 hexosediphosphate + adenylic acid (+24,000 g. cal.).

III. 2 Hexosediphosphate → 4 triosephosphate (−28,000 g. cal.).

IV. 4 Triosephosphate + 4 pyruvic acid → 4 phosphoglyceric acid + 4 lactic acid (+32,000 g. cal. + 24,000[1] g. cal.).

V. 4 Phosphoglyceric acid → 4 phosphopyruvic acid.

[1] This portion of the heat output is due to neutralization by protein of the acid formed.

VI. 4 Phosphopyruvic acid + 2 adenylic acid → 4 pyruvic acid + 2 adenylpyrophosphate (-2×7400 g. cal.).

VII. Adenylpyrophosphate + 2creatine → 2creatinephosphate + adenylic acid ($+1000$ g. cal.).

There are two criticisms to make of this scheme. In the first place it gives resynthesis of only one molecule of creatinephosphate for two molecules of lactic acid formed from glycogen, whereas the figures actually found by Lundsgaard [1931] during the anaerobic recovery period showed about four molecules of creatinephosphate for two molecules of lactic acid. In the second place, this scheme conflicts with the thermal data; it would lead us to expect a large output of heat during anaerobic recovery, whereas the observed output is very small. We may suppose that the heat of reaction II is used in overcoming the cooling effect of reaction III; but about 75% of the heat of reaction IV would be expected to appear as heat, only 25% being needed to overcome the cooling effect of reaction VI. There is also another difficulty. The relation of heat to tension is the same in the early contractions of muscle poisoned with iodoacetic acid as in a normal muscle [Fischer, 1930]. Presumably in the poisoned muscle as in the normal, the fibrils draw first (to a degree depending on the tension produced) upon the adenylpyrophosphate; in the poisoned muscle this is restored only by creatinephosphate breakdown, breakdown of carbohydrate further than triosephosphate being impossible. The resynthesis of adenylpyrophosphate by means of creatinephosphate breakdown is thermally very efficient; therefore in normal muscle the resynthesis by means of carbohydrate breakdown must be very efficient also, for no greater heat formation occurs per unit of tension.

These difficulties are very largely removed by taking into consideration the coupled oxido-reduction and phosphorylation of adenylic acid described in this paper. This coupling means that two molecules of adenylpyrophosphate would be formed during reaction IV. This, together with the adenylpyrophosphate formed in reaction VI, gives the possibility of the synthesis of six molecules of creatinephosphate, as well as the restoration of the original adenylpyrophosphate concentration. Thus three molecules of creatinephosphate are synthesized for every two molecules of lactic acid formed from glycogen—a figure much nearer Lundsgaard's observed results. As regards the thermal data, the heat produced in reaction IV is not a very easily computed amount, as a large part of it depends upon the neutralization of the acid formed by protein, and this fraction will vary according to the *p*H of the muscle at the time. But it seems very likely that the heat produced in reaction IV would be just about enough to overcome the cooling effect of reaction VII and to provide for the requirements of the endothermic synthesis of adenylpyrophosphate ($-48{,}000$ cal.). Thus the observed results during anaerobic recovery—the small "delayed anaerobic heat" amounting to about 9% of the whole anaerobic heat [see Blaschko, 1930] and the small negative heat [Hartree, 1933]—are easily understood; and resynthesis of adenylpyrophosphate from carbohydrate breakdown becomes as efficient as resynthesis from creatinephosphate.

Summary

1. The existence in muscle extract of a coupled mechanism whereby synthesis of adenylpyrophosphate (from adenylic acid and free phosphate) can accompany oxido-reduction has been demonstrated.

2. That the synthesis of adenylpyrophosphate depends on the oxido-reduction is shown by the fact that any condition which prevents the latter (absence of cozymase, presence of iodoacetate) prevents also the synthesis.

3. Phloridzin has no effect on the synthesis.

4. Arsenate has no inhibitory effect on the oxido-reduction, but entirely prevents the coupled synthesis of adenylpyrophosphate.

5. The activating effect of arsenate upon adenylpyrophosphatase (in enzyme preparations carefully freed from other substrates) was small. It is suggested that the inhibition by arsenate of the coupled phosphorylation of adenylic acid may be an important factor in the activating effect of arsenate upon hexosediphosphate breakdown.

6. Oxido-reductions which can be coupled with adenylic phosphorylation in muscle extract are: triosephosphate + pyruvic acid; 2 triosephosphate; triosephosphate + oxaloacetic acid; α-glycerolphosphate + pyruvic acid. The results suggest that for each molecule oxidized or reduced one atom of P is esterified. Oxido-reduction between glyceraldehyde and pyruvic acid or oxaloacetic acid is not coupled with adenylpyrophosphate synthesis.

7. No evidence could be found for transfer in muscle extract of phosphate from hexosediphosphate to adenylic acid.

8. The establishment of this synthesis of adenylpyrophosphate coupled with oxido-reduction between triosephosphate and pyruvate makes much clearer (i) the origin of the Harden & Young equation, (ii) the events of anaerobic recovery, explaining (*a*) the observed ratio of creatinephosphate synthesis to lactic acid production; (*b*) the absence of any significant heat output during the anaerobic recovery period.

REFERENCES

Blaschko (1930). *J. Physiol.* **70**, 96.
Braunstein & Vyshepan (1937). *Bull. Biol. Med. exp.* **3**, 70.
Dewan & Green (1937). *Biochem. J.* **31**, 1074.
Dische (1934). *Naturwissenschaften*, **22**, 776.
—— (1936). *Naturwissenschaften*, **24**, 462.
Dixon & Lutwak-Mann (1937, 1). *Nature, Lond.*, **139**, 548.
—— (1937, 2). (In the Press.)
Euler, Albers & Schlenk (1936). *Hoppe-Seyl. Z.* **240**, 113.
Fischer (1930). *Naturwissenschaften*, **18**, 736.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
—— —— (1929). *J. biol. Chem.* **81**, 629.
Friedemann & Graeser (1933). *J. biol. Chem.* **100**, 291.
Green, Dewan & Leloir (1937). *Biochem. J.* **31**, 934.
Hartree (1933). *J. Physiol.* **77**, 104.
Innes (1937). (In the Press.)
Kiessling (1935). *Ber. dtsch. chem. Ges.* **68**, 597.
Lehmann (1935). *Biochem. Z.* **281**, 271.
Lohmann (1928). *Biochem. Z.* **202**, 466.
—— (1931). *Biochem. Z.* **233**, 460.
—— (1934). *Biochem. Z.* **271**, 264.
Lutwak-Mann & Mann (1935). *Biochem. Z.* **281**, 140.
Lundsgaard (1931). *Biochem. Z.* **233**, 322.
Mann (1935). *Biochem. Z.* **279**, 82.
Meyerhof (1937). *Naturwissenschaften*, **25**, 443.
—— & Kiessling (1935, 1). *Biochem. Z.* **281**, 449.
—— —— (1935, 2). *Biochem. Z.* 283, 83.
—— —— (1936). *Naturwissenschaften*, **24**, 361.
—— & Lohmann (1934, 1). *Biochem. Z.* **271**, 89.
—— —— (1934, 2). *Biochem. Z.* **273**, 415.
—— & Schulz (1935). *Biochem. Z.* **281**, 292.

Meyerhof & Schulz (1936). *Biochem. Z.* **289**, 87.
Myrbäck (1933). *Ergebn. Enzymforsch.* **2**, 139.
Needham & Pillai (1937). *Nature, Lond.*, **140**, 64.
—— & van Heyningen (1935). *Biochem. J.* **29**, 2040.
Nilsson (1933). *Biochem. Z.* **258**, 198.
Ohlmeyer (1935). *Biochem. Z.* **283**, 114.
Ostern, Guthke & Tersakowec (1936). *Hoppe-Seyl. Z.* **243**, 9.
Parnas & Baranowski (1935). *C.R. Soc. Biol., Paris*, **120**, 307.
—— & Heller (1924). *Biochem. Z.* **152**, 5.
—— & Lutwak-Mann (1935). *Biochem. Z.* **278**, 11.
—— & Mochnacka (1936). *C.R. Soc. Biol., Paris*, **123**, 1173.
—— Sobczuk & Nejbaum (1936). *C.R. Soc. Biol., Paris*, **122**, 701.
Pett (1933). *Biochem. J.* **27**, 1672.
—— & Wynne (1934). *Biochem. J.* **28**, 365.
Schäffner & Berl (1936). *Hoppe-Seyl. Z.* **238**, 111.
—— & Krumey (1936). *Hoppe-Seyl. Z.* **243**, 149.