CCXL. THE LINKAGE OF CHEMICAL CHANGES IN MUSCLE EXTRACT.

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THIS work was started as a result of the first paper of a series by Parnas *et al.* [1934] on the inhibition of ammonia production resulting from addition of phosphoglyceric acid to muscle "brei" poisoned with iodoacetate. Lohmann [1934] had shown that in dialysed muscle extract adenylic acid reacts with phosphocreatine to give adenylpyrophosphate; Parnas and his colleagues pointed out that muscle deaminase attacks only adenylic acid, not adenylpyrophosphate. They argued therefore from the NH_3 inhibition that synthesis of phosphocreatine had taken place at expense of the energy and the phosphate of phosphoglyceric acid breakdown, and had been followed by conversion of adenylic acid into adenylpyrophosphate by Lohmann's reaction. Since that time they [Parnas *et al.*, 1935, 1] have actually demonstrated in this muscle "brei" an increase in phosphocreatine (especially when creatine is added), and an increase in adenylpyrophosphate, accompanying the ammonia inhibition. In Parnas's experiments the iodoacetate prevented the breakdown of the carbohydrate of the "brei" by inhibiting the dismutation of dihydroxyacetonephosphate to give glycerophosphate and phosphoglyceric acid [Meyerhof and Kiessling, 1933, 1; Embden and Deuticke, 1934; Ostern, 1934]. Barrenscheen and his colleagues [1935] have also shown that, during the disappearance of phosphoglyceric acid from muscle "brei" or extract, there is an increase in easily hydrolysable organic P, although, apart from showing some increase in phosphopyruvic acid, they did not identify the compounds formed.

Our own work was carried out on dialysed muscle extracts, which have the advantage of containing no carbohydrate or carbohydrate breakdown products, so that the addition of any poison is unnecessary in showing the effect of added phosphoglyceric acid. The extract can also be obtained free from creatine, adenylic acid, adenylpyrophosphate and magnesium, and the effect of adding any of these can be observed. A preliminary report of the present work was sent to *Nature* [Needham and van Heyningen, 1935]; a few days before this was published a preliminary account of similar results with dialysed muscle extracts by Ostern *et al.* [1935, 1] appeared, and shortly afterwards Meyerhof and Lehmann [1935] described similar work.

TECHNIQUE.

Preparation of extracts. The frog muscle extract was made as described by Meyerhof [1926], by crushing the muscles in water at 0°. For the rabbit muscle extract, the animal was anaesthetised with Numal Roche, killed by bleeding and skinned; the hind limbs were cooled in ice, then the muscle was removed, minced in a cooled mincer, and mixed with 3 vols. of ice-cold water, being

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allowed to extract for 30 min. before straining and centrifuging. Dialysis was carried out in a cellophane bag, holding 15–30 ml. extract, which was arranged to move slowly backwards and forwards in the vessel containing 5 litres of ice-cold 0·5% KCl solution. The KCl solution was kept well stirred.

The extracts were buffered with varying amounts of phosphate and bicarbonate. In Exps. 1 and 2 the p_H was about 9·0; in the later experiments it was always maintained at 7·2 by bubbling nitrogen containing 5% CO_2 through the bicarbonate-containing mixture.

The barium was removed from the barium adenylpyrophosphate and barium phosphoglycerate before adding to the extract. This was done by dissolving in as little HCl as possible, adding the calculated quantity of sodium sulphate, spinning off the precipitate and neutralising.

Methods of analysis. All the phosphate estimations were done by Fiske and Subbarow's [1929] method. The separation of the phosphocreatine fraction, with soluble calcium salt, from the free phosphate and adenylpyrophosphate was made by means of Fiske and Subbarow's $CaCl_2$-$Ca(OH)_2$ reagent. Pyrophosphate values were found by 7 minutes' hydrolysis in *N* HCl at 100°; the difficultly hydrolysable phosphate (phosphoglyceric acid-P) was found by taking the difference between the total acid-soluble phosphate and the free phosphate after 2 hours' hydrolysis at 100° in *N* HCl (an allowance must be made for some adenylic acid-P unhydrolysed after 2 hours). All these hydrolyses were done in tubes fitted with water condensers. Total P was estimated by the method of Eggleton and Eggleton [1929–30]. Lactic acid was estimated by the method of Friedemann *et al.* [1927], and pyruvic acid by Case's [1932] method with one modification (for which we are indebted to Prof. A. Szent-Györgyi). After the formation of the dinitrophenylhydrazones, the excess of dinitrophenylhydrazine is removed by addition of 1 ml. of 10% $NaNO_2$ solution to each 25 ml. sample. Without this precaution, high blanks were always obtained. After 1 minute's action of the $NaNO_2$ the extraction with ethyl acetate is begun; with longer delay the hydrazones may be acted upon, and low pyruvic acid values result.

The samples of extract were as a rule deproteinised by running into trichloroacetic acid of such a strength that the final concentration of trichloroacetic acid was 4%; where phosphocreatine estimations were to be done, the sample and the acid were cooled to 0° before mixing. Where lactic acid was to be estimated, protein was removed with phosphotungstic acid. All experimental results are expressed in mg.

Preparation of barium adenylpyrophosphate. This was made from rabbit muscle according to the shorter method (method *a*) described by Lohmann [1931, 1]. The barium precipitate was twice redissolved in acid and reprecipitated with alcohol and barium acetate, and the final precipitate was washed many times with 50% alcohol. The resulting salt was 97·5% pure. For the adenylic acid used we are indebted to Dr P. Ostern, and for the barium salt of phosphoglyceric acid to Prof. O. Meyerhof and Prof. C. Neuberg.

Synthesis of phosphocreatine.

Two typical experiments to show the conditions under which phosphocreatine may be synthesised in frog muscle extract are given below.

Exps. 1 and 2. In each case the extract stood 1 hour at room temperature and was dialysed 5 hours at 0°. It was made 0·04 *M* with regard to bicarbonate and 0·03 *M* with regard to phosphate. 1 ml. extract was made up to 2 ml.; each sample contained 0·38 mg. phosphoglyceric acid-P and 0·1 mg. Mg as $MgCl_2$. All stood 1 hour at room temperature. (See Table I.)

Table I.

Exp. 1.	Inorganic P	Change in inorganic P	Phospho-creatine-P
Without addition	0·880	—	0
+2 mg. creatine	0·853	−0·027	0
+adenylpyrophosphate containing 0·023 mg. pyrophosphate-P	1·090	+0·210	0
+2 mg. creatine +0·4 mg. adenylic acid	1·090	+0·210	0·067
Exp. 2.			
Without addition	0·690	—	0
+6 mg. creatine	0·660	−0·030	0
+0·4 mg. adenylic acid	0·880	+0·190	0
+6 mg. creatine +0·4 mg. adenylic acid	0·850	+0·160	0·054

These experiments show: (*a*) That phosphoglyceric acid is broken down, with production of inorganic phosphate, when either adenylic acid or adenylpyrophosphate is present. (*b*) That the presence of creatine, in the absence of these adenylic compounds, does not assist breakdown. (*c*) That when breakdown occurs, provided creatine is present, phosphocreatine is synthesised.

These dialysed frog extracts did not show great activity, and the experiments were repeated and extended, using rabbit extract.

Exp. 3. The extract stood 1 hour at 20°, then was dialysed for 4 hours at 0°. It was made 0·032 *M* with regard to bicarbonate and 0·01 *M* with regard to phosphate. For each sample 4 ml. of the alkaline extract were used and made up with the various additions to 8 ml. Each sample had 0·6 mg. Mg. All stood 1 hour at room temperature. (See Table II.)

Table II.

A = +24 mg. creatine +1·6 mg. adenylic acid +1·1 mg. phosphoglyceric acid-P.
B = +24 mg. creatine +1·6 mg. adenylic acid.
C = +24 mg. creatine +1·1 mg. phosphoglyceric acid-P.
D = + 1·6 mg. adenylic acid +1·1 mg. phosphoglyceric acid-P.

	Inorganic P	Phospho-creatine-P	Pyrophos-phate-P	(2 hours' hydrolysable P) −(7 min. hydrolysable P)	Difficultly hydrolysable P	Total P
A	2·10	0·320	0·384	0·112	0·448	3·36
A	1·89	0·000	0·032	0·112	0·240	2·27
C	2·08	0·496	0·080	0·352	0·304	3·31
D	2·08	0·016	0·384	0·256	0·576	3·46

This experiment shows: (*a*) The disappearance of phosphoglyceric acid-P; in A only 19%, in C only 13% and in D only 30% remained at the end of the time. (Allowance was made in A and D for the adenylic P unhydrolysed in 2 hours, about 60%.) (*b*) The very slight increase in inorganic P accompanying the breakdown. (*c*) The large increase in phosphocreatine, especially in C. This last fact is probably to be explained by incomplete removal of the adenylic acid of the extract by deamination and dialysis, as rabbit extract is well known to retain its activity much longer than frog extract [Lohmann, 1931, 2]. In A, where extra adenylic acid was added, this has probably reacted with some of the phosphocreatine formed, with the result that the phosphocreatine content is decreased. The incompleteness of the dialysis is further shown by the formation of a small amount of phosphocreatine in D, where no creatine was added. (*d*) The synthesis of adenylpyrophosphate. The fact that a synthesis, though small, has gone on in C confirms the view that adenylic acid is present in this sample; in A and D an amount of adenylpyrophosphate equivalent to the whole of the adenylic acid added has appeared.

In A 61%, in C 50% and in D 33% of the phosphoglyceric acid-P appears as phosphocreatine-P+adenylpyrophosphate-P, whilst only about 18% appears in each as inorganic P.

There seems to be no phosphopyruvic acid present, as the pyruvic acid concentration was not altered by 1 hour's hydrolysis in *N* acid at 100°.

Exp. 4. Another experiment was made with extract which stood 2 hours at 37° and was dialysed 6 hours at 0°. Samples were prepared as in Exp. 3, except that 1·3 mg. phosphoglyceric acid-P were added in each case. (See Table III.)

Table III.

	Inorganic P	Phosphocreatine-P	Pyrophosphate-P	(2 hours' hydrolysable P) − (7 min. hydrolysable P)	Difficultly hydrolysable P	Total P	Pyruvic acid	Change in inorganic P	Change in phosphocreatine-P	Change in pyrophosphate-P	Change in phosphoglyceric acid-P
A	2·16	0·064	0·144	0·560	0·176	3·04	1·54	+0·75	+0·064	+0·144	−1·24
B	1·41	0	0	0·192	0·112	1·71	—	—	—	—	—
C	1·47	0·048	0	0·512	0·736	2·72	Trace	+0·006	+0·048	0	−0·76
D	2·34	0	0	0·464	0·224	2·94	1·50	+0·93	0	0	−1·19

This experiment bears out the results of the last, but the phosphocreatine and pyrophosphate syntheses are much less and the production of inorganic phosphate much greater. In the light of the work described later, it seems likely that the enzyme responsible for the interaction between adenylpyrophosphate and creatine has been damaged by keeping, whilst the enzymes responsible for interaction between phosphoglyceric (or phosphopyruvic) acid and adenylic acid, and for the breakdown of adenylpyrophosphate to adenylic acid and orthophosphate, retain their activity. Creatine seems to have been dialysed away completely, but a trace of adenylic acid seems still to be present in C.

The nature of the co-enzyme function of the adenylic compounds.

The fact that creatine alone cannot bring about dephosphorylation of phosphoglyceric acid in muscle extract, while in presence of adenylic acid or adenylpyrophosphate as well such dephosphorylation occurs and phosphocreatine is produced, leads naturally to the idea that the adenylpyrophosphate added or formed from the adenylic acid phosphorylates creatine by a reverse Lohmann reaction. According to this view, the phosphate of the phosphoglyceric acid is transferred to adenylic acid and the inorganic P appearing as a final product of phosphoglyceric acid breakdown is due to the action of yet another enzyme which converts adenylpyrophosphate into adenylic acid and free phosphate. Lohmann and Meyerhof [1934] have stated that the formation of pyruvic acid from phosphoglyceric acid in extract needs adenylpyrophosphate as co-enzyme, adenylic acid working much less efficiently; they have also stated that phosphoglyceric acid breakdown has no effect in maintaining the adenylpyrophosphate concentration of an extract. Both these facts would militate against the view that the essential step in dephosphorylation of phosphoglyceric acid is the reaction with adenylic acid, giving adenylpyrophosphate. Our own experiments on these two points give opposite results, as is shown below, and Meyerhof and Lehmann also now take the view that adenylic acid reacts with phosphopyruvic acid, formed from the added phosphoglyceric acid. Barrenscheen and Beneschovsky [1935] too have shown that adenylic acid is as potent as adenylpyrophosphate in restoring the power of an extract to form pyruvic acid from phosphoglyceric acid.

Exp. 5. Frog muscle extract, kept 1 hour at room temperature, dialysed 4 hours. 10 ml. extract were made 0·04 *M* as regards bicarbonate and 0·004 *M* with regard to phosphate. 4 ml. of this extract were in each case diluted to 8 ml.; both solutions contained adenylpyrophosphate (0·96 mg. pyrophosphate-P) and 1·2 mg. Mg. A contained also 1·6 mg. phosphoglyceric acid-P. Both solutions were kept at room temperature, and samples were removed at 0, 5, 30 and 60 min. for the estimation of inorganic phosphate and pyrophosphate. The decrease in phosphoglyceric acid was calculated by subtracting the decrease in pyrophosphate from the increase in inorganic phosphate. (See Table IV.)

Table IV.

	Time min.	Inorganic P	Increase in inorganic P	Pyrophosphate-P	Decrease in pyrophosphate-P	Inorganic P from phosphoglyceric acid	Phosphoglyceric acid-P remaining
A	0	1·44	—	0·96	—	—	1·60
	5	1·97	0·53	0·89	0·07	0·46	1·14
	30	2·78	1·34	0·58	0·38	0·96	0·64
	60	3·50	2·06	0·17	0·79	1·27	0·33
B	0	1·44	—	0·96	—	—	—
	5	1·70	0·26	0·58	0·38	—	—
	30	2·08	0·64	0·13	0·83	—	—
	60	2·18	0·74	0·17	0·79	—	—

This experiment shows the effect which phosphoglyceric acid has in preventing the disappearance of adenylpyrophosphate. An attempt was now made to compare the activities of adenylpyrophosphate and adenylic acid as the co-enzyme of phosphoglyceric acid breakdown.

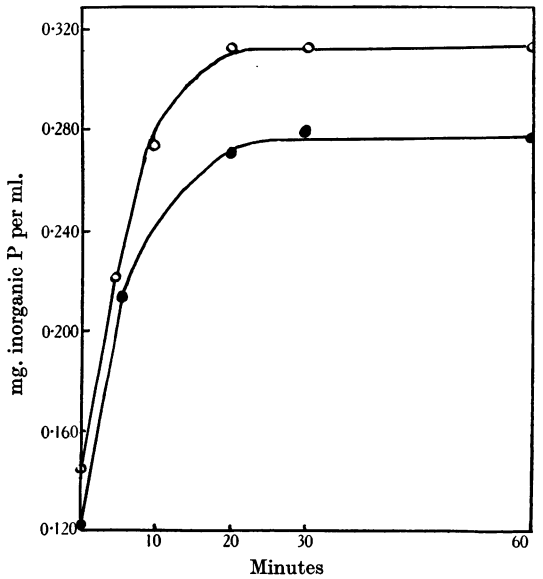

Fig. 1. o—o with adenylpyrophosphate; •—• with adenylic acid.

Exp. 6. The alkaline extract was prepared exactly as in the last experiment. In each case 5 ml. of this solution were made up to 10 ml.; each solution contained 2 mg. phosphoglyceric acid-P and 1·5 mg. Mg. A contained 0·29 mg. pyrophosphate-P, and B an amount of adenylic acid equivalent to this adenylpyrophosphate. Both were kept at room temperature and samples were removed at 0, 5, 10, 20, 30, 60 min.; inorganic phosphate was estimated. (See Fig. 1.)

Fig. 1 shows that no difference in rate of breakdown of phosphoglyceric acid was observable in the two cases. The fact that the inorganic phosphate in A starts 0·24 mg. higher than in B suggests that the whole of the adenylpyrophosphate has been broken down to adenylic acid and orthophosphate in the first minute during mixing, before the first sample was taken, so that thereafter both samples were in the same situation with regard to co-enzyme.

In order to overcome this difficulty of rapid conversion of the adenylpyrophosphate into adenylic acid it was decided to use long-dialysed rabbit muscle extracts, as Lohmann [1934] has shown that such extracts lose their power to form inorganic phosphate from adenylpyrophosphate.

Exp. 7. Rabbit extract, kept 1 hour at room temperature, dialysed 15 hours at 0°. Two solutions were made up; 8 ml. of the extract were diluted to 16 ml., the final mixtures being 0·02 *M* with regard to bicarbonate and 0·002 *M* with regard to phosphate. Each solution contained 2·4 mg. phosphoglyceric acid-P and 2·4 mg. Mg. Solution A had adenylpyrophosphate (4·64 mg. pyrophosphate-P), and B an amount of adenylic acid equivalent to this adenylpyrophosphate. The solutions were kept at 28°, and samples were removed at 0, 5, 15, 30, 60 min. (See Table V.)

Table V.

	Time min.	Inorganic P	Pyrophosphate-P + inorganic P	Pyrophosphate-P	Adenylic acid-P	Inorg. P after 2 hours' hydrolysis	(2 hours' hydrolysable P) − (7 min. hydrolysable P)	Total P	Phosphoglyceric acid-P
A	0	1·09	5·70	4·61	2·32	6·66	0·96	10·25	2·23
	5	1·15	5·82	4·67	,,	6·96	1·14	,,	2·10
	15	1·28	5·90	4·62	,,	6·96	1·06	,,	2·04
	30	1·41	6·02	4·61	,,	7·30	1·28	,,	1·92
	60	1·73	6·60	4·87	,,	7·55	0·95	,,	1·22
B	0	1·41	3·08	1·67	1·96	3·97	0·89	6·01	0·97
	5	1·47	3·90	2·43	,,	4·60	0·70	,,	0·15
	15	1·57	3·90	2·33	,,	4·60	0·70	,,	0·15
	30	1·83	3·97	2·15	,,	4·80	0·83	,,	0·08
	60	2·59	4·16	1·57	,,	4·73	0·57	,,	0

In this experiment rapid interaction between adenylic acid and phosphoglyceric acid can be very clearly seen. About half the phosphoglyceric acid-P has disappeared before the first sample could be removed, and an amount of adenylpyrophosphate-P roughly equivalent has appeared. During the next 5 min. there is a further increase in adenylpyrophosphate-P and a decrease in phosphoglyceric acid-P. By the end of this time the phosphoglyceric acid is practically exhausted, and then disappearance of pyrophosphate with corresponding increase in inorganic phosphate begins. It is interesting to notice the differences in events in A. Here in the course of the whole hour less than half of the phosphoglyceric acid disappears. There is a slow steady increase in inorganic P, probably due to the slow breakdown of adenylpyrophosphate, the enzyme concerned not having been completely destroyed; but the adenylpyrophosphate concentration does not change, presumably being kept up by interaction of the adenylic acid formed with the phosphoglyceric (or phosphopyruvic) acid.

This experiment gives convincing proof of the reaction between adenylic acid and phosphoglyceric (or phosphopyruvic) acid, and shows the superiority of adenylic acid over adenylpyrophosphate in assisting the breakdown of phosphoglyceric acid. It is now necessary to consider the evidence for the reverse Lohmann reaction.

Meyerhof and Lohmann [1932] showed some years ago that in rabbit extracts at p_H 9·0, which had been inactivated by standing 2 hours at room temperature, a synthesis of phosphocreatine took place when adenylpyrophosphate was added, and the increase in phosphocreatine was equivalent to the pyrophosphate disappearance. It is now necessary, however, to show that this reverse reaction can go on under the conditions used in the experiments just described, *i.e.* with dialysed extracts and at p_H 7·2.

Exp. 8. Frog muscle extract was used; it was kept 1 hour at room temperature and was dialysed 4 hours at 0°. 7 ml. were made up to 14 ml.; the solution was made 0·02 *M* with regard to bicarbonate and 0·004 *M* with regard to phosphate. 25 mg. of creatine were added and adenylpyrophosphate (4·4 mg. pyrophosphate-P). It was kept at room temperature for 1 hour and samples were removed at 0, 2, 15, 30 and 60 min. (See Table VI.)

Table VI.

Time min.	Phospho-creatine-P	Inor-ganic P	Pyrophos-phate-P +inor-ganic P	Pyrophos-phate-P	Decrease in pyrophos-phate-P	Increase in phospho-creatine-P	Increase in phospho-creatine-P +inor-ganic P
0	—	3·36	7·05	3·69	—	—	—
2	0·294	3·70	7·05	3·35	0·34	0·294	0·634
15	0·672	4·60	7·12	2·52	1·17	0·672	1·912
30	0·658	5·60	6·89	1·29	2·40	0·658	2·898
60	Trace	6·72	7·00	0·28	3·41	0	3·360

With this dialysed extract the observable synthetic activity is very small; the breakdown of adenylpyrophosphate to adenylic acid and free phosphate goes on very rapidly and the adenylic acid thus formed must react with the phosphocreatine synthesised. The phosphocreatine concentration reaches a maximum in about 30 min., after which the concentration of adenylic acid has become high enough to cause its complete disappearance.

The experiment was repeated with frog muscle extract which had been dialysed 16 hours (after standing 1 hour), as this treatment was shown by Lohmann [1934] to damage or destroy the enzyme responsible for adenylpyrophosphate breakdown, while not greatly affecting the enzyme responsible for reaction between adenylic acid and phosphocreatine.

Exp. 9. The solutions were made up exactly as in the last experiment, but no phosphate was added, in order that changes in pyrophosphate-P and phosphocreatine-P might show up better. No calcium precipitation was performed, but the phosphocreatine was estimated by the extrapolation method of Eggleton and Eggleton [1927], and the pyrophosphate was determined by finding the increase after 7 minutes' hydrolysis in the trichloroacetic acid extract, instead of in the solution of the calcium precipitate as usual. Special precautions were taken to keep the samples very cold, −12°, until the estimations could be carried through; the protein precipitation was carried out by adding the trichloroacetic acid to the frozen samples just before each extrapolation was done and thawing rapidly. Samples were taken at 0, 15, 30, 60, 90 and 120 min. (See Table VII and Fig. 2.)

In these experiments the increase in inorganic phosphate is much slower, although not entirely inhibited. During the first 30 min., however, 75% of the pyrophosphate-P disappearing is transferred to creatine. The phosphocreatine concentration reaches a maximum after about an hour; it then falls off, as the concentration of adenylic acid increases. If the breakdown of adenylpyrophosphate to adenylic acid and free phosphate had been entirely inhibited,

no doubt an equilibrium would have been reached at some definite concentration of the reactants, as in Lohmann's experiment starting with phosphocreatine and adenylic acid.

Table VII.

Time min.	Phospho-creatine-P + inorganic P	Inorganic P	Phospho-creatine-P	Inorg. P after 7 min. hydrolysis	Pyrophos-phate-P	Decrease in pyrophos-phate-P	Increase in phospho-creatine-P + inorganic P
0	1·33	1·33	0	5·95	4·62	—	—
15	2·58	1·50	1·08	5·95	3·37	1·25	1·25
30	3·30	1·90	1·40	6·05	2·75	1·87	1·97
60	4·13	2·41	1·72	6·05	1·92	2·70	2·80
90	4·65	3·10	1·55	6·17	1·52	3·10	3·32
120	5·23	3·70	1·53	6·17	0·94	3·68	3·90

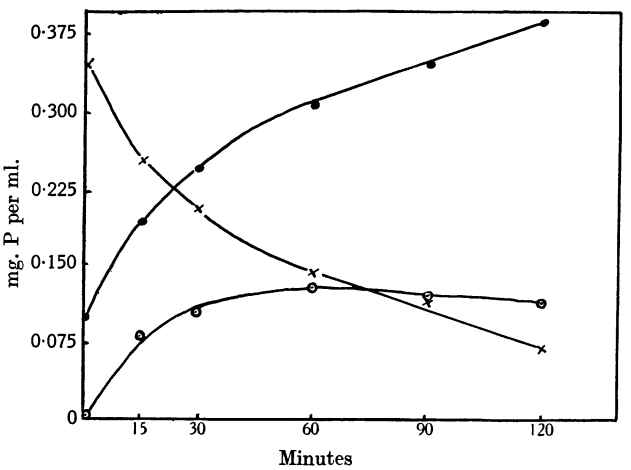

Fig. 2. •—• Phosphocreatine-P + inorganic P; ⊙—⊙ Phosphocreatine-P; ×—× Adenylpyrophosphate-P.

Discussion.

From the work of Meyerhof's school it seems likely that the co-enzyme is only necessary during glycogen breakdown to lactic acid at the stages where a change in phosphorylation takes place. It has been shown to be unnecessary in: (*a*) The change from hexosediphosphate to triosephosphate [Meyerhof and Lohmann, 1934]. (*b*) The change from phosphoglyceric acid to phosphopyruvic acid [Lohmann and Meyerhof, 1934]. (*c*) The interaction of pyruvic acid with glycerophosphate to give one equivalent of lactic acid with no setting free of phosphate [Meyerhof and Kiessling, 1933, 2]. There seem to be no data as to the necessity of co-enzyme for the dismutation of triosephosphate into phosphoglyceric acid and glycerophosphate. The remaining stages, at which co-enzyme is necessary are: (*a*) The phosphorylation of carbohydrate. (*b*) The dephosphorylation of phosphopyruvic acid. Evidence is provided in this paper for the co-enzyme function of adenylic acid as a phosphate acceptor in the formation of pyruvic acid from phosphopyruvic acid.

That not adenylic acid, but adenylpyrophosphate, is necessary as a phosphate donator at the first stage is indicated by many earlier experiments in which, for the production of lactic acid from glycogen, adenylpyrophosphate is

much more efficient than adenylic acid [*cf.* Lohmann, 1931, 2]. We may quote here one experiment of our own in which frog muscle extract, inactivated by standing 1 hour and dialysed 3 hours, showed no power of esterification of glycogen or lactic acid production when adenylic acid was added but regained both activities to some extent when adenylpyrophosphate was added.

The synthesis of 1 g. molecule of adenylpyrophosphate from 1 of adenylic acid and 2 of free phosphate requires energy to the value of 25,000 cals. Hydrolysis of 2 g. molecules of phosphopyruvic acid provides only 16,000 cals. [Meyerhof and Lehmann, 1935]. It seems then, that synthesis of adenylpyrophosphate at the expense of phosphopyruvic acid breakdown must be an endothermic reaction. Nevertheless, as shown in Exp. 7, the reaction appears to go to completion, for the phosphoglyceric acid disappeared completely while adenylpyrophosphate was still present.

The reversibility of the Lohmann reaction seems to be established, not only from our own experiments described here, but also from experiments of Meyerhof and Lehmann not yet published in full. In this connection the work of Lohmann [1935] on the presence of both adenylpyrophosphate and adenosinediphosphate in heart and smooth muscle is of great interest. To suppose a direct reaction according to the equation:

$$1 \text{ adenylpyrophosphate} + 2 \text{ creatine} \rightleftharpoons 2 \text{ phosphocreatine} + 1 \text{ adenylic acid}$$

would involve the very unlikely assumption of a trimolecular reaction in each direction. It seems more reasonable to suggest that the reaction goes in two stages, first with production of adenosinediphosphate, which then reacts with a second molecule of creatine or phosphocreatine. The reaction between adenosinediphosphate and phosphoarginine has been demonstrated by Lohmann in crab muscle. In heart and smooth muscle the change from the triphosphate to the diphosphate is very rapid, making the triphosphate difficult to isolate. It may be that in skeletal muscle it is the diphosphate which is unstable and so has escaped detection.

Finally, we should like to discuss an important deduction which has been drawn by Parnas and his co-workers [1935, 2; Mann, 1935] from certain of their experiments. They found: (*a*) That, whilst in a "brei" made with water ammonia formation takes place very rapidly, in a "brei" made with $M/10$ phosphate solution this ammonia formation may be inhibited for 30–60 min. (*b*) This phosphate inhibition of ammonia production disappears if fluoride or iodoacetic acid is added to the "brei". (*c*) In a phosphate "brei" with fluoride, the ammonia inhibition takes place if phosphoglyceric or pyruvic acid is added, whilst in a phosphate "brei" with iodoacetic acid, the ammonia inhibition only takes place if phosphoglyceric acid is added, pyruvic acid having no effect. The conclusion was drawn that a substance intermediate between pyruvic acid and phosphoglyceric acid, *i.e.* phosphopyruvic acid, performs the phosphorylation of adenylic acid or creatine, and further, that this phosphopyruvic acid can be synthesised by the "brei" from pyruvic acid (whether added to or formed in the muscle) and free phosphate, if the phosphate concentration is high enough. This last reaction, if correct, is very interesting; it is endothermic, and the question of the energy provision arises; also it is unusual amongst all the reactions we have been considering, in involving the esterification of free phosphate. It seems to us, however, that the evidence so far brought forward is hardly conclusive. In the case of the phosphate alone, the evidence is not very convincing that the effect is not due simply to a more favourable milieu for prolonged carbohydrate breakdown. It seemed possible, also, that a high inorganic

phosphate concentration might slow the formation of adenylic acid and free phosphate from adenylpyrophosphate. This turned out not to be the case, however. When adenylpyrophosphate was added to a muscle extract (after standing 1 hour at room temperature and 2 hours' dialysis at 0°) the formation of free phosphate from it went on even more rapidly in presence of 0·05 *M* phosphate than in absence of any added phosphate.

In the case of the pyruvic acid effect in fluoride "brei" it seems at present difficult to exclude the possibility that this is due to its interaction with glycerophosphate already in the muscle leading to the formation of an equivalent amount of triosephosphate and ultimately to phosphoglyceric acid. If added phosphoglyceric acid can have the inhibitory effect on NH_3 formation, as in Parnas's experiments, then it seems that pyruvic acid must have this effect also, since fluoride does not prevent formation of glycerophosphate or reaction between pyruvic acid and glycerophosphate. That phosphoglyceric acid can inhibit means that the fluoride poisoning (which prevents the change of phosphoglyceric acid into phosphopyruvic acid [Embden and Deuticke, 1934; Lohmann and Meyerhof, 1934]) is incomplete, or else that phosphoglyceric acid itself can phosphorylate adenylic acid. The fact that pyruvic acid has no effect in iodoacetate "brei" is easily understood on these lines, as iodoacetate, besides preventing formation of phosphoglyceric acid, also prevents the reaction together of pyruvic acid and glycerophosphate [Embden and Deuticke, 1934; Meyerhof and Kiessling, 1933, 2]. There seems no reason why it should inhibit synthesis of phosphopyruvic acid if this were going on, as it does not inhibit dephosphorylation of phosphopyruvic acid.

Light has thus been shed by much recent work on the rôle of the adenylic component of the co-enzyme system; the part played by magnesium, however, remains completely obscure. Another obscure question is the mode of entry of inorganic P into the cycle of reactions. This may not occur to any great extent *in vivo*, but *in vitro* it certainly does; for instance in normal glycolysis in muscle extract with the complete co-enzyme system present a mixture of hexosediphosphate and hexosemonophosphate temporarily accumulates. Without co-enzyme a small amount of esterification goes on [Lohmann, 1931, 3], leading to formation of some difficultly hydrolysable hexose esters, and in presence of fluoride or iodoacetic acid too, carbohydrate is esterified. As we have seen, the esterification of pyruvate remains problematical, and the interaction with free phosphate seems chiefly to concern the hexose stage.

Note. Just as this paper was ready for the press, the detailed account of the experiments of Ostern *et al.* [1935, 2] appeared, together with a theoretical discussion by Parnas and Ostern [1935].

Summary.

1. Using dialysed muscle extracts, it has been shown that, in presence of adenylic acid and magnesium, added creatine can be converted into phosphocreatine during the breakdown of phosphoglyceric acid.

2. The two reactions involved in this synthesis have been demonstrated: (*a*) The reaction between phosphoglyceric acid (or phosphopyruvic acid) and adenylic acid to form adenylpyrophosphate and pyruvic acid. (*b*) The reaction between adenylpyrophosphate and creatine to form phosphocreatine and adenylic acid.

3. Evidence is brought forward for the view that the co-enzyme function of the adenylic acid and adenylpyrophosphate in muscle glycolysis consists in their ability to act respectively as phosphate acceptor and phosphate donator.

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