XLVIII. COMPETITION BETWEEN PHOSPHORYLATING ENZYMES IN MUSCLE EXTRACT

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It has been shown [Lohmann, 1934; Ostern *et al.*, 1935; Needham & van Heyningen, 1935; Lehmann, 1935] that the following reactions, involving the transfer of phosphate from one organic molecule to another, can go on in muscle extract:

The second reaction has been very carefully investigated, and it was shown that an equilibrium occurs [Lehmann, 1935; 1936]. The equilibrium point depends on *pH*. It was further found that the reaction is not trimolecular. It can be split up into two bimolecular reactions in which adenosinediphosphate (a substance discovered by Lohmann [1935] in crab muscle) figures in vertebrate muscle also, as an intermediate between adenylpyrophosphate and adenylic acid. The equilibrium formula may thus be written

$$\frac{[\text{Adenylpyrophosphate}] [\text{Creatine}]^2}{[\text{Adenylic acid}] [\text{Creatinephosphate}]^2} = K_1 \cdot K_2$$

made up from the two formulae

$$\frac{[\text{Adenylpyrophosphate}] [\text{Creatine}]}{[\text{Adenosinediphosphate}] [\text{Creatinephosphate}]} = K_1$$

and

$$\frac{[\text{Adenosinediphosphate}] [\text{Creatine}]}{[\text{Adenylic acid}] [\text{Creatinephosphate}]} = K_2.$$

As the estimation of K_2 is very difficult, this value is best calculated from $K_2 = \frac{(K_1 \cdot K_2)}{K_1}$. In crab muscle extract only the K_1 reaction is usually found (arginine of course replacing creatine). In vertebrate muscle also the K_1 reaction is far faster than the K_2 reaction, and probably in normal muscle the latter scarcely occurs. K_1 and K_2 decrease with increasing *pH* and vice versa.

The fact that adenylic acid is so rapidly deaminated has also caused some workers to doubt whether this substance plays any important role in muscle *in vivo*. Adenosinediphosphate is much less readily deaminated than adenylic acid, its deamination is completely inhibited at a slightly alkaline reaction, while for inhibition of adenylic acid deamination the unphysiological *pH* of 11 is necessary. Adenosinediphosphate is therefore to be recommended for use in *in vivo* experiments on the adenylic compounds, as a more physiological substance than adenylic acid itself.

It seems very probable that the formation of adenylpyrophosphate from phosphopyruvic acid also takes place in two stages since both adenosinediphosphate and adenylic acid can act as phosphate carriers in rabbit muscle extract for the Parnas reaction.

In the following work, the part played by adenosinediphosphate was assumed, but no attempt was made to distinguish in practice between adenylypyrophosphate and adenosinediphosphate.

There is still another phosphorylation, the formation of hexosediphosphate from glycogen. From work on muscle brei, this was believed to occur more quickly at the expense of the phosphate of adenylypyrophosphate [Parnas *et al.*, 1934] although inorganic phosphate could also play a part. Recently Parnas and his colleagues [Parnas & Baranowski, 1935; Ostern & Guthke, 1936; Ostern *et al.*, 1936; Parnas & Ostern, 1936] have shown that inorganic phosphate can react with glycogen to form hexosemonophosphate. They are of the opinion that glycogen itself does not react with adenylypyrophosphate, but that the latter phosphorylates hexosemonophosphate. The results given in this paper confirm the finding that glycogen can be esterified by inorganic P; they do, however, also confirm the earlier view that there is also a direct reaction between adenylypyrophosphate and glycogen.

As there exist, therefore, a number of different directions in which phosphate may be transferred, it is of interest to examine which possibility is actually realized under various conditions. The case chosen for particular study was that of phosphopyruvic acid as donator, with creatine and glycogen as competing acceptors and adenylic acid as carrier. The competition between two phosphorylating agents, phosphopyruvic acid and creatinephosphate, competing for the same acceptor, adenylic acid, was also investigated.

TECHNIQUE

Preparation of extracts

The extracts were made in the usual way [*vide* Meyerhof, 1926]. Rabbit and frog muscle were used. To get rid of creatine, inorganic phosphate, inosinic acid, lactic acid etc. the extracts were dialysed at 4° in cellophane tubes against 20 volumes of distilled water or 0.5% KCl solution; the dialysis liquid was frequently changed. An important condition for the success of the experiments described is the absence of adenylypyrophosphatase. This enzyme can be inactivated in two ways without affecting the other enzymes concerned: (1) by long dialysis (15 hours); in this case complete inactivation seems only to be reached if the extract is moved during the dialysis, and inactivation by shaking may play some part; (2) by keeping the extract several days at 0°.

The extracts used were practically free from phosphate and the adenylypyrophosphatase had been completely inactivated.

To obtain pH 8.8, a mixture of *M*/2 sodium bicarbonate and *M*/2 sodium carbonate was added to the extract, the final concentration being adjusted to *M*/20.

To obtain pH 6.5, the extract was made *M*/20 with respect to sodium bicarbonate and saturated with CO₂.

Methods of analysis

All phosphate estimations were done by the method of Fiske & Subbarow [1925], and the various phosphate fractions were assessed in the following way, using the hydrolysis method of Lohmann [1928]. Estimations were made: (a) On the trichloroacetic filtrate after standing 40 min. with the Fiske & Subbarow reagents (Fiske P); this gives the creatinephosphate P and any inorganic P which may be present. In complete absence of adenylypyrophosphatase and exclusion of

development of inorganic P, Fiske P after n min. — Fiske P initial = creatine-phosphate P formed. (b) After 7 min. hydrolysis at 100° with N HCl (7' P). (c) After 60 min. hydrolysis at 100° with N HCl (60' P).

Adenylic acid P is 20 % hydrolysed after 60 min. Phosphopyruvic acid is 50 % hydrolysed in 7 min. and 100 % in 60 min.; therefore 60' P — 7' P — 20 % of adenylic P (if present) = 50 % of the phosphopyruvic P. In using this method for estimating phosphopyruvic acid it is, of course, necessary to know the amount of adenylic acid present.

The pyrophosphate of adenylypyrophosphate is completely hydrolysed in 7 min.; if phosphopyruvic acid is also present, 7' P — Fiske P — 50 % phosphopyruvic P = pyrophosphate P. Phosphoglyceric acid and glycerophosphate are practically unhydrolysed in 60 min.; an increase in either will therefore be observed as a decrease in the amount of P hydrolysable in 60 min. In all the experiments described here fluoride was added. In this way, when esterification of glycogen occurs, the observed result will be an increase in glycerophosphoric and phosphoglyceric acids, the fluoride preventing further breakdown of the phosphoglyceric acid. When pyruvic acid is present, the glycerophosphoric can be converted into phosphoglyceric acid. In these experiments, therefore, esterification of glycogen is measured in terms of increase in difficultly hydrolysable P, i.e. 60' P initial — 60' P after n min.

Preparations used. The adenylic acid was obtained from the firm of Henning, Berlin. The adenylypyrophosphate was made from rabbit muscle by Lohmann's method [1931]; the creatinephosphate by Lehmann's method [1935]; and the phosphopyruvic acid by the synthetic method of Kiessling [1935].

COMPETITION BETWEEN CREATINE AND GLYCOGEN

Exp. 1. Rabbit muscle extract 6 days old, dialysed 15 hours.

1 ml. extract + 3 ml. additions.

Each sample contained 0.5 mg. adenylic P, 1.28 mg. phosphopyruvic P, 0.12 mg. Mg., 0.13 % NaHCO_3 .

Fluoride was added, making the concentration $M/50$.

The samples were kept at 20° for 6 min.

Added	Creatine-phosphate P/mg.	Creatine-phosphate P as % of phospho- pyruvic P added	Phospho- glyceric P/mg.	Phospho- glyceric P as % of phospho- pyruvic P added
—	0	—	0.07	6*
10 mg. creatine	0.87	68	0.07	6*
10 mg. glycogen	0	—	0.93	74
10 mg. creatine + 10 mg. glycogen	0.79	62	0.04	3

* The F inhibition does not seem complete; the effect is always very irregular. Usually $M/50$ F gives 100 % inhibition, and very often $M/100$ is enough.

These results, and those of Exp. 2, show that either creatine or glycogen separately is readily esterified. If, however, both creatine and glycogen are added together, the phosphorylation occurs first almost entirely in favour of the creatine. This is the case both at pH 8.8 and 6.5. If the samples are kept a longer time, 20 min. instead of 5 min., further rearrangement goes on, phosphate passing from creatinephosphate (via adenylic acid) to glycogen. This is shown by the much greater increase in difficultly hydrolysable P after 20 min., and the corresponding decrease in creatinephosphate P. This latter process is not quite so

Exp. 2. Frog muscle extract, 4 days old, dialysed 8 hours.

1 ml. extract + 3 ml. additions.

Each sample contained 0.5 mg. adenylic P, 1.48 mg. phosphopyruvic P, 0.8 mg. Mg.

Fluoride was added, making the concentration $M/50$.

Temperature = 20°.

pH	Time min.	Acceptor	Formed creatine-phosphate P/mg.	Creatine phosphate P as % of phosphopyruvic P added	Formed phosphoglyceric P/mg.	Phosphoglyceric P as % of phosphopyruvic P added
8.8	5	10 mg. creatine	1.31	88	0	—
8.8	5	10 mg. glycogen	0	—	1.05	71
8.8	5	10 mg. creatine + 10 mg. glycogen	1.05	71	0.20	14
8.8	20	10 mg. creatine + 10 mg. glycogen	0.61	41	0.64	43
6.5	5	10 mg. creatine	1.22	83	0	—
6.5	5	10 mg. glycogen	0	—	1.15	77
6.5	5	10 mg. creatine + 10 mg. glycogen	0.95	64	0.14	10
6.5	20	10 mg. creatine + 10 mg. glycogen	0.42	28	0.64	43

Fig. 1. *Exp. 2.* Esterification of competing acceptors for P from phosphopyruvic acid with adenylic acid as P carrier. Black, creatinephosphate P formed; white, phosphoglyceric P formed from glycogen esterification.

quick at pH 8.8 as at pH 6.5, because the latter pH favours the transport from creatinephosphoric to adenylic acid.

The fact that pH has little effect on the original reaction is interesting. It might have been supposed that at a lower pH, where the equilibrium has shifted in favour of creatinephosphate breakdown, the rate of glycogen esterification might have compared more favourably with that of creatine esterification. The results show that at acid as well as alkaline reaction, the transfer of phosphate from adenylypyrophosphate to creatine is a much more rapid reaction than the transfer to glycogen. As, however, the transfer to creatine is a reversible reaction, while that to glycogen is not, as time goes on glycogen esterification gradually gains.

That two separate enzymes are concerned is made likely by the following experiment with a very old extract, which was found to be incapable of phosphorylating glycogen, although it showed considerable activity with creatine.

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Exp. 3. Rabbit muscle extract, 19 days old, dialysed 15 hours.

2 ml. extract with 2 ml. additions.

Each sample contained 0.1 mg. adenylypyrophosphate P (7' P), 1.12 mg. phosphopyruvic P, 1.8 mg. Mg.

Fluoride was added, making the concentration $M/100$.

The samples were kept for 30 min. at 20°.

pH	Acceptor	Formed mg. creatine- phosphate P	Formed mg. phospho- glyceric P
8.8	+ 10 mg. creatine	0.7	0
8.8	+ 10 mg. glycogen	0	0
8.8	+ 10 mg. creatine + 10 mg. glycogen	0.6	0
6.5	+ 10 mg. creatine	0.7	0
6.5	+ 10 mg. glycogen	0	0
6.5	+ 10 mg. creatine + 10 mg. glycogen	0.7	0

That the first step in the phosphate transference (from phosphopyruvic to adenylic acid) is not affected either by the presence of fluoride or by change in pH was shown separately.

Exp. 4. Rabbit muscle extract, 12 days old, dialysed 15 hours.

1 ml. extract + 1 ml. additions.

Each sample contained 1.0 mg. adenylic P, 0.72 mg. phosphopyruvic P, 0.6 mg. Mg.

The samples were kept for 20 min. at 20°.

pH		Formed mg. adenylypyro- phosphate P	% of phospho- pyruvic P
8.8	$M/50$ NaF	0.66	94
8.8	—	0.72	100
6.5	$M/50$ NaF	0.72	100
6.5	—	0.64	91

COMPETITION BETWEEN PHOSPHOPYRUVIC ACID AND CREATINEPHOSPHATE

Exp. 5. Frog muscle extract, 5 days old, dialysed 8 hours.

1 ml. extract + 3 ml. additions.

Each sample contained 0.5 mg. adenylic P, 1.48 mg. phosphopyruvic P, 1.32 mg. creatinephosphate P, 0.5 mg. Mg.

Fluoride was added making the concentration $M/50$.

Room temperature.

pH	Time	Creatine- phosphate P in mg.	% decrease
8.8	45"	1.34	—
8.8	5'	1.28	—
6.5	45"	0.95	22
6.5	5'	1.37	—

These results show that, at pH 8.8, the reaction between phosphopyruvic and adenylic acids is more rapid than that between creatinephosphate and adenylic acid. Even after 5 min. the creatinephosphate content has scarcely diminished. At pH 6.5, on the other hand, the rate of creatinephosphate reaction with adenylic acid is much greater, and 40–80% of the adenylic acid has been phosphorylated at the expense of creatinephosphate in 45 sec. But as time goes on,

because the creatinephosphate breakdown is reversible, while that of phosphopyruvic is irreversible, resynthesis of some creatinephosphate takes place. The action of the adenylic deaminase also favours creatinephosphate resynthesis.

MECHANISM OF GLYCOGEN ESTERIFICATION

In the experiments so far described the extracts used were practically free from inorganic phosphate; a little was contained in the phosphopyruvate added, but the final concentration was not more than 0.003 *M*. From their manner of preparation the extracts can have contained very little, if any, adenylypyrophosphatase, so that no inorganic P formation was to be expected. Some experiments were now done to test the effect of varying concentrations of inorganic P upon the course of glycogen esterification.

Exp. 6. Rabbit muscle extract, 8 days old, dialysed 8 hours.

2 ml. extract + 3 ml. additions.

Each sample contained 0.61 mg. phosphopyruvic P, 0.5 mg. Mg., 0.13 % NaHCO₃.

Fluoride was added making the concentration *M*/100.

The phosphopyruvic acid added contained a certain amount of inorganic P and the extract also contained traces; altogether 0.25 mg. inorganic P was present. The samples were kept for 30 min. at 37°.

	Further additions	Change in Fiske P in mg. P	Change in Fiske P in % of phosphopyruvic P added (%)	Change in phosphopyruvic P + adenylypyrophosphate P in mg. P	Change in phosphopyruvic P + adenylypyrophosphate P in % of phosphopyruvic P added (%)	Change in difficultly hydrolysable P (phosphoglyceric P formation) in mg. P	Phosphoglyceric P formation in % of phosphopyruvic P added (%)
1	—	+0.01	+ 2	±0	—	±0	—
2	1 mg. adenylic P	+0.05	+ 8	-0.04	- 7	+0.01	—
3	1 mg. adenylic P + 10 mg. creatine	+0.21	+34	-0.22	-36	±0	—
4	1 mg. adenylic P + 10 mg. glycogen	±0	0	-0.37	-61	+0.37	+61
5	1 mg. adenylic P + 10 mg. creatine + 10 mg. glycogen	-0.05	+ 8	-0.26	-43	+0.14	+28

These results show that practically no inorganic P is formed when phosphopyruvic and adenylic acids are added (sample 2), and the absence of adenylypyrophosphatase is thus demonstrated. All the glycogen esterification here is at the expense of phosphopyruvic acid P. It must therefore have been transferred by adenylic acid, and esterification by adenylypyrophosphate has gone on, although nearly 0.002 *M* inorganic phosphate is present. This is in striking contrast to *Exp. 7*, where the concentration of inorganic P was increased six times.

In *Exp. 7* we see (a) that adenylypyrophosphatase is absent (sample 2); (b) that esterification of free phosphate has gone on where glycogen is present (decrease in Fiske P, samples 4 and 5); (c) that the increase in difficultly hydrolysable P is almost entirely at the expense of the inorganic P, only about 12 % coming from phosphopyruvic P. Under these conditions, then, where the inorganic P concentration is about 0.01 *M*, esterification of glycogen goes on more readily from inorganic P than from phosphopyruvic P.

Exp. 7. The same experiment as 6, with addition of 1.25 mg. inorganic P.

In [] figures without addition of inorganic P

Further additions	Change in Fiske P in mg. P	Change in Fiske P in % of phosphopyruvic P added (%)	Change in phosphopyruvic P and adenylypyrophosphate P in mg. P	Change in phosphopyruvic P and adenylypyrophosphate P in % of phosphopyruvic P added (%)	Change in phosphoglyceric P in mg. P	Change in phosphoglyceric P in % of phosphopyruvic P added (%)
1	+0.03 [+0.01]	+ 5 [+2]	0 [0]	0 [0]	±0 [±0]	0 [-]
2 + Adenylic P	+0.06 [+0.05]	+10 [+8]	-0.03 [-0.04]	- 5 [-7]	+0.01 [+0.01]	+ 2 [-]
3 + Adenylic P + Creatine	+0.18 [+0.21]	+30 [+34]	-0.18 [-0.22]	-30 [-36]	+0.02 [±0]	+ 3 [-]
4 + Adenylic P + Glycogen	-0.42 [±0]	-69 [0]	-0.05 [-0.37]	- 8 [-61]	+0.47 [+0.37]	+77 [+61]
5 + Adenylic P + Creatine + Glycogen	-0.47 [+0.05]	-71 [+8]	-0.08 [-0.26]	-13 [-43]	+0.55 [+0.14]	+90 [+28]

In *Exp. 8* a comparison is made of degrees of glycogen esterification with different sources of phosphate. The conditions were the same as in *Exp. 6* including, of course, *M*/50 fluoride etc.

	Difficultly hydrolysable P formed
(1) +10 mg. glycogen +0.61 mg. phosphopyruvic P +1 mg. adenylic P	0.37
(2) +10 mg. glycogen +1.25 mg. inorganic P	0.14
(3) +10 mg. glycogen +1.25 mg. inorganic P +0.61 mg. phosphopyruvic P +1 mg. adenylic P	0.47

That adenylic acid plays some part also in the transfer of inorganic P to glycogen is suggested by the next experiment.

Exp. 9. Rabbit muscle extract, 14 days old, 8 hours dialysed.

2 ml. extract + 2 ml. additions.

Each sample contained 10 mg. glycogen, 1.88 mg. inorganic P, 0.3 ml. 1.3 % NaHCO₃, 1 mg. Mg. Sample 3 contained 1 mg. adenylic P.

One was precipitated at once; the other two were kept 60 min. at 37°.

- (1) 0' 1.88 inorganic P.
- (2) 60' 1.67 "
- (3) 60' 1.49 "

Here twice as much inorganic P is esterified in presence of adenylic acid as in its absence.

The reaction between glycogen and inorganic phosphate is very much slower than the reaction between glycogen and adenylypyrophosphate. In *Exp. 8* only 0.14 mg. inorganic P was esterified in 30 min. at 37° in absence of phosphopyruvic and adenylic acids; in their presence, 0.47 mg. of inorganic P was esterified. On the other hand, in *Exp. 2*, with a low concentration of inorganic P, in the presence of adenylypyrophosphate and phosphopyruvic acid, more than 1 mg. of difficultly hydrolysable P was formed in 5 min.

We have, then, two reasons for believing that reaction with inorganic P is not essential for glycogen esterification. (1) In presence of phosphopyruvic acid + adenylic acid large amounts of glycogen are esterified, although the

inorganic P content does not change; these results were obtained in conditions where formation of inorganic P (which might have masked esterification) is not going on; also a simultaneous decrease in phosphopyruvic P occurs. (2) From the available data, the rate of reaction of free phosphate with glycogen is very much lower than the rate of esterification by means of phosphopyruvic + adenylic acids. This makes it difficult to regard the reaction with inorganic phosphate as an essential preliminary step.

The mechanism of the part played by adenylic acid in the esterification of free phosphate will be further investigated. It is interesting to notice that a similar effect was obtained by Cori & Cori [1936], using washed muscle residue in place of muscle extract. Here esterification of glycogen (with formation of a new hexosemonophosphate) occurred at the expense of inorganic P and the degree of esterification was increased by adding adenylic acid.

DISCUSSION

The possible directions of phosphate transfer can be summarized as follows:

Competition can occur between I, 1 and 2b; II, 2a, 3 and 4; III, 4 and 5; IV, 2a and 2b.

I. $1 < 2b$ in acid milieu; $1 > 2b$ in alkaline milieu.

II. $2a > 4$, independent of pH. With long experiments, conditions change, because 2a is reversible while 4 is not, and the result appears $2a < 4$. $2a < 3$ in acid milieu; $2a > 3$ in alkaline milieu.

3 and 4 will be further investigated. 4 appears to be dependent on the amount of inorganic P present. With much inorganic P $4 > 3$, with little $4 < 3$.

III. In presence of sufficient inorganic P $5 > 4$; in absence of sufficient inorganic P $5 < 4$.

IV. $2a < 2b$ in acid milieu; $2a > 2b$ in alkaline milieu. As these two transferences form a reversible equilibrium, following the law of mass action, the observed result depends in practice not only on pH, but on donors and acceptors also present.

These results, obtained *in vitro*, show possible directions of phosphate transfer and some of the factors which may influence this transfer. It is interesting to consider the bearing of these results, as far as may be possible, on the complex relationships in living muscle. In the following, only anaerobic contraction is considered.

In a resting muscle, pH about 7.2, containing adenylypyrophosphate, creatinephosphate and glycogen, the stimulus arrives, initiating adenylypyrophosphate breakdown. Glycogen is phosphorylated; there is no competition with creatine at this stage, because no free creatine is present. Whether some adenylypyrophosphate produces inorganic P at this time, we do not know.

As soon as free adenylic acid is formed, reaction with creatinephosphate begins; results on pH changes [Lipmann & Meyerhof, 1930], volume changes [Meyerhof & Möhle, 1936] and transparency changes [v. Muralet, 1934] in intact

muscle seem to show that no phosphopyruvic acid is present at this stage to compete with creatinephosphate in this phosphorylation. As the adenylypyrophosphate is reformed, some of its phosphate is transferred to glycogen. During this stage the reaction is slightly alkaline, and the rate of creatinephosphate breakdown is less, the possible rate of its resynthesis greater, than at a lower pH ; nevertheless, because the phosphorylation of glycogen, though comparatively slow, is irreversible, the formation of hexosephosphate and breakdown of creatinephosphate go steadily forward.

By some point of time after the stimulus, further breakdown of the hexosediphosphate will lead to a sufficient concentration of phosphopyruvic acid to be of importance in competition with the creatinephosphate, present now in low concentration. The pH now falls, owing to the formation of various acids, and the rate of creatinephosphate breakdown is thereby increased. As a high concentration of free creatine is now present, there will be competition between this and the glycogen for adenylypyrophosphate P . The net result, depending (*a*) on the greater rate of creatine esterification than of glycogen esterification, (*b*) on the reversibility of the former reaction and the irreversibility of the latter, will be a maintenance of creatinephosphate concentration at some low level and a steady transfer of phosphate to glycogen. Another reaction which probably becomes more important at this stage, owing to the low pH , is the formation of inorganic P from adenylypyrophosphate.

To sum up:

During the first stage, phosphate is passing in directions 4 and 5, and possibly 3.

During the second stage, phosphate is passing in directions 2*b*, 4 and possibly 3.

In the third stage, phosphate begins to pass in direction 1; it is also probably passing in direction 3, and in directions 2*a* and 2*b* at equal rates.

SUMMARY

1. When dialysed muscle extract is used as the enzyme system, phosphopyruvic acid as the phosphate donator, adenylic acid as the phosphate carrier, with creatine and glycogen as competing acceptors, phosphorylation occurs almost entirely in favour of the creatine. This effect is obtained both at alkaline and acid reactions.

2. Two enzyme systems seem to be concerned, as extracts can be obtained which will phosphorylate creatine and not glycogen.

3. At high pH values the reaction between phosphopyruvic acid and adenylic acid is more rapid than that between creatinephosphate and adenylic acid; vice versa at low pH values.

4. Evidence is given in favour of the view that glycogen can react both with inorganic phosphate and also directly, even in absence of adenylypyrophosphatase, with adenylypyrophosphate. The former, if plentiful when both are present, is preferred.

5. The esterification of inorganic phosphate can be increased by the presence of adenylic acid.

6. Suggestions are made for the correlation of these results with happenings *in vivo*.

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