77. The Effect of Amino-acids on Phosphate Transfer in Muscle Extract*

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(Received 3 July 1942)

Lohmann [1934] demonstrated in vertebrate muscle extract phosphate transfer from creatinephosphate to adenylic acid resulting in adenylypyrophosphate and creatine. Ostern, Baranowski & Reis [1935], D. M. Needham & van Heyningen [1935], and Meyerhof & Lehmann [1935] all showed in vertebrate muscle extract phosphate transfer from adenylypyrophosphate to creatine resulting in creatinephosphate and adenylic acid. Meyerhof & Lehmann [1935] also reported phosphate transfer from adenylypyrophosphate to arginine in invertebrate muscle extract.

The two directions of phosphate transfer were investigated more closely by Lehmann [1935; 1936] who showed that the same equilibrium point was reached from both sides; it depended on pH. Alkalinity favoured phosphagen synthesis, neutrality and acidity favoured the formation of adenylypyrophosphate. The reversible transfer of 2 mol. phosphate between 1 mol. adenylic acid and 2 mol. creatine was shown not to be a trimolecular reaction but could be split up into two bimolecular reactions in which adenosine diphosphate (a substance discovered by Lohmann [1934] in crayfish) figured in vertebrate muscle also, as an intermediate between adenylypyrophosphate and adenylic acid.

Lehmann [1935] reported that vertebrate muscle extract phosphorylated only creatine and invertebrate muscle extract transferred phosphate only to arginine. Baldwin & D. M. Needham [1937] showed that those invertebrates which according to J. Needham, D. M. Needham, Baldwin & Yudkin [1932] contained both phosphagens, also had both the enzymes needed for phosphorylating creatine and arginine. In the work now reported we sought to find out whether there was competitive inhibition of creatine phosphorylation by arginine in vertebrate muscle extract or of arginine phosphorylation by creatine in muscle extract of invertebrates. We found however that arginine did not inhibit creatine phosphorylation but accelerated and even initiated it under certain conditions. Various other reactions, most of them involving phosphate transfer, were investigated, and the effect on them of arginine and other compounds was studied.

Methods

Enzymes used. The enzyme used was rabbit muscle extract prepared according to Meyerhof [1926]. To investigate the transfer of phosphate from adenylypyrophosphate to creatine and arginine, or the phosphorylation of adenylic acid from a phosphate donor, conditions had to be established in which enzymic side reactions were abolished as much as possible. They were mainly dephosphorylation of adenylypyrophosphate to inorganic phosphate and adenylic acid by adenylypyrophosphatase and deamination of adenylic acid to NH_3 and inosinic acid by adenylic acid deaminase. The activity of adenylypyrophosphatase was mainly abolished by letting the extract stand in the ice chest for several days or weeks. Combination of ageing with prolonged dialysis led to complete inactivation of adenylypyrophosphatase [Lehmann, 1935]. These facts were tested again and confirmed, since experiments on phosphate transfer by Lehmann & D. M. Needham [1937] had been criticized on this point, and it was claimed that one could never obtain complete destruction of adenylypyrophosphatase by ageing and dialysis only. The absence of adenylypyrophosphatase was demonstrated in the present work in each extract used for a series of experiments and checked again from time to time. The interference caused by adenylic acid deaminase could be completely disregarded in those of our experiments which were carried out at pH 10, at which reaction the deaminase is inactive [Lehmann, 1935]. In the experiments at a neutral or slightly alkaline reaction advantage was taken of the observation of G. T. Cori,

* A preliminary report appeared in the Proceedings of the Physiological Society, 14 February 1942, *J. Physiol.* 100.

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Colowick & C. F. Cori [1939] that adenylic acid deaminase was removed from an aged extract by filtering. We confirmed these findings.

The extracts were aged by leaving at $+4^\circ$ for at least 5 days. Toluene was added. If it is not specially mentioned otherwise they also underwent dialysis through a cellophane membrane. The membrane was washed previously with dilute HCl to remove traces of Cu. The dialysis was carried out for a period of at least 48 hr. at $+4^\circ$. The dialysis fluid was frequently changed and was in turn distilled water and 0.45% KCl. The last dialysis was always performed against 0.45% KCl.

Compounds used. The phosphorylated compounds used were prepared by us except the adenylic acid which was obtained from a Lwow (Poland) factory, and the hexosediphosphate which was the product of a U.S. firm. The adenylypyrophosphate was prepared by a modification of Lohmann's method [Lohmann, 1931] which was communicated to us by Dr D. M. Needham. Later we also used the modifications recommended by Kerr [1939]. Adenosinediphosphate was prepared from adenylypyrophosphate by means of dilute myosin. This, according to a personal communication of Dr K. Bailey, only dephosphorylates adenylypyrophosphate but does not attack adenosinediphosphate—the enzyme responsible for dephosphorylation of adenosinediphosphate being the myokinase [Kalckar, 1942]. The myosin was given to us by Dr J. Needham and Dr Shen. Creatinephosphate was prepared by the enzymic phosphorylation of creatine from phosphoglyceric acid [Lehmann, 1935] and phosphoglyceric acid was prepared from glucose and phosphate in fluoride-poisoned yeast extract [Meyerhof & Kiessling, 1933]. All the phosphate esters were used as the K or Na salts.

Most of the amino-acids and related compounds used were commercial products. Dr Bach provided us with ornithuric acid, glycocyamine and citrulline. The cystine was obtained from Mr Cole and the α -ketoglutaric acid was prepared by Mr Williamson. Prof. Sir F. G. Hopkins gave us reduced and oxidized glutathione.

Experimental procedure

If it is not stated otherwise each sample contained 20% muscle extract, 0.13% Mg (as $MgCl_2$) and 0.1% $NaHCO_3$. The resulting mixture was just neutral or slightly alkaline (up to pH 7.6). In most experiments Na_2CO_3 was added to give a final concentration of $M/20$, resulting in a pH of about 10. If Na_2CO_3 was present, the various solutions were added in such order as to avoid the enzyme being at any moment in a Na_2CO_3 concentration of $>M/20$. Higher concentrations were apt to destroy the enzyme. Some inorganic phosphate was always present. The dialysed extract contained usually 1–2 mg. per 100 ml. P as inorganic phosphate and the Ba salt of the adenylypyrophosphate used contained 0.6–0.8% P as inorganic P. This resulted in the presence of 0.6–0.8 mg. per 100 ml. P as inorganic phosphate in that majority of the experiments in which adenylypyrophosphate and dialysed extract were used. All the compounds were, if necessary, neutralized beforehand. If they were buffers, their pH was adjusted to that of the experiments. Further particulars will be given in the text. At the end of the experiments trichloroacetic acid was added to give a final concentration of 5% and the extract used for analysis. The phosphate was measured colorimetrically using the modification of the method of Fiske & Subbarow [1925] given by Lohmann & Jendrassik [1926]. A standard of 200 μ g. P in 25 ml. was used and if the sample to be estimated contained a much lower concentration, a measured amount of inorganic phosphate was added to make it comparable with the standard. The creatine phosphate was measured after Lohmann [1928] as the difference between the directly estimated phosphate (inorganic phosphate + phosphagen phosphate) and the true inorganic phosphate precipitable by ammoniacal Mg citrate [Mathison, 1909]. The determination of arginine phosphate followed the same principle, but owing to the greater stability of arginine phosphate against acid the 'direct' value was measured after 20 hr. previous incubation in $N/15$ HCl at 24° .

The easily hydrolysable phosphate of adenylypyrophosphate and adenosinediphosphate was measured as the increase in inorganic phosphate after 7 min. hydrolysis in N HCl at 100° . The change of 3-phosphoglyceric acid into 2-phosphopyruvic acid was observed by measuring the increase of inorganic phosphate after 60 min. hydrolysis in N HCl at 100° [Lohmann & Meyerhof, 1934]. The formation of triosephosphate from hexosediphosphate was followed by measuring the increase of inorganic phosphate after 20 min. incubation in N NaOH at room temperature [Meyerhof & Lohmann, 1934]. The free NH_3 was measured colorimetrically using Nessler's reagent, if necessary after a previous distillation in the Parnas apparatus.

All figures will be given for a volume of 10 ml. The volume employed in our experiments was usually 4 ml.

Transfer of phosphate from adenylypyrophosphate to creatine

The effect of arginine. Our original aim was to find if arginine inhibited creatine phosphorylation, but it showed itself to be an activator. This could be seen when the activity of the extract had diminished owing to prolonged dialysis. An extract which before dialysis transferred phosphate from adenylypyrophosphate to creatine in a few minutes, after dialysis needed 20–30 min. for final equilibrium. The experiments were performed under the same conditions each time and Mg was added to the dialysed extract. Arginine was seen to accelerate the transfer in feebly active extracts, though it did not change the final equilibrium (Table 1).

Table 1. *Activation of a weak extract by arginine*

Dialysed rabbit muscle extract, 21 days old. Adenylpyrophosphate containing 825 μ g. transferable P. 7.5 mg. creatine. pH 10. 37°.

Time in min.	Creatine phosphate formed, μ g. P	
	No addition	75 mg. arginine
3	147	560
30	545	525

Table 2. *Reactivation of an inactive extract by arginine*

Dialysed rabbit muscle extract, 18 days old. Adenylpyrophosphate containing 825 μ g. transferable P. 7.5 mg. creatine. pH 10. 37°.

Time in min.	Addition	Creatine phosphate formed, μ g. P
1	—	15
1	75 mg. arginine	290
30	—	12
30	75 mg. arginine	395

Some of the extracts which had been dialysed longer became completely inactive and did not produce a transfer of phosphate even over a prolonged period. In such cases, arginine largely restored the transfer (Table 2).

The inactivity of the extract and the effect of arginine upon it were studied in two ways: (a) the reason for activation by arginine, whether it was a specific effect of the arginine molecule or of a particular part of it which it might share with other compounds; (b) whether changed conditions (pH, dialysis etc.) could restore activity to the extract.

1. Comparison with the effects of other compounds

To see whether the guanidine group of arginine was responsible for the activation, guanidine itself was tried as well as urea and NH_3 (Table 3) and also guanidoacetic acid (glycocyanine) (Table 4). These substances were inactive.

Table 3. *No influence of guanidine, urea and NH_3*

Dialysed rabbit muscle extract, 8 days old. Adenylpyrophosphate containing 875 μ g. transferable P. 7.5 mg. creatine. pH 10. 33°. 10 min.

Addition	Creatine phosphate formed, μ g. P
—	245
23 mg. guanidine	235
125 mg. urea	225
33 mg. NH_3	240

Table 4. *No influence of glycocyanine*

Dialysed rabbit muscle extract, 30 days old. Adenylpyrophosphate containing 825 μ g. transferable P. 7.5 mg. creatine. pH 10. 33°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	0
25 mg. glycocyanine	0
25 mg. arginine	190
50 mg. arginine	200

The experiment with glycocyanine (Table 4) demonstrates that 25 mg. arginine are sufficient for full activation.

To test whether the α -aminovaleric acid part of the arginine molecule was responsible for its effect, citrulline was tried which differs from arginine only in that the imino group of guanidine is replaced by oxygen (Table 5), and ornithine (α : δ -diaminovaleric acid, Table 6).

Table 5. *Activation by citrulline*

Dialysed rabbit muscle extract, 7 days old. Adenylpyrophosphate containing 825 μ g. transferable P. 7.5 mg. creatine. pH 10. 30°.

Time in min.	Creatine phosphate formed, μ g. P	
	No addition	50 mg. citrulline
5	125	300
20	280	388
40	306	375

Table 6. *Activation by ornithine*

Dialysed rabbit muscle extract, 11 days old. Adenylpyrophosphate containing 740 μ g. transferable P. 7.5 mg. creatine. pH 10. 33°. 20 min.

Addition	Creatine phosphate formed, μ g. P
—	0
12.5 mg. ornithine	80
25 mg. ornithine	185
50 mg. arginine	170

Of all the compounds used so far, only the amino-acids were found to be active regardless of whether a guanidine group was present or not. The question arose now which particular group of the aminovaleric acid was the active one. Ornithine was thus compared with ornithuric acid in which the two amino groups are blocked with benzoic acid. No influence of ornithuric acid could be demonstrated (Table 7).

Table 7. *Activation by ornithine, no activation by ornithuric acid*

Dialysed rabbit muscle extract, 48 days old. Adenylpyrophosphate containing 830 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	25
M/60 ornithine	365
M/60 ornithuric acid	25

Table 8. *Activation by glutamic acid, no activation by α -ketoglutaric acid*

Dialysed rabbit muscle extract, 17 days old. Adenylpyrophosphate containing 1450 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	35
100 mg. glutamic acid	680
100 mg. α -ketoglutaric acid	30

The last experiments made it almost sure that a free amino group was the effective group, and comparison with other α -amino-acids showed that they all activated. The essential nature of the α -amino group could be demonstrated by replacing it by O, OH or H. Thus alanine was active but not pyruvic, lactic or propionic acid. Glycine was effective but not acetic acid; aspartic acid but not succinic or fumaric acid. In Table 8 a comparison is given of the effects of glutamic acid and of α -ketoglutaric acid.

The various amino-acids were compared with one another. Table 9 shows that the diaminohexoic acid lysine was not more active than the monoamino-*iso*hexoic acid leucine, if the usual concentration of activators was used.

Table 9. *Comparison of lysine and leucine*

Dialysed rabbit muscle extract, 6 days old. Adenylpyrophosphate containing 1450 μ g. transferable P. 7.5 mg. creatine. pH 10. 34°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	37
M/15 lysine	365
M/15 leucine	380

Table 10. *Comparison of d(−)-alanine and l(+)-alanine*

Dialysed rabbit muscle extract, 5 days old. Adenylpyrophosphate containing 1450 μ g. transferable P. 7.5 mg. creatine. pH 10. 34°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	65
60 mg. d(−)-alanine	430
60 mg. l(+)-alanine	455

There was no difference as regards their activating effects between d(−)-alanine and l(+)-alanine (Table 10).

Cysteine was still active at concentrations which were too low for other amino-acids to show an effect (Table 11).

The higher activity at low concentrations of cysteine suggested that sulphhydryl had an effect similar to that of the α -amino group. This was confirmed by the activation brought about by thiolacetic acid (Table 12).

The tripeptide glutathione was more active than cysteine (Table 13). The action of S—S compounds like cystine and oxidized glutathione was not inhibitory. They activated slightly, but much less than their reduced forms. This may have been due to their forming some sulphhydryl under the conditions of our experiment, and also to their containing the activating α -amino group. They did however diminish the influence of activating amino-acids, regardless of whether these contained an SH group or not (Table 13).

Table 11. *Comparison of cysteine with glycine and lysine*

Dialysed rabbit muscle extract, 7 days old. Adenylpyrophosphate containing 1450 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Molarity of addition	Creatine phosphate formed, μ g. P		
	30		
	Cysteine	Glycine	Lysine
—	—	295	—
1/30	—	295	—
1/60	260	270	260
1/120	170	35	25
1/240	155	—	20

Table 12. *Activation by thiolacetic acid**

Dialysed rabbit muscle extract, 32 days old. Adenylpyrophosphate containing 1450 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Addition	Creatine phosphate formed, μ g. P
—	120
38 mg. thiolacetic acid	305

* Much sulphhydryl in the experiments interfered with the phosphate estimation. The trichloroacetic extract was therefore treated with chloramine T. A slight excess of the calculated amount needed was added and the precipitate which appeared was filtered off. We are obliged to Mr J. R. Bendall for advice on this method.

Table 13. *Activation by reduced glutathione (GSH): the effect of oxidized glutathione (GS-SG) and cystine on the activation by other amino-acids*

Dialysed rabbit muscle extract, 37 days old. Adenylpyrophosphate containing 830 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Additions	Creatine phosphate formed, μ g. P	Increase in creatine phosphate formed, μ g. P	Additions	Creatine phosphate formed, μ g. P	Increase in creatine phosphate formed, μ g. P
—	155	—	M/50 cysteine, M/50 cystine	220	65
M/50 GSH	525	370	M/20 glycine	455	300
M/80 GS-SG	195	40	M/20 glycine, M/80 GS-SG	195	40
M/50 GSH, M/80 GS-SG	435	280	M/20 glycine, M/50 cystine	200	45
M/50 cysteine	280	125	M/30 leucine	385	230
M/50 cystine	190	35	M/30 leucine, M/50 cystine	185	30

Simple primary amines were effective, but acetamide, the only amide we tested, had no influence. In Table 14 a comparison is made between acetamide and ethylamine.

Table 14. *No influence of acetamide: activation by amines*

Dialysed rabbit muscle extract, 21 days old. Adenylpyrophosphate containing 850 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 5 min.

Addition	Creatine phosphate formed, μ g. P	Addition	Creatine phosphate formed, μ g. P
—	0	α -phenylethylamine	160
M/12 acetamide	0	M/35 histamine	455
M/12 ethylamine	422		

In Table 15 are recorded substances which were tested for their effect on transfer of phosphate in dialysed muscle extract at pH 10.

Table 15. *Compounds tested for their effect upon phosphate transfer in dialysed muscle extract at alkaline pH*

Effective	Not effective
Glycine, thiolacetic acid	Acetamide, acetic acid
d(-)-Alanine, l(+)-alanine, cysteine	Pyruvic acid, lactic acid, propionic acid
Aspartic acid	Succinic acid, fumaric acid
Glutamic acid	α -Ketoglutaric acid
Arginine, citrulline, ornithine	Urea, guanidine, creatinine, glycocyamine
Leucine, lysine	—
Histidine	—
Glutathione (GSH)	—
Methylamine, ethylamine	Ammonia, choline
α -Phenylethylamine, piperidine, histamine	—

2. *Conditions necessary for activation*

(a) *Alkaline reaction.* No activation by amino-acids was found at neutrality. To obtain time curves of the enzyme action, diluted extracts were used, but no rise in the velocity of phosphate transfer could be observed when amino-acids were present. Table 16 shows an experiment where the extract was not 20 % of the total volume as usual in our experiments, but only 0.3 %. It will be seen that at pH 7.5 the equilibrium was much in favour of adenylypyrophosphate, and that it was necessary to add a great surplus of creatine to obtain an equilibrium point which involved a significant shift of phosphate.

Table 16. *No influence of amino-acids at low pH*

Dialysed rabbit muscle extract, 76 days old. Adenylypyrophosphate containing 820 μ g. transferable P. 34°.

Time in min.	pH	Extract % of total vol.	Creatine, mg.	Addition	Creatine phosphate formed, μ g. P
15	10	20	7.5	—	48
15	10	20	7.5	50 mg. glycine	450
15	7.5	0.3	7.5	—	63
15	7.5	0.3	7.5	50 mg. glycine	63
1	7.5	0.3	100	—	52
1	7.5	0.3	100	50 mg. glycine	30
1	7.5	0.3	100	50 mg. arginine	40
1	7.5	0.3	100	50 mg. thiolacetic acid	65
15	7.5	0.3	100	—	220
15	7.5	0.3	100	50 mg. glycine	230
15	7.5	0.3	100	50 mg. arginine	195
15	7.5	0.3	100	50 mg. thiolacetic acid	235

The fact that at pH 7.5 large amounts of creatine had to be used made it possible that the increase in creatine and not the lower pH was responsible for the insensitivity of the reaction towards amino-acids. Large amounts of creatine might have been able to take on the role of the amino-acids. In testing this point we had to take into account that creatine, unlike the amino-acids, shifted the equilibrium point and large amounts of it would be expected to increase the phosphate transfer at neutral and at alkaline reaction. Table 17 shows however that an added excess of creatine at an alkaline reaction did not

Table 17. *Activation by glycine in the presence of excess of creatine*

Dialysed rabbit muscle extract, 14 days old. Adenylypyrophosphate containing 1000 μ g. transferable P. pH 10. 35°.

Time in min.	Extract % of total vol.	Creatine, mg.	Addition	Creatine phosphate formed, μ g. P
5	20	7.5	—	125
5	20	7.5	25 mg. glycine	290
5	20	75	—	325
1	1	7.5	—	150
1	1	7.5	25 mg. glycine	250
5	1	75	—	300
5	1	75	25 mg. glycine	480

prevent the amino-acids from exerting their influence. Compared with low amounts of creatine an excess had a beneficial influence, but this could be greatly improved upon by addition of glycine. The absolute proof of the inability of creatine to replace amino-acids in their activation will be given when it will be shown that it did not activate the phosphorylation of arginine (see Table 21).

(b) *Dialysis.* Undialysed extract was more active than its dialysed equivalent and could not be improved by addition of glycine (Table 18). Undialysed extract seemed thus

to contain a factor which was lost on dialysis and which could be replaced by an amino-acid. This factor was heat-stable, and boiled undialysed extract could be used for the activation of dialysed extract (Table 18). (The boiled extract was prepared by 15 min. heating on a boiling water bath. It was seen to be inactive by itself. This had to be tested as Curtius & Ohlmeyer [1938] have shown that a number of enzymes transferring phosphate are not completely destroyed by short boiling.) The activating principle was present in the dialysate but not in the ash of the dialysate. The dialysate was prepared by dialysing successively four equal samples of 1 vol. muscle extract for 4 hr. against the same sample of 4 vol. water. The dialysate was then concentrated and contained inorganic phosphate = 30 mg. per 100 ml. P, as compared with 50 mg. per 100 ml. P in the undialysed extract. The similarity of the actions of the effective principle in undialysed muscle extract and of the amino-acids added to dialysed extract is borne out by the fact that cystine inhibited the undialysed extract. It was shown previously (Table 13) that it did not inhibit dialysed extract, but counteracted the activation by added amino-acids.

Table 18. *No influence of amino-acids on undialysed extract. Activation of dialysed extract by boiled undialysed extract and dialysate*

Rabbit muscle extract, 14 days old. Adenylpyrophosphate containing 840 μ g. transferable P. 7.5 mg. creatine added. pH 10. 35°. 6 min.

Enzyme	Addition	Creatine phosphate formed, μ g. P
20% dialysed extract	—	125
20% dialysed extract	50 mg. glycine	350
20% undialysed extract	—	665
20% undialysed extract	50 mg. glycine	640
20% undialysed extract	50 mg. cystine	195
—	25% boiled undialysed extract	0
20% undialysed extract	25% boiled undialysed extract	467
20% dialysed extract	25% dialysate	280
20% dialysed extract	5% dialysate	150
20% dialysed extract	Ash equivalent to 25% dialysate	135

Transfer of phosphate from adenosinediphosphate to creatine

The amino-acid effect was not restricted to the shift of one phosphate group of adenylpyrophosphate to creatine; an influence was also observed on the transfer of the second phosphate group. Using adenylpyrophosphate as a donator we rarely obtained a shift of more than 50% of the transferable phosphate. Experiments were therefore carried out in which adenosinediphosphate served as the phosphate donator. The effect of amino-acids on phosphate transfer from adenosinediphosphate to creatine is shown in Table 19.

Table 19. *Activation of phosphate transfer from adenosinediphosphate to creatine*

Dialysed rabbit muscle extract, 38 days old. Adenyldiphosphate containing 625 μ g. transferable P. 7.5 mg. creatine. pH 10. 35°. 10 min.

Addition	Creatine phosphate formed, μ g. P
—	50
50 mg. glycine	413
37 mg. cysteine	438

Table 20. *Activation of phosphate transfer from creatine phosphate to adenylic acid*

Dialysed rabbit muscle extract, 28 days old. 1.25% of total volume. Creatine phosphate containing 600 μ g. transferable P. 7.5 mg. adenylic acid. 36°. 1 min.

pH	Addition	Transfer of phosphate μ g. P
8	—	62
8	30 mg. cysteine	325
8	25 mg. glycine	200
10	—	42
10	25 mg. glycine	93

Transfer of phosphate from creatine phosphate to adenylic acid

The transfer of phosphate from creatine phosphate to adenylic acid is lowest at an alkaline reaction. Thus high *pH*, which was most suitable for demonstrating the amino-acid effect, was most unsuitable for the reaction itself. We succeeded however with a very inactive extract, used in great dilution (1.25 % of the total volume instead of the usual 20 %), in demonstrating an activation at *pH* 8 and a small but definite one even at *pH* 10 (Table 20).

Transfer of phosphate from adenylypyrophosphate to arginine

An amino-acid effect on arginine phosphorylation in crab muscle extract was also observed. Glycine and cysteine greatly activated a crab muscle extract, which was itself completely inactive at *pH* 10 (Table 21). Creatine had no effect, as was to be expected, for its amino-group is substituted.

Table 21. *Activation of phosphate transfer from adenylypyrophosphate to arginine*

Dialysed crab muscle extract, 3 days old. Adenylypyrophosphate containing 800 μ g. transferable P. 7.5 mg. arginine. *pH* 10. 37°. 5 min.

Addition	Arginine phosphate formed, μ g. P
—	0
50 mg. creatine	0
50 mg. glycine	245
37 mg. cysteine	278

Table 22. *No inhibition of arginine phosphorylation by creatine*

Dialysed crab muscle extract, 8 days old. Adenylypyrophosphate containing 1250 μ g. transferable P. 75 mg. arginine. *pH* 7.5. 38°. 15 min.

Addition	Arginine phosphate formed, μ g. P
—	260
50 mg. creatine	243

The same extract which was inactive at *pH* 10 worked perfectly at *pH* 7.5. For the same reasons that required addition of more creatine at *pH* 7.5, more arginine had to be used at this reaction than would have been needed at *pH* 10. Table 22 shows that the improved transfer of phosphate was not inhibited by creatine; thus just as arginine was no competitive inhibitor of creatine phosphorylation, creatine was no competitive inhibitor of the phosphorylation of arginine.

Formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid

The formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid involves an intramolecular shift of phosphate. An effect of amino-acids on the reaction was demonstrated (Table 23). The enzyme system involved was—unlike the one which phosphorylated creatine—almost inactive at *pH* 10 even when undialysed extract was used. Again no amino-acid effect could be seen at a neutral reaction.

Table 23. *Activation of the formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid*

Dialysed rabbit muscle extract, 5 days old. 40 % of the total volume. Phosphoglyceric acid containing 4000 μ g. P. 25°. 20 min.

<i>pH</i>	Addition	Phosphopyruvic acid formed, μ g. P
7	—	640
7	80 mg. glycine	640
10	—	0
10	80 mg. glycine	160

Table 24. *Influence of glycine on the speed of formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid*

Dialysed rabbit muscle extract, 11 days old. 40 % of the total volume. Phosphoglyceric acid containing 4000 μ g. P. *pH* 8.5. 25°.

Time in min.	Phosphopyruvic acid formed, μ g. P		Conversion %	
	—	80 mg. glycine	—	80 mg. glycine
15	0	520	0	13
30	460	760	11.5	19
60	968	948	24	23

The equilibrium between phosphoglyceric acid and phosphopyruvic acid is reached at room temperature when 75–80% of the total P belongs to phosphoglyceric acid and 25–20% to phosphopyruvic acid [Lohmann & Meyerhof, 1934]. We thus investigated again whether the amino-acid effect shifted the equilibrium or whether it only increased the speed of its attainment. Table 24 shows that the equilibrium itself was not changed by glycine, but that the velocity was increased. It will be remembered that the same was seen when the equilibrium between adenylypyrophosphate and creatine phosphate was investigated.

Formation of triosephosphate from hexosediphosphate

The question arose whether the amino-acid effect applies to reactions of phosphoric esters in general or whether it is confined to reactions involving phosphate transfer. We therefore investigated whether amino-acids affect the formation of triosephosphate from hexosediphosphate, a reaction which does not involve a shift of phosphate [Meyerhof & Lohmann, 1934]. No influence could be observed (Table 25).

Table 25. *No influence of glycine on triosephosphate formation from hexosediphosphate*

Dialysed rabbit muscle extract, 14 days old. Hexosediphosphate containing 4000 μ g. P. pH 8. 36°.

Time	Concentration of extract %	Triosephosphate formed, μ g. P		Conversion, %	
		Addition ... —	40 mg. glycine	—	40 mg. glycine
3	40.00	1020	1020	26	26
6	40.00	1020	1040	26	27
20	40.00	1000	1040	25	27
3	0.40	875	800	22	20
15	0.40	1000	1020	25	26
1	0.20	600	630	15	16
12	0.20	980	940	25	24
3	0.04	250	100	6	3
15	0.04	510	560	13	14

The influence of amino-acids on the solubility of slightly soluble salts

In most of our experiments which were carried out at an alkaline reaction the Mg was added to the muscle extract first and the adenylypyrophosphate and the Na_2CO_3 followed afterwards. In some the Mg, adenylypyrophosphate and Na_2CO_3 were first mixed, and afterwards the muscle extract was added. In the latter case a precipitation of Mg adenylypyrophosphate and MgCO_3 occurred. This precipitation could not be seen in the first procedure even when filtered and clear extract was used, this lack of visible precipitation was obviously due to the presence of colloids. In the second case the precipitate of Mg adenylypyrophosphate and MgCO_3 was completely dissolved in the samples where amino-acids were added. This could not have been due to the buffering effect of the amino-acids as they were adjusted to the alkaline reaction.

The effect of amino-acids was not restricted to the solubility of Mg adenylypyrophosphate and MgCO_3 , but could also be observed with Mg phosphate and Ca salts.* If for instance two samples of 1 ml. of $M/10$ MgHPO_4 in $N/20$ HCl were treated with three drops of N NaOH , a precipitate of Mg phosphate appeared. To one sample 2 ml. of water were added and to the other 2 ml. of a 20% glycine solution which had been adjusted to pH 8.5 (9 mol. glycine : 1 mol. NaOH). The second sample became a clear solution. Phytin which is a commercial product consisting of a mixture of Ca and Mg phytates is sparingly soluble in pure water, but 100 ml. of M glycine solution dissolve up to 0.5 g.

Fig. 1 shows the effect of various glycine concentrations on the solubility of Mg phosphate, and Table 26 shows the effect of glycine on the solubility of Ca phosphate.

* The solubility of Ca oxalate is not altered by amino-acids and addition of excess Na oxalate will precipitate all Ca in a solution containing amino-acids and Ca salts of organic or inorganic phosphate.

The same compounds which activated phosphate transfer also had an effect on the solubility of sparingly soluble salts.

Fig. 1. The effect of glycine on the solubility of Mg phosphate ($\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$).

Table 26. *Solubility of Ca phosphate in a glycine buffer at pH 8.5 (9 mol. glycine: 1 mol. NaOH)*

Room temperature	
Conc. of buffer	µg. P dissolved in 10 ml.
<i>M</i> /10	206
<i>M</i> /5	250
<i>M</i> /2	450
<i>M</i>	504

Table 27. *Different effects of glycine and cysteine on the solubility of Ca phosphate (CaHPO_4). Neutral pH*

Room temperature		
Conc. of amino-acid	µg. P dissolved in 10 ml.	
—	118	
	Glycine	Cysteine
<i>M</i> /10	145	180
<i>M</i> /5	176	292

Thus glycine and thiolacetic acid produced the effect but not acetamide and acetate. Amines like piperidine and histamine redissolved sparingly soluble salts, but urea, guanidine etc. did not do so. Cysteine was more effective than glycine (Table 27).

DISCUSSION

The accelerating and activating effect of amino-acids on phosphate transfer in muscle extract is obviously due to their amino group. This is proved by the observation that amines were likewise effective, but not amino-acids or amines in which the amino group was substituted (ornithine and choline). The SH group seems to act similarly on phosphate transfer, as is indicated by the effect of thiolacetic acid. Cysteine which contains both an amino and an SH group was found to be active at lower concentrations than S-free amino-acids. The fact that at alkaline reaction undialysed extract is more active than its dialysed equivalent may be due at least partly to the loss of amino-acids and related

compounds. It has been demonstrated that dialysed extracts could be activated by addition of boiled undialysed extract or dialysate. The behaviour of cystine points in the same direction. It inhibits undialysed extract but shows no effect on dialysed extract. If, however, the latter is activated by amino-acids, their effect can be suppressed by addition of cystine.

There are some facts in the literature which may possibly have a bearing on our findings. Boiled muscle extract was shown by Kendal & Stickland [1937] to contain activators other than the known coenzymes. Some effects are known of amino-acids on the activity of amylase, urease, lipase, phosphatase. Sumner & Hand [1928] protected urease from inactivation by adding amino-acids, and they give a long list of instances where previous authors found a protective action of amino-acids on enzymes; Bodansky [1936] in particular observed an activation of bone phosphatase by amino-acids which he also attributes to their protective function. However, the concentrations used by Bodansky were much below those employed by us.

Amino-acids are also often used to protect enzymes from metals. We could find no particular sensitivity of creatine phosphorylation towards Zn and Cu, the two metals which were most inhibitory in the formation of Robison ester from glycogen [Gill & Lehmann, 1939]. We found no appreciable inhibition of undialysed extract with ZnSO_4 up to $M/400$. There is always a certain danger of Cu contamination where cellophane tubes are used for dialysis. Thus Kubowitz & Lüttgens [1941] dialysed their cocarboxylase against a glycine solution to protect the enzyme against Cu. We washed our dialysis tubes with dilute HCl, and using the usual qualitative methods could find no Cu in our extracts. Moreover the transfer of phosphate from adenylypyrophosphate to creatine is inhibited only by relatively large amounts of Cu. In Table 28 the effect of high concentrations of Cu is demonstrated. It is reversed by glycine and the transfer is activated beyond the normal figure. However, Cu in the comparatively high concentration of $M/40,000$ had no effect on the reaction.

Table 28. *Inhibition by Cu and its suppression by glycine. No inhibition at $M/40,000$ Cu*

Dialysed rabbit muscle extract, 36 days old. 850 μg . transferable P of adenylypyrophosphate. pH 10. 36°.

Time in min.	Creatine, mg.	Added CuSO_4	Creatine phosphate formed, μg . P	
			No addition	50 mg. glycine
3	60	0	323	—
3	60	$M/40,000$	300	—
3	7.5	0	178	500
3	7.5	$M/400$	35	302
3	7.5	$M/4000$	105	440
3	7.5	$M/40,000$	185	—

There is a possibility of other metals being present which may have a specific inhibitory effect. However, if the amino-acid effect were simply a removal of metal it would be difficult to understand why it takes place only at an alkaline reaction. Furthermore the ash of the dialysate did not inhibit when added to muscle extract (Table 18).

It may be suggested that the effect of amino-acids on the solubility of sparingly soluble salts could well be connected with their activation of phosphate transfer. This would explain why no activation by amino-acids could be observed at a neutral reaction. Magnesium salts particularly are easily soluble at neutrality and therefore no increase in solubility would be necessary. The amino-acids may also influence the combination of the phosphate with the enzyme itself. This would agree with the fact that the amino-acids influence reactions involving phosphate transfer, but not for instance the formation of triosephosphate from hexosediphosphate.

The interaction of divalent ions, amino-groups, and phosphate, revealed in the experiments on solubility, may be a model for the activation of phosphatases by divalent ions such as Mg, Ca, Mn. These ions may link the phosphorus with the amino group of the enzyme molecule.

The higher solubility of sparingly soluble Ca salts in presence of amino-acids may be of importance for the intestinal absorption of these compounds. It has been shown that Ca, when taken by man as the soluble chloride, is largely converted into sparingly soluble CaCO_3 in the intestine [J. B. S. Haldane, R. Hill & Luck, 1923]. It is particularly remarkable that the solubility of Ca phytate is influenced by amino-acids. The question whether amino-acids play a part in the absorption of Ca and Mg has been taken up by one of us (H. L.) with Dr McCance and Dr Widdowson, and the influence of protein on Ca absorption in man could be demonstrated [McCance, Widdowson & Lehmann, 1942].

The effect of amino-acids particularly on the solubility of calcium salts may of course play a part in many physiological events.

The salutary effect of proteolytic enzymes on wound healing may be connected with it, a relation between calcium and cell growth being well known.

Plant seeds are particularly rich in protein and also in sparingly soluble calcium and magnesium salts. The breakdown to amino-acids may help to make the store of mineral salts available for the growing plant.

R. Hill & Lehmann [1941] observed that there was in the leaves of *Euonymus* a parallelism between iron and phosphorus contents. Experiments by the same authors on iron in various leaves showed that only a small proportion of it was 'available'. Our present observations demonstrate that it cannot be decided whether a salt is physiologically available in animal or plant tissues by testing its solubility in an aqueous or even acid extract. The extracts must contain the same compounds which might increase the solubility of the salts inside the cell. We could find no influence of glycine on the solubility of ferric salts. Of the compounds used in the present investigation both cysteine and thiolacetate increased considerably the solubility of ferric phosphate, ferric adenylypyrophosphate and even of ferric phytate at a neutral reaction. The soluble compound was purple, a sign that the stable thiol complex was formed. Citrate, preferably at a slightly alkaline reaction, showed a similar effect. Thus if amino-acids in general should play a role in making calcium and magnesium salts available for the growing plant, no exactly parallel mechanism can be assumed for ferric salts.

A soluble combination of the sparingly soluble BaCO_3 with amino-acids has previously been observed by Siegfried [1905]. He suggested that carbamino-acids were formed. Complex compounds of amino-acids with soluble salts such as CaCl_2 and BaCl_2 were found by Pfeifer & Modelski [1912; 1913] and crystallized by King & Palmer [1920]. Our findings are new in so far as they show that the addition of amino-acids raises the solubility of otherwise sparingly soluble salts, particularly at an alkaline reaction. A mutual effect on solubility of highly charged proteins or amino-acids and neutral salts such as NaCl and LiCl has been described by Cohn [1935]. It differs from our observations in its order of magnitude.

SUMMARY

1. The effect of arginine on phosphorylation of creatine by adenylypyrophosphate has been studied in rabbit muscle extract. It increases the velocity of creatine phosphate formation, but does not shift the final equilibrium.

2. The effect of arginine has been compared with that of similar compounds (guanidine, glycocyamine, ornithine, ornithuric acid, citrulline). Only those containing an α -amino group were effective.

3. Amino-acids in general were tested and found to be effective. The corresponding keto-acids were ineffective.

4. Cysteine was still effective at $M/240$ but in the cases of glycine and lysine the activation disappeared between $M/60$ and $M/120$. Thiolacetic acid was effective.
5. Amines were effective but not acetamide.
6. The amino-acid effect could only be demonstrated at alkaline reaction, and on dialysed extract.
7. Dialysed extract could be activated by boiled undialysed extract and by dialysate but not by the ash of dialysate.
8. Cystine inhibited undialysed extract but not its undialysed equivalent. In dialysed extract it suppressed the activating effect of added amino-acids.
9. The following reactions were activated by amino-acids: phosphorylation of creatine by adenylypyrophosphate and by adenosinediphosphate. Phosphorylation of adenylic acid by creatine phosphate. Phosphorylation of arginine by adenylypyrophosphate in invertebrate muscle extract. Formation of 2-phosphopyruvic acid from 3-phosphoglyceric acid. No effect could be shown on the formation of triosephosphate from hexosediphosphate.
10. Amino-acids have an effect on the solubility of sparingly soluble salts such as magnesium phosphate, magnesium adenylypyrophosphate, magnesium carbonate, calcium phosphate, magnesium and calcium phytates. This effect is particularly well marked at an alkaline reaction. It is brought about by the same compounds which activate phosphate transfer and all the compounds shown to have no effect there have no effect on the solubility of sparingly soluble salts.
11. Though ferric phosphate, ferric adenylypyrophosphate and ferric phytate formed soluble thiol complexes with cysteine, no effect could be observed with glycine.
12. It is pointed out that the effect of amino-acids on sparingly soluble salts may have a bearing on a number of physiological processes and should be kept in mind when the availability of a mineral *in vivo* is thought to be measured by its solubility in aqueous or acid extracts.

One of us (L. P.) wishes to thank Prof. Sir F. G. Hopkins and Dr T. S. Hele for the hospitality of the Biochemical Laboratory; the other (H. L.) was assisted by a research grant from the Ella Sachs Plotz Foundation. Dr R. Hill helped us in the experiments on iron salts.

We are greatly indebted to Dr D. M. Needham for her interest in this investigation, and for her helpful advice.

REFERENCES

- Baldwin, E. & Needham, D. M. [1937]. *Proc. roy. Soc. B*, **122**, 197.
 Bodansky, O. [1936]. *J. biol. Chem.* **114**, 273.
 Cohn, E. J. [1935]. *Ann. Rev. Biochem.* **4**, 93.
 Cori, G. T., Colowick, S. P. & Cori, C. F. [1939]. *J. biol. Chem.* **127**, 771.
 Curtius & Ohlmeyer, P. [1938]. *Biochem. J.* **298**, 412.
 Fiske, C. H. & Subbarow, Y. [1925]. *J. biol. Chem.* **66**, 375.
 Gill, P. M. & Lehmann, H. [1939]. *Biochem. J.* **33**, 1151.
 Haldane, J. B. S., Hill, R. & Luck, J. M. [1923]. *J. Physiol.* **57**, 301.
 Hill, R. & Lehmann, H. [1941]. *Biochem. J.* **35**, 1190.
 Kalckar, H. M. [1942]. *J. biol. Chem.* **143**, 299.
 Kendal, L. P. & Stickland, L. H. [1937]. *Biochem. J.* **31**, 1758.
 Kerr, S. E. [1939]. *J. biol. Chem.* **139**, 121.
 King, H. & Palmer, A. D. [1920]. *Biochem. J.* **14**, 574.
 Kubowitz, F. & Lüttgens, W. [1941]. *Biochem. Z.* **307**, 170.
 Lehmann, H. [1935]. *Biochem. Z.* **281**, 271.
 — [1936]. *Biochem. Z.* **286**, 336.
 Lehmann, H. & Needham, D. M. [1937]. *Biochem. J.* **31**, 1210.
 Lohmann, K. [1928]. *Biochem. Z.* **194**, 306.
 — [1931]. *Biochem. Z.* **233**, 460.
 — [1934]. *Biochem. Z.* **271**, 264.

- Lohmann, K. & Jendrassik, L. [1926]. *Biochem. Z.* **178**, 419.
Lohmann, K. & Meyerhof, O. [1934]. *Biochem. Z.* **273**, 60.
McCance, R. A., Widdowson, E. & Lehmann, H. [1942]. *Biochem. J.* **36**, 686.
Mathison, G. C. [1909]. *J. Physiol.* **38**, xx.
Meyerhof, O. [1926]. *Biochem. Z.* **178**, 395.
Meyerhof, O. & Kiessling, W. [1933]. *Biochem. Z.* **267**, 313.
Meyerhof, O. & Lehmann, H. [1935]. *Naturwissenschaften*, **23**, 337.
Meyerhof, O. & Lohmann, K. [1934]. *Biochem. Z.* **271**, 89.
Needham, D. M. & van Heyningen, W. E. [1935]. *Biochem. J.* **29**, 2040.
Needham, J., Needham, D. M., Baldwin, E. & Yudkin, J. [1932]. *Proc. roy. Soc. B*, **110**, 260.
Ostern, P., Baranowski, T. & Reis, J. [1935]. *C.R. Soc. Biol., Paris*, **118**, 1414.
Pfeifer, P. & v. Modelski, J. [1912]. *Hoppe-Seyl. Z.* **81**, 329.
—— — [1913]. *Hoppe-Seyl. Z.* **85**, 5.
Siegfried, M. [1905]. *Hoppe-Seyl. Z.* **44**, 85.
Sumner, J. B. & Hand, D. B. [1928]. *J. biol. Chem.* **76**, 149.