The Effects of Ammonium, Inorganic Phosphate and Potassium Ions on the Activity of Phosphofructokinases from Muscle and Nervous Tissues of Vertebrates and Invertebrates

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1. The effect of NH_4^+ , P_i and K^+ on phosphofructokinase from muscle and nervous tissues of a large number of animals was investigated. The activation of the enzyme from lobster abdominal muscle by NH_4^+ was increased synergistically by the presence of P_i or SO_4^{2-} . In the absence of K^+ , NH_4^+ plus P_i markedly activated phosphofructokinase from all tissues studied. In the presence of 100 mM- K^+ , NH_4^+ plus P_i activated phosphofructokinase from nervous tissue and muscle of invertebrates and the enzyme from brain of vertebrates, but there was no effect of NH_4^+ plus P_i on the enzyme from the muscles of vertebrates. Nonetheless, NH_4^+ plus P_i increased the activity of vertebrate muscle phosphofructokinase in the presence of 50 mM- K^+ at inhibitory concentrations of ATP, i.e. these ions de-inhibited the enzyme. In the absence of NH_4^+ plus P_i , K^+ activated phosphofructokinase from vertebrate tissues at non-inhibitory ATP concentrations, but the effect was less marked with the enzyme from invertebrate tissues. Indeed, high concentrations of K^+ (>50 mM) caused inhibition of invertebrate tissue phosphofructokinase. Of the other alkali-metal ions tested, only Rb^+ activated phosphofructokinase from lobster abdominal muscle and rat heart muscle. 2. The properties of lobster abdominal-muscle phosphofructokinase were studied in detail. This muscle was chosen as representative of invertebrate muscle because large quantities of tissue could be obtained from one animal and the enzyme was considerably more stable in tissue extracts than in extracts of insect flight muscle. In general, the properties of the enzyme from this tissue were similar to those of the enzyme from many other tissues: ATP concentrations above an optimum value inhibited the enzyme and this inhibition was decreased by raising the fructose 6-phosphate or the AMP concentration. In particular, NH_4^+ plus P_i activated the enzyme at non-inhibitory concentrations of ATP and they also relieved ATP inhibition (see above). 3. It is suggested that increases in the concentration of NH_4^+ and P_i , under conditions of increased ATP utilization in certain muscles and/or nervous tissue, may play a part in the stimulation of glycolysis through the effects on phosphofructokinase (the effect may be a direct activation and/or a relief of ATP inhibition). Changes in the concentration of NH_4^+ and P_i are consistent with this theory in nervous tissue and the anaerobic type of muscles. The role of AMP deaminase in production of NH_4^+ from AMP in these tissues is discussed in relation to the control of glycolysis.

NH_4^+ increases the activity of phosphofructokinase (EC 2.7.1.11) from yeast and various mammalian tissues (see, e.g., Muntz, 1947, 1953a; Muntz & Hurwitz, 1951; Passonneau & Lowry, 1963, 1964). There is, however, some confusion in the literature about the action of NH_4^+ on phosphofructokinase *in vitro*. Passonneau & Lowry (1964) consider that NH_4^+ does not relieve the ATP inhibition of phospho-

fructokinase from nervous tissue or liver. On the other hand, Sols & Salas (1966) consider that NH_4^+ increases the activity of yeast phosphofructokinase by relief of ATP inhibition. A detailed study of the effects of NH_4^+ and K^+ on the activity of purified rabbit muscle phosphofructokinase was reported by Abrahams & Younathan (1971). They concluded that low concentrations of NH_4^+ or high concentrations of K^+ activate the enzyme at non-inhibitory concentrations of ATP but only NH_4^+ relieves the ATP inhibition. It is also known that P_i activates phosphofructokinase at non-inhibitory concentrations of ATP and that it relieves ATP inhibition (Passonneau & Lowry, 1962, 1964). However, a detailed study of the effects of NH_4^+ plus P_i on phosphofructokinase in the

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presence of physiological concentrations of K^+ in relation to the control of the activity of this enzyme in muscle and nervous tissue has not been reported.

The inhibition of the activity of phosphofructokinase by ATP and the relief of this inhibition by AMP and fructose diphosphate are properties that are considered to be important in the regulation of glycolysis (see Newsholme, 1970, for review). However, in some muscles, changes in the concentrations of adenine nucleotides or fructose diphosphate may not be sufficient to account for the increase in glycolytic flux observed during mechanical activity (Karpatkin *et al.*, 1964; Helmreich & Cori, 1965; Helmreich *et al.*, 1965; Sacktor & Hurlbut, 1966; Wilson *et al.*, 1967; see also Newsholme & Start, 1973b). It has been suggested that a substrate cycle between fructose 6-phosphate and fructose diphosphate in muscle, catalysed by phosphofructokinase and fructose diphosphatase, increases the sensitivity of the control mechanism at the level of fructose 6-phosphate phosphorylation in muscle. Thus very small changes in the concentrations of the regulators of phosphofructokinase (see above) could be responsible for the observed changes in glycolytic flux (Newsholme & Crabtree, 1970, 1973). However, in some muscles (Newsholme & Crabtree, 1973) and in nervous tissue (Krebs & Woodford, 1966; Sugden, 1973) fructose diphosphatase activities are not detectable, so that a substrate cycle cannot exist in these tissues. It seemed possible that in some muscles and in nervous tissue the activity of phosphofructokinase could be regulated by effectors in addition to adenine nucleotides and fructose diphosphate. Possible candidates for such regulators appeared to be NH_4^+ , P_i and K^+ . Therefore the effects of physiological concentrations of these ions on phosphofructokinase from muscle and nervous tissue from a large number of animals were tested. The results are presented in this paper.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from the Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., except for the following: 2-heptyl-4-hydroxyquinoline *N*-oxide was obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Sources of animals

Animals were obtained from the sources given in Newsholme & Taylor (1969) and Sugden & Newsholme (1973) with the addition of the pig roundworm,

Ascaris lumbricoides, which was obtained from a local slaughterhouse, and the scallop, *Pecten maximus*, and the common periwinkle *Littorina littorea*, which were obtained from the Marine Biological Association, Plymouth PL1 2PB, U.K.

Preparation of homogenates

Except for the pig roundworm, the scallop and the winkle (from which muscles were removed directly), all animals were killed by cervical section. Muscle or nervous tissue was removed by careful dissection as quickly as possible. (Mice and rats were anaesthetized with diethyl ether before cervical sections.) Muscles from the lobster and scallop were stored at -15°C until required. Preliminary experiments demonstrated that there was very little loss of phosphofructokinase activity under these conditions. Tissues were homogenized in ground-glass homogenizers with 10–20 vol. of extraction medium (50 mM-Tris-HCl, 1 mM-EDTA and 5 mM-MgCl₂ at pH 8.2). In some experiments the homogenates were dialysed against extraction medium before use, but this was found to have no effect on the properties of the enzyme. Homogenates were diluted with extraction medium just before assay of phosphofructokinase.

Dialysis of coupling enzymes

$(NH_4)_2SO_4$ activates phosphofructokinase (Underwood & Newsholme, 1965; Frenkel, 1968). Consequently, it was necessary to remove $(NH_4)_2SO_4$ from the coupling enzymes (aldolase, triose phosphate isomerase, glycerol 1-phosphate dehydrogenase and phosphoglucose isomerase) used in the assay of phosphofructokinase. Dialysis against a 400-fold excess of dialysis buffer (10 mM-Tris-HCl and 1 mM-EDTA at pH 7.5) was carried out at 4°C for 5 h with at least six changes of buffer.

Assay of phosphofructokinase

The assay medium consisted of 50 mM-Tris-HCl, 6.7 mM-MgCl₂, 0.13 mM-NADH and $5\text{ }\mu\text{g}$ of 2-heptyl-4-hydroxyquinoline *N*-oxide (an inhibitor of mitochondrial NADH oxidation). Dialysed glycerol 1-phosphate dehydrogenase–triose phosphate isomerase (not less than $10\text{ }\mu\text{g}$) and aldolase (not less than $100\text{ }\mu\text{g}$) were added to each cuvette. Suitable volumes (for details, see the Results section) of ATP (neutralized with $NaHCO_3$) and fructose 6-phosphate (Na^+ salt) were added. In addition, suitable volumes of possible effectors and suitable volumes of homogenates ($1\text{--}10\text{ }\mu\text{l}$) were added (for details, see the Results section). All cations were added as their Cl^- salts, and all anions were added as their Na^+ salts. At the concentrations used, these counter-ions did not affect the activity of phosphofructokinase. P_i was added as a mixture of Na_2HPO_4 and NaH_2PO_4 .

(solutions of 200mM- Na_2HPO_4 and 200mM- NaH_2PO_4 were mixed to give a final pH of 7.5). The final volume in the cuvette was 2ml and the final pH was 7.5. The reaction was usually initiated by the addition of fructose 6-phosphate, homogenate or occasionally K^+ (for details of individual assays, see the Results section).

In some assays of phosphofructokinase from lobster abdominal muscle, fructose 6-phosphate concentrations were 'buffered' by the addition of glucose 6-phosphate (Na^+ salt) and phosphoglucose isomerase (not less than 10 μg) to the assay medium. Hexose phosphates were added as a mixture of glucose 6-phosphate and fructose 6-phosphate with concentrations at the equilibrium ratio of 3.3:1 respectively (Kahana *et al.*, 1960). The reaction was initiated by the addition of homogenate (10 μl) after preincubation of the assay medium to allow equilibration of hexose 6-phosphates. In all assays the rate of change of extinction was followed at 340 nm in a Gilford recording spectrophotometer (model 240) at 25°C and the rate of change of extinction was linear for at least 10 min. Blank assays were run concurrently with test assays by omitting fructose 6-phosphate. In assays of phosphofructokinase in lobster muscle homogenates NADH oxidation in the blank cuvettes was negligible. In other assays NADH oxidation in the blank cuvette was never more than 5% of that in the test cuvette.

To show that a modification in the rate of NADH oxidation was due to effects on phosphofructokinase rather than effects on the coupling enzymes, the activity was assayed by measuring fructose diphosphate formation in a sampling assay. Lobster abdominal-muscle homogenate was incubated at 25°C with 50mM-Tris-HCl, 1mM-ATP (neutralized with NaHCO_3) and 2mM-fructose 6-phosphate. Suitable volumes of various solutions were also added (for details see the Results section). The final pH was 7.5 and the final volume 2ml. Duplicate samples (200 μl) were removed at various intervals (0–5 min) and added to a solution of 10% (v/v) HClO_4 to terminate the reaction. The solution was neutralized by the addition of 3M- KHCO_3 . Fructose diphosphate was measured by the method of Bücher & Hohorst (1965) in a Gilford recording spectrophotometer (model 240). The experiment was repeated twice with identical results.

Expression of results

All phosphofructokinase activities are expressed in terms of μmol of product formed/min per g fresh wt. of tissue at 25°C. In each experiment, the results are representative of at least three similar experiments. The biological variation in the activity of the enzyme from the same tissue, but from different individual animals, at specific concentrations of ATP, fructose

6-phosphate and effector ions was less than 15% in all results reported in this paper. Results of representative experiments are reported rather than mean values of activities from several experiments with ranges or S.E.M., since it was considered that the former method of presentation would produce more comprehensible tables. Further, it is not the purpose of this paper to deduce precise quantitative correlations but rather to demonstrate the possibility that phosphofructokinase is regulated by effectors in addition to its substrates, fructose diphosphate, AMP or citrate.

Results

Effects of anions and cations

Preliminary experiments established that the activation of phosphofructokinase by NH_4^+ was greater in the presence of P_i (see also Table 3). Since it is known that P_i is present in the cell at millimolar concentrations (see, e.g., Lowry *et al.*, 1964; Newsholme & Randle, 1964; Regen *et al.*, 1964), and since the work was directed towards an understanding of the physiological importance of properties of the enzyme, the effects of NH_4^+ were usually studied in the presence of P_i . A study of the effects of NH_4^+ plus P_i and K^+ on the activities of phosphofructokinase from nervous tissue and muscle from different animals was carried out at non-inhibitory concentrations of ATP and the results are shown in Table 1. In the absence of NH_4^+ plus P_i , the enzyme from both invertebrate and vertebrate tissues was activated by K^+ , but the ion had a much smaller effect on phosphofructokinase from invertebrate tissues (Table 1). In the absence of K^+ , the enzyme from both vertebrate and invertebrate tissues was markedly activated by NH_4^+ plus P_i . At physiological intracellular concentrations of K^+ [i.e. 50–100mM (see Steinbach, 1962)] NH_4^+ plus P_i activated phosphofructokinase from invertebrate tissues and from nervous tissues of vertebrates, but the enzyme from vertebrate muscle was much less affected by these ions (Table 1).

High concentrations of K^+ inhibited the enzyme from lobster abdominal muscle but not that from rat heart (see Table 2). An inhibitory effect was also seen in invertebrate tissues with high concentrations of Na^+ or K^+ (approx. 100mM) in the presence of 1mM- NH_4^+ and 0.5mM- P_i (results not shown). The inhibition may be due to changes in ionic strength, since it was greater when the homogenate was preincubated for 5 min in the cuvette with K^+ and the reaction was initiated by the addition of fructose 6-phosphate (e.g. compare results for lobster abdominal muscle in Tables 1 and 2). Such a trend was also seen with phosphofructokinase from other invertebrate muscles or nervous tissue (results not shown). When the reaction was initiated by the addition of K^+ , activities

Table 1. *Effects of K^+ , NH_4^+ and P_i on the activities of phosphofructokinase from vertebrate and invertebrate tissues*
 For details of assay, see the Materials and Methods section. ATP and fructose 6-phosphate concentrations were 1 mM. Reactions were initiated by addition of homogenate or fructose 6-phosphate.

		Phosphofructokinase activities (μ mol of substrate transformed/min per g of fresh tissue)									
		No added K^+			50 mM- K^+			100 mM- K^+			
		No added NH_4^+ or P_i	1 mM- NH_4^+ + 0.5 mM- P_i	2 mM- NH_4^+ + 1 mM- P_i	No added NH_4^+ or P_i	1 mM- NH_4^+ + 0.5 mM- P_i	2 mM- NH_4^+ + 1 mM- P_i	No added NH_4^+ or P_i	1 mM- NH_4^+ + 0.5 mM- P_i	2 mM- NH_4^+ + 1 mM- P_i	2 mM- NH_4^+ + 1 mM- P_i
Nematoda											
Pig roundworm (<i>Ascaris lumbricoides</i>)	Body wall muscles	0.2	7.2	11.3	0.6	—	—	0.6	—	—	—
Mollusca											
Scallop (<i>Pecten maximus</i>)	Striated 'fast' muscle	0.3	7.4	7.0	1.8	7.3	7.4	3.6	5.8	—	6.4
Periwinkle (<i>Littorina littorea</i>)	Pedal retractor muscle	<0.1	2.4	2.6	0.4	—	—	0.5	—	—	—
Crustacea											
Lobster (<i>Homarus vulgaris</i>)	Cerebral ganglion	<0.1	4.1	6.3	0.2	—	—	0.2	—	—	—
	Abdominal muscle	0.7	9.1	11.9	0.9	8.6	12.9	1.0	6.6	7.9	—
Insecta											
Locust (<i>Schistocerca gregaria</i>)	Cerebral ganglion	<0.1	4.5	4.9	0.8	2.4	2.9	0.6	1.1	0.9	—
	Flight muscle	0.1	19.9	21.4	4.1	16.6	19.7	2.2	18.4	18.1	—
	Leg muscle	0.9	7.6	8.2	3.6	5.9	6.1	3.6	4.6	5.3	—
	Flight muscle	<0.1	9.1	24.8	1.6	—	—	1.9	—	—	—
Rosehafer (<i>Pachnoda ephippiata</i>)	Flight muscle	0.5	8.5	11.0	0.6	—	—	2.7	—	—	—
Poplar-hawk moth (<i>Laothae populi</i>)	Flight muscle	<0.1	6.6	34.2	0.5	—	—	1.9	—	—	—
Silver-Y moth (<i>Plusia gamma</i>)	Flight muscle	0.8	25.4	42.3	4.4	—	—	7.7	—	—	—
Yellow-underwing moth (<i>Noctua pronuba</i>)	Flight muscle	1.9	30.9	40.2	8.4	—	—	7.1	—	—	—
Blowfly (<i>Calliphora erythrocephala</i>)	Flight muscle	8.7	90.0	103.3	36.7	—	—	36.3	—	—	—
Bumble-bee (<i>Bombus terrestris</i>)	Flight muscle	0.5	3.8	8.9	0.5	—	—	0.3	—	—	—
Cockroach (<i>Blaberus discoidalis</i>)	Flight muscle	0.7	21.4	23.6	19.5	37.3	36.3	24.0	36.8	36.3	—
Pisces											
Rainbow trout (<i>Salmo gairdneri</i>)	White muscle	1.4	30.3	29.3	25.7	30.1	29.3	27.2	29.3	27.8	—
Amphibia											
Frog (<i>Rana temporaria</i>)	Gastrocnemius muscle	<0.1	2.0	2.6	1.8	1.9	2.2	1.0	2.2	2.3	—
Aves											
Domestic pigeon (<i>Columba livia</i>)	Pectoral muscle	3.2	54.0	55.2	56.9	65.6	61.7	65.6	70.7	66.9	—
	Heart muscle	0.8	13.3	12.9	11.7	14.0	14.0	14.4	15.6	15.6	—
	Whole brain	0.2	8.7	9.6	2.3	9.1	10.8	2.4	9.3	11.0	—
Mammalia											
Laboratory rat	Heart	<0.1	9.1	8.8	8.0	9.9	9.6	8.7	10.2	9.8	—
	Whole brain	<0.1	8.9	11.5	3.9	11.9	14.2	4.2	11.5	16.5	—
Laboratory mouse	Gastrocnemius muscle	3.2	40.1	39.8	46.3	50.2	45.7	50.8	54.0	50.8	—
	Heart	0.5	7.5	7.8	9.9	10.8	10.4	9.0	9.4	9.8	—
	Whole brain	0.5	8.2	9.1	5.3	9.6	10.7	5.8	9.2	9.8	—

Table 2. *Effect of K^+ on the activities of lobster muscle and rat heart phosphofructokinases*

The reaction was initiated by the addition of K^+ . ATP and fructose 6-phosphate concentrations were both 1 mM. The maximum activities of phosphofructokinase from lobster muscle and rat heart were 18.5 and 6.4 $\mu\text{mol/min per g}$ fresh wt. respectively. (Maximum activity of phosphofructokinase is taken as the activity observed in the presence of 5 mM- NH_4^+ , 2.5 mM- P_i and in the absence of K^+ .) For details of assay, see the Materials and Methods section. Activities are reported as $\mu\text{mol/min per g}$ fresh wt. of muscle.

K^+ concn. (mM)	Lobster muscle phosphofructokinase activities	Activity (% of maximum)	Rat heart phosphofructokinase activities	Activity (% of maximum)
0	1.2	6	0.9	9
0.5	2.1	11	1.1	13
1	3.1	17	1.3	15
2.5	4.8	26	1.8	24
5	7.1	38	2.9	42
10	9.1	49	3.7	55
25	10.8	58	5.0	76
50	10.7	58	5.6	92
75	6.0	33	6.3	97
100	5.3	29	6.4	100
150	3.5	19	6.2	96
200	2.1	12	6.4	100

Table 3. *Effects of NH_4^+ on the activities of lobster muscle phosphofructokinase at different concentrations of P_i and K^+*

The reaction was initiated by the addition of homogenate. ATP and fructose 6-phosphate concentrations were 1 mM. For details of assay, see the Materials and Methods section.

NH_4^+ concn. (mM)	K^+ concn. (mM)	$[\text{P}_i]$ (mM) ...	Phosphofructokinase activities				
			0	0.1	0.25	0.5	2.5
0	0		<0.1	0.42	0.79	1.15	1.19
0.1	0		1.3	2.4	5.8	8.0	9.6
0.5	0		1.4	2.3	8.0	9.6	11.4
1.0	0		5.4	5.4	9.5	13.4	13.2
5.0	0		6.7	7.1	11.7	13.7	12.4
0	100		0.32	0.59	1.17	4.5	5.8
0.1	100		1.1	2.0	6.4	10.2	15.9
0.5	100		0.60	1.2	7.0	7.4	9.7
1.0	100		2.0	2.3	6.2	11.6	10.5
5.0	100		3.5	4.2	8.7	10.7	10.9

of the enzyme from invertebrate muscle were never maximum (although greater than in experiments where extracts were preincubated with K^+ , see Table 1), whereas those from vertebrate muscle were maximum (Table 2).

Other alkali-metal ions were tested for their ability to activate phosphofructokinase from lobster muscle or rat heart. Only Rb^+ was observed to have any effect: it was 60–70% as effective as K^+ (results not shown). This ion has a similar ionic radius to K^+ and NH_4^+ (Pauling, 1960).

Effects of NH_4^+ , P_i and other compounds on phosphofructokinase from lobster abdominal muscle

The activation of phosphofructokinase from invertebrate tissues by NH_4^+ plus P_i (in the absence of K^+) was investigated in more detail. Lobster abdominal muscle was chosen as the source of enzyme for this study, since large amounts of muscle could be obtained from a single animal, the tissue could be stored without loss of enzyme activity and the enzyme was found to be reasonably stable in extracts of this

Table 4. *Effects of ATP, AMP and NH_4^+ plus P_i on the activities of lobster muscle phosphofructokinase*

For details of assay, see the Materials and Methods section. The ratio activity in the absence of AMP/activity in the presence of AMP provides an indication of the extent of ATP inhibition of phosphofructokinase. Ratios approaching unity indicate little or no inhibition by ATP, whereas very small ratios indicate marked ATP inhibition. In Expt. A the fructose 6-phosphate concentration is 1 mM, in Expt. B the fructose 6-phosphate concentration is 0.1 mM. Fructose 6-phosphate concentrations were buffered by the addition of glucose 6-phosphate and phosphoglucose isomerase.

Phosphofructokinase activities 5 mM- NH_4^+ + 2.5 mM- P_i				Phosphofructokinase activities 0.5 mM- NH_4^+ + 0.25 mM- P_i			
ATP concn. (mM)	No AMP added	2 mM- AMP	Activity in absence of AMP Activity in presence of AMP	No AMP added	2 mM- AMP	Activity in absence of AMP Activity in presence of AMP	Activity in absence of AMP Activity in presence of AMP
Expt. A							
0.5	18.8	18.8	1.00	13.6	14.4	0.95	
1	18.0	17.8	1.01	14.2	13.6	1.04	
2	19.8	21.4	0.93	13.9	16.2	0.86	
3	15.7	17.0	0.92	15.5	16.5	0.94	
4	15.7	17.0	0.92	—	—	—	
5	16.2	17.5	0.93	13.4	15.7	0.85	
7.5	22.9	24.4	0.94	2.6	18.0	0.14	
10	14.9	24.2	0.62	0.71	14.7	0.05	
12.5	3.4	22.4	0.15	0.45	10.6	0.04	
15	2.5	20.6	0.12	0.32	8.2	0.04	
Expt. B							
0.2	4.3	13.9	0.31	—	—	—	
0.5	5.0	17.6	0.28	0.84	13.4	0.06	
1	5.0	20.1	0.25	0.39	12.8	0.03	
2	5.9	20.1	0.29	<0.1	13.9	<0.01	
3	3.4	17.5	0.20	0.45	13.2	0.03	
4	1.9	17.5	0.11	—	—	—	
5	1.3	17.0	0.08	—	—	—	
7.5	0.45	15.7	0.03	—	—	—	
10	0.32	4.4	0.07	—	—	—	

muscle. It is suggested that the results obtained with the enzyme from this tissue are representative of other invertebrate muscles and nervous tissue.

The activation of phosphofructokinase by NH_4^+ was increased by the presence of P_i (Table 3). In the absence of P_i , 5 mM- NH_4^+ produced about 50% of the maximum activity (approximately a 50-fold activation). However, in the presence of P_i (0.5 or 2.5 mM), low concentrations of NH_4^+ (e.g. 0.1 mM) produced a marked activation of phosphofructokinase (Table 4). Choline, acetylcholine or L(-)-carnitine (at 2 mM) did not activate the enzyme (in the presence of 1 mM- P_i). In the absence of NH_4^+ , P_i produced very little activation (less than 10% maximum value at 2.5 mM- P_i). However, SO_4^{2-} could replace P_i , so that in some experiments $(\text{NH}_4)_2\text{SO}_4$ was used to study the activation of phosphofructokinase by NH_4^+ .

From a variety of experiments, no evidence was obtained to support the possibility that NH_4^+ activated phosphofructokinase by the stimulation of a covalent modification of the enzyme. For example, preincubation of the muscle homogenate with $(\text{NH}_4)_2\text{SO}_4$ (5 mM) followed by assay of phosphofructokinase in a small volume of homogenate

(<5 μl) did not increase the activity above that predicted by 'carry-over' of $(\text{NH}_4)_2\text{SO}_4$ in the sample of homogenate. Similarly, a short period of dialysis of the homogenate, which had been previously activated by the addition of $(\text{NH}_4)_2\text{SO}_4$, removed activation completely.

It was established that this observed effect of NH_4^+ plus P_i on phosphofructokinase was not due to activation of the coupling enzymes, since the effect was demonstrated in a sampling assay for phosphofructokinase [see Newsholme *et al.* (1970) for problems associated with coupled enzyme assays for phosphofructokinase].

Effects of K^+ and NH_4^+ plus P_i on the response of phosphofructokinase to changes in the concentrations of fructose 6-phosphate, ATP and AMP

Relatively simple kinetic experiments were undertaken to investigate the effects of NH_4^+ plus P_i on phosphofructokinase in homogenates of lobster muscle.

At low concentrations of NH_4^+ plus P_i , the plot of phosphofructokinase activity from lobster abdominal muscle against fructose 6-phosphate concentration

Fig. 1. Hofstee (1959) plots (i.e. activity is plotted against activity/fructose 6-phosphate concentration) of phosphofructokinase activity from lobster muscle at two different concentrations of NH_4^+ plus P_i

Fructose 6-phosphate concentrations were buffered by the addition of glucose 6-phosphate and phosphoglucose isomerase. The concentration of ATP was 1 mM in both experiments. The activity was assayed as described in the Materials and Methods section. $\circ$, NH_4^+ was 1 mM and P_i was 0.5 mM; $\bullet$, NH_4^+ was 10 mM and P_i was 5.0 mM.

Fig. 2. Plot of the activity of lobster muscle phosphofructokinase against the concentration of ATP in the presence and absence of AMP

The concentrations of fructose 6-phosphate was 1 mM (buffered at this concentration by the addition of glucose 6-phosphate and phosphoglucose isomerase), NH_4^+ was 0.5 mM and P_i was 0.25 mM. The activity was assayed as described in the Materials and Methods section. $\circ$, No added AMP; $\bullet$, 2 mM-AMP was present.

is sigmoid. At higher concentrations of NH_4^+ plus P_i , the plot is shifted to the left and sigmoidicity is decreased (plots not shown). These results suggest that NH_4^+ plus P_i can increase phosphofructokinase activity by relieving ATP inhibition. If the data are plotted in the form of a Hofstee (1959) plot, concave-downwards curves are obtained (Fig. 1). Concave-downwards Hofstee (1959) plots are analogous to concave-downwards Scatchard equilibrium binding plots (Scatchard, 1949) and indicate positive co-operative interactions (see Dalziel, 1973). At the higher concentration of NH_4^+ plus P_i , the concave-downwards nature of the Hofstee (1959) plot is decreased, indicating an activation of the enzyme by NH_4^+ plus P_i . The 'apparent K_m ' of phosphofructokinase for fructose 6-phosphate (calculated from the straight-line negative-slope portions of Hofstee plots) is decreased from approximately 0.09 to 0.01 mM by increasing the NH_4^+ plus P_i concentration. There is no evidence that NH_4^+ plus P_i increases the maximum activity of phosphofructokinase, since the intercepts on the ordinate are similar at both concentrations of these ions.

The suggestion that NH_4^+ plus P_i can relieve the ATP inhibition of phosphofructokinase is supported by the results reported in Table 4. The activity of lobster muscle phosphofructokinase is measured at increasing ATP concentrations and the effect of AMP is shown at two concentrations of NH_4^+ plus P_i and two concentrations of fructose 6-phosphate. The enzyme is inhibited by ATP: there is an optimum ATP concentration above which the enzyme is inhibited (Table 4 and Fig. 2). Inhibition by ATP occurs at a higher concentration of ATP when AMP (2 mM) is present and/or when the concentration of NH_4^+ plus P_i is increased (Table 4). At 1 mM-fructose 6-phosphate, 0.5 mM- NH_4^+ and 0.25 mM- P_i , ATP inhibition is observed at about 5 mM-ATP, whereas, at 5 mM- NH_4^+ and 2.5 mM- P_i , inhibition is observed at 10 mM-ATP (in the absence of AMP). AMP (2 mM) relieves ATP inhibition, but inhibition can be observed again at increased ATP concentrations. At 0.1 mM-fructose 6-phosphate, inhibition is observed at all concentrations of ATP tested as demonstrated by the ratio of activity in the absence of AMP/activity in the presence of AMP, but the ratio of activities (in the absence of AMP/in the presence of AMP) is very much smaller at the lower concentration of NH_4^+ plus P_i , and is also much smaller than at 1 mM-fructose 6-phosphate (Table 4). Further, it was established that, although NH_4^+ plus P_i both relieves the ATP inhibition and activates the enzyme, AMP only relieves the ATP inhibition; it does not activate the enzyme at non-inhibitory ATP concentrations (Table 5). Finally it was established that, at inhibitory concentrations of ATP, K^+ did not relieve ATP inhibition of phosphofructokinase from vertebrate heart muscle, whereas inhibition could be

Table 5. *Effects of AMP and NH_4^+ plus P_i on the activities of phosphofructokinase from lobster muscle, rat heart and brain*

ATP and fructose 6-phosphate concentrations were 1 mM. The reaction was initiated by the addition of fructose 6-phosphate. For details of assay, see the Materials and Methods section.

Additions	Phosphofructokinase activities		
	Lobster muscle	Rat heart	Rat brain
None	0.5	1.2	0.1
1 mM- NH_4^+ plus 0.5 mM- P_i	12.1	8.7	11.6
2 mM-AMP	1.2	1.5	0.77
1 mM- NH_4^+ plus 0.5 mM- P_i and 2 mM-AMP	12.6	9.5	13.4

Table 6. *Effects of K^+ on the inhibition of phosphofructokinase from pigeon heart by ATP*

Fructose 6-phosphate (with which the reaction was initiated) was present at a concentration of 1 mM. For details of assay, see the Materials and Methods section.

NH_4^+ concn. (mM)	P_i concn. (mM)	ATP concn. (mM)	K^+ concn. (mM)	AMP concn. (mM)	Phosphofructokinase activity
1	0.5	1	—	—	13.3
1	0.5	7.5	—	—	0.5
1	0.5	7.5	50	—	0.5
1	0.5	7.5	50	2	4.7
6	3	7.5	50	2	10.6

relieved by both AMP and by NH_4^+ plus P_i (see Table 6). It should be noted that an increase in NH_4^+ plus P_i concentration increases phosphofructokinase activity in the presence of an inhibitory concentration of ATP and an optimum concentration of K^+ (see Tables 1 and 6): i.e. these ions relieve ATP inhibition.

Discussion

At non-inhibitory concentrations of ATP, NH_4^+ plus P_i caused a marked activation of phosphofructokinase from muscle and nervous tissue of all species examined. In the presence of physiological concentrations of K^+ , and non-inhibitory ATP concentration, NH_4^+ plus P_i activated phosphofructokinase from invertebrate muscle and nervous tissue and from vertebrate nervous tissue.

The properties of phosphofructokinase from lobster abdominal muscle were studied in detail. This study showed that invertebrate muscle phosphofructokinase is similar to the enzyme from many other tissues; it is inhibited above an optimum concentration of ATP and this inhibition is relieved by AMP, fructose 6-phosphate, and NH_4^+ plus P_i . Thus NH_4^+ plus P_i can relieve ATP inhibition of both invertebrate muscle (lobster) phosphofructokinase and pigeon heart phosphofructokinase (Table 6). Since it is generally accepted that in most resting muscles phosphofructokinase activity is inhibited by the concentration of ATP in the muscle, this effect of NH_4^+ plus P_i may be more important, quantitatively,

than the activation effect at non-inhibitory ATP concentrations.

The results reported in this paper suggest that changes in the intracellular concentration of NH_4^+ plus P_i could modify the activity of phosphofructokinase in muscle or nervous tissue from many different animals, and therefore such changes could be important in the regulation of glycolysis. A problem in the interpretation of the effects of NH_4^+ in relation to the control of phosphofructokinase activity in the intact cell has been whether the effects are significant at physiological concentrations of K^+ . This work has shown that in both muscle and nervous tissues of the animals investigated, an increase in the intracellular concentration of NH_4^+ plus P_i could lead to an increase in the activity of phosphofructokinase: at non-inhibitory concentrations of ATP and physiological concentrations of K^+ , NH_4^+ plus P_i activated phosphofructokinase from invertebrate muscle and nervous tissue and from vertebrate brain; at inhibitory concentrations of ATP and physiological concentrations of K^+ , NH_4^+ plus P_i activated phosphofructokinase from the vertebrate and invertebrate muscles that were studied.

The concentration of NH_4^+ is increased in certain tissues, particularly 'white' anaerobic muscles and nervous tissues, under conditions that cause an increase in glycolytic flux. In the rat leg and locust leg muscles (which are more anaerobic than aerobic) the concentration of NH_4^+ is increased from 0.28 to 0.98 mM during a tetanic stimulation (rat leg), and

from 0.28 to 1.8 mM during jumping (locust leg) (Gerez & Kirsten, 1965). However, in pigeon pectoral, locust and blowfly flight muscles there is no significant change in the NH_4^+ concentration during mechanical activity: these are aerobic muscles (Gerez & Kirsten, 1965; Sacktor & Wormser-Shavit, 1966). The NH_4^+ concentration in mammalian brain is increased during conditions that increase glycolytic flux: the NH_4^+ concentration in 'normal' mammalian brain is reported to be approximately 0.2–0.3 mM (Weil-Malherbe, 1962), and it may be increased to 5 mM by prolonged ischaemia. The concentration of NH_4^+ is also increased during convulsions (Richter & Dawson, 1948; Folbergrova *et al.*, 1969). The concentrations of P_i in both muscle and nervous tissue increase under conditions when the glycolytic flux is stimulated [e.g. increased contractile activity, increased ion transport, anoxia (see Karparkin *et al.*, 1964; Lowry *et al.*, 1964; Regen *et al.*, 1964; Helmreich & Cori, 1965; Rolleston & Newsholme, 1967; Opie *et al.*, 1971)]. The results of the present work raise the possibility that the increases in the intracellular concentrations of NH_4^+ and P_i in some muscle and nervous tissues may play some part in the stimulation of glycolysis through the changes in the activity of phosphofructokinase by the mechanisms discussed above (i.e. activation and/or de-inhibition).

Since the increases in concentration of NH_4^+ occur in the more anaerobic muscles during mechanical activity, it is possible that small changes in the concentrations of NH_4^+ plus P_i could have marked effects on the rate of glycolysis, through small changes in phosphofructokinase activity affecting the rate of the substrate cycle between fructose 6-phosphate and fructose diphosphate (which is catalysed by the simultaneous activities of phosphofructokinase and fructose diphosphatase) in these muscles (see Newsholme & Crabtree, 1973).

The importance of NH_4^+ plus P_i in affecting the activity of phosphofructokinase from the aerobic type of muscle (e.g. heart muscle) is not known. It is possible that the concentration of NH_4^+ decreases when substrates other than glucose are used by the muscle so that the inhibition of phosphofructokinase that occurs under these conditions may be due both to an increase in the concentration of citrate (Newsholme & Randle, 1964; Garland & Randle, 1964), and a decrease in the concentration of NH_4^+ .

The possible importance of changes in concentrations of NH_4^+ plus P_i regulating the rate of glycolysis in the 'anaerobic' muscles and nervous tissue raises the question of the source of the NH_4^+ plus P_i in these tissues. The increase in P_i concentration is probably the result of increased ATP hydrolysis and creatine phosphate degradation (see Newsholme, 1970). This increased hydrolysis of ATP will lower the concentration of the latter and raise that of AMP, via the action of adenylate kinase (see Newsholme, 1970).

AMP is probably the major source of NH_4^+ in muscle (Needham, 1971), and it may also be important in nervous tissue (see Muntz, 1953b). The enzyme responsible for the production of NH_4^+ is probably AMP deaminase. It is noteworthy that the activity of AMP deaminase is very high in the anaerobic type of muscle and in nervous tissue, but it is low in the aerobic type of muscle (Lowenstein, 1972; Leech, 1973). It is suggested that under conditions of increased ATP utilization in muscle or nervous tissue, the activity of AMP deaminase is increased owing to changes in the adenine nucleotide concentrations (Ronca-Testoni *et al.*, 1970). Thus AMP will be converted into NH_4^+ which, in combination with increased concentration of P_i , will activate phosphofructokinase and stimulate glycolysis. However, the problem arises that one activator of phosphofructokinase (AMP) is converted into another (NH_4^+), which would seem to have little advantage in metabolic control. The magnitude of the change in phosphofructokinase activity will depend on the magnitude of the change in concentration of the regulator, AMP, and because of the reversibility of the adenylate kinase reaction the latter change is limited (see Newsholme & Start, 1973a). The conversion of AMP into NH_4^+ and IMP is irreversible (see Dydynska & Wilkie, 1966; Sugden, 1973), so that the change in NH_4^+ concentration is not necessarily proportional to that of AMP. Indeed the increases in NH_4^+ concentration that have been reported in muscle and brain (see above) are greater than the increases that have been reported in the concentrations of AMP. It is suggested that the increase in NH_4^+ concentration in anaerobic muscle or nervous tissue under conditions of increased ATP utilization is much greater than the change in AMP concentration, and this is the physiological significance of the conversion of AMP into NH_4^+ and the effects of the NH_4^+ plus P_i on phosphofructokinase in these tissues, since this causes a large increase in the rate of glycolysis.

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