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Properties of Pyruvate Kinase and Phosphoenolpyruvate Carboxykinase in Relation to the Direction and Regulation of Phosphoenolpyruvate Metabolism in Muscles of the Frog and Marine Invertebrates

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1. The properties of pyruvate kinase and, if present, phosphoenolpyruvate carboxykinase from the muscles of the sea anemone, scallop, oyster, crab, lobster and frog were investigated. 2. In general, the properties of pyruvate kinase from all muscles were similar, except for those of the enzyme from the oyster (adductor muscle); the pH optima were between 7.1 and 7.4, whereas that for oyster was 8.2; fructose bisphosphate lowered the optimum pH of the oyster enzyme from 8.2 to 7.1, but it had no effect on the enzymes from other muscles. Hill coefficients for the effect of the concentration of phosphoenolpyruvate were close to unity in the absence of added alanine for the enzymes from all muscles except oyster adductor muscle; it was 1.5 for this enzyme. Alanine inhibited the enzyme from all muscles except the frog; this inhibition was relieved by fructose bisphosphate. Low concentrations of alanine were very effective with the enzyme from the oyster (50% inhibition was observed at 0.4mM). Fructose bisphosphate activated the enzyme from all muscles, but extremely low concentrations were effective with the oyster enzyme (0.13μM produced 50% activation). 3. In general, the properties of phosphoenolpyruvate carboxykinase from the sea anemone and oyster muscles are similar: the K_m values for phosphoenolpyruvate are low (0.10 and 0.13mM); the enzymes require Mn^{2+} in addition to Mg^{2+} for activity; and ITP inhibits the enzymes and the inhibition is relieved by alanine. These latter compounds had no effect on enzymes from other muscles. 4. It is suggested that changes in concentrations of fructose bisphosphate, alanine and ITP produce a co-ordinated mechanism of control of the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase in the sea anemone and oyster muscles, which ensures that phosphoenolpyruvate is converted into oxaloacetate and then into succinate in these muscles under anaerobic conditions. 5. It is suggested that in the muscles of the crab, lobster and frog, phosphoenolpyruvate carboxykinase catalyses the conversion of oxaloacetate into phosphoenolpyruvate. This may be part of a pathway for the oxidation of some amino acids in these muscles.

In muscles of marine invertebrates that are able to withstand sustained periods of anaerobiosis, the maximal activities of pyruvate kinase are severalfold greater than those of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) [e.g. in phasic and catch adductor muscles of the oyster (*Ostrea*), phosphoenolpyruvate carboxykinase activities are 1.5 and 0.2 μmol/min per g of muscle and pyruvate kinase activities are 4.2 and 1.1 μmol/min per g of muscle respectively]. However, in other muscles, pyruvate kinase activities are approximately 100-fold greater than those of phosphoenolpyruvate carboxykinase [e.g., in the abdominal flexor and the claw adductor muscles of the lobster, phosphoenolpyruvate carboxykinase activities are 1.3 and 0.6 μmol/min per g and pyruvate kinase activities are 149 and 59.8 μmol/min per g respectively; compare results reported by Zammit & Newsholme (1976) with those by Zammit *et al.* (1978)].

In the muscles of the marine bivalves *Crassostrea* (oyster) and *Mytilus* (mussel) the role of phosphoenolpyruvate carboxykinase is considered to be the conversion of phosphoenolpyruvate into oxaloacetate, a reaction that initiates the formation of succinate and other end products of anaerobic carbohydrate metabolism [(for reviews, see de Zwaan *et al.* (1976) and Hochachka (1976)]. The advantage of this pathway, in comparison with the formation of lactate, is that an additional molecule of ATP is regenerated per molecule of glucose or glucose residue utilized. The properties of phosphoenolpyruvate carboxykinase and pyruvate kinase from adductor muscle of *Crassostrea* and of pyruvate

kinase from the adductor muscles of *Mytilus* (Mustafa & Hochachka, 1971, 1973*a*,*b*; de Zwaan, 1972) provide the basis for a theory of control of the direction of phosphoenolpyruvate metabolism in these muscles (see Hochachka & Somero, 1973). It is suggested that, under aerobic conditions, the high concentration of ITP inhibits the activity of phosphoenolpyruvate carboxykinase so that phosphoenolpyruvate is converted into pyruvate which can be subsequently oxidized [see Alp *et al.* (1976) for activities of the citric acid-cycle enzymes in these muscles]. Under anaerobic conditions, pyruvate cannot be oxidized so that it is converted into alanine via the pyruvate–glutamate transaminase reaction [the activity of lactate dehydrogenase is low in these muscles (Zammit & Newsholme, 1976)]. The resultant increase in concentration of alanine inhibits the activity of pyruvate kinase and relieves the ITP inhibition of phosphoenolpyruvate carboxykinase. In addition, the decrease in intracellular pH due to the accumulation initially of CO_2 and subsequently of acidic end products of anaerobic metabolism should also inhibit pyruvate kinase activity. Consequently, glycolytic residues are directed into the succinate pathway.

Since the above theory is based on the properties of the enzymes from only one muscle, the oyster adductor muscle (the data from *Mytilus* are only for pyruvate kinase), it was considered important to investigate how far these properties are common to enzymes from other invertebrate muscles and to compare the properties of pyruvate kinase from the muscle of a facultative anaerobe with those of the enzyme from muscle of a bivalve mollusc that does not contain detectable activitities of phosphoenolpyruvate carboxykinase (scallop). In addition, the properties of both enzymes from the muscle of the common oyster (*Ostrea edulis*) were compared with those of the enzymes from muscles of animals representative of the coelenterates, the arthropods and the vertebrates in order to provide information on the role of phosphoenolpyruvate carboxykinase in all these muscles.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and all inorganic reagents were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Sources of animals

Animals were obtained from the sources given by Beis & Newsholme (1975) and Zammit & Newsholme (1976).

Preparation of homogenates

Homogenates for assay of pyruvate kinase activity were prepared as described by Zammit *et al.* (1978). The homogenates were fractionated with $(NH_4)_2SO_4$ (40–60% saturation; approx. 95% of the total activity was precipitated), and the precipitate was taken up in buffer and dialysed for 2h against excess extraction buffer. The medium for extraction of phosphoenolpyruvate carboxykinase was the same as that used for pyruvate kinase. For phosphoenolpyruvate carboxykinase assays, homogenates were used without further treatment.

Assay of enzyme activities

Pyruvate kinase was assayed as described by Zammit *et al.* (1978), and phosphoenolpyruvate carboxykinase was assayed as described by Zammit & Newsholme (1976). When the kinetic properties of the enzymes were studied, the concentrations of some of the components of the assay medium were varied (see the Tables and Figures for details). Kinetic studies were performed at the pH optimum of the individual enzyme being studied.

Expression of results

In Table 1 and some Figures, the activities of the enzymes are expressed as a fraction of the maximal activity obtained, to facilitate comparison between the properties of the enzymes from different muscles. In order that the original activities can be compared, the activity in the control condition (in μmol/min per g of fresh muscle) is given in the legend.

Results and Discussion

Properties of pyruvate kinase

pH optima. The effects of pH on the activity of pyruvate kinase from the basilar muscle of the sea anemone, the phasic adductor muscles of the scallop and the oyster, the leg muscle of the horse-shoe crab, the abdominal muscle of the lobster and the sartorius muscle of the frog were studied in the absence of effectors such as fructose bisphosphate and alanine. Under these conditions, the pH optima of pyruvate kinase from all muscles, except for the oyster, fall within the narrow range, pH7.1–7.4 (see Table 1). The optimal pH for the enzyme from oyster adductor muscle is 8.2; the activity at pH7.1 is only about 10% of that at pH8.2. This pH optimum is similar to that for the enzyme from the adductor muscle and mantle tissue of another oyster *Crassostrea virginica* (Mustafa & Hochachka, 1971). The high pH optimum for the enzyme from the oyster is decreased to pH7.1 by addition of fructose bisphosphate (Table 1); this compound did not have any effect on the pH optimum of the enzymes from any of the other muscles studied (including the scallop, see

Table 1. *Effect of pH on the maximal activities of phosphoenolpyruvate carboxykinase and pyruvate kinase from muscles of invertebrates and the frog*
Acitivities are expressed on an arbitrary scale, a value of 1.0 being assigned to the activity at the pH optimum. Systematic names are given in Table 2. The effect of fructose bisphosphate (0.2mM) on response to pH is shown for the enzymes from scallop and oyster adductor muscles; it had no effect on pH optima of the enzyme from muscles of other animals. The results presented are representative of three similar experiments using extracts from different animals. The mean activities at the optimal pH values were as follows: pyruvate kinase activities were 8.1, 72.1, 4.8, 141, 132 and 375 μmol/min per g of muscle for sea anemone, scallop, oyster, lobster, horse-shoe crab and frog respectively; phosphoenolpyruvate carboxykinase activities were 1.1, 1.4, 1.4, 1.7 and 1.2 μmol/min per g of muscle for sea anemone, oyster, lobster, horse-shoe crab and frog respectively.

	Enzyme activities (arbitrary units)												
	Sea anemone Basilar muscle		Scallop Phasic adductor muscle		Oyster Phasic adductor muscle			Lobster Abdominal muscle		Horse-shoe crab Leg muscle		Frog Sartorius muscle	
			Pyruvate kinase		Pyruvate kinase								
pH	Pyruvate kinase	Phosphoenol-pyruvate carboxy-kinase	No added fructose bisphos-phate	0.2mM-Fructose bisphos-phate	No added fructose bisphos-phate	0.2mM-Fructose bisphos-phate	Phosphoenol-pyruvate carboxy-kinase	Pyruvate kinase	Phosphoenol-pyruvate carboxy-kinase	Pyruvate kinase	Phosphoenol-pyruvate carboxy-kinase	Pyruvate kinase	Phosphoenol-pyruvate carboxy-kinase
6.6	—	0.95	0.95	0.95	—	0.92	0.96	—	0.70	0.88	0.88	0.85	0.85
6.8	0.90	1.00	1.00	1.00	0.10	1.00	1.00	0.95	0.80	0.91	0.95	0.92	0.90
7.0	0.90	1.00	1.00	1.00	0.10	0.98	1.00	0.98	1.00	0.95	1.00	1.00	1.00
7.2	1.0	0.70	0.98	0.98	0.34	0.90	0.95	1.0	0.90	1.00	0.85	1.00	0.65
7.4	0.90	—	0.90	0.90	0.55	0.83	0.90	1.0	0.50	1.00	0.68	0.85	0.60
7.6	0.85	0.50	0.85	0.85	0.80	0.78	—	0.96	—	0.95	—	0.76	—
7.8	0.80	—	0.70	0.70	0.88	0.74	0.80	0.70	—	0.90	0.60	—	—
8.0	—	—	0.55	0.55	1.00	0.70	—	—	—	0.71	—	0.35	—
8.2	—	—	—	—	1.00	0.60	—	0.55	—	0.50	—	—	—
8.4	—	—	—	—	0.95	—	—	—	—	—	—	—	—

Table 2. *Maximal activities and K_m values for substrates of pyruvate kinase and phosphoenolpyruvate carboxykinase and concentrations of phosphoenolpyruvate, fructose bisphosphate and ADP in various muscles*

Hill coefficients (*h*) are given in parentheses for plots of pyruvate kinase activity against concentration of phosphoenolpyruvate under different conditions. Phosphoenolpyruvate carboxykinase activities were taken from Zammit & Newsholme (1976); pyruvate kinase activities were taken from the following paper (Zammit *et al.*, 1978). Concentrations of phosphoenolpyruvate, ADP and fructose bisphosphate were taken from Beis & Newsholme (1975).

		Enzyme activities (μmol/min per g of muscle)		K_m values (mM)						Concentrations in muscle (μmol/g fresh wt.)		
				Pyruvate kinase				Phosphoenolpyruvate-carboxykinase				
					Phosphoenolpyruvate							
Animal	Muscle	Phosphoenolpyruvate carboxykinase	Pyruvate kinase	ADP	No added fructose bisphosphate	0.2 mM-Fructose bisphosphate	2mM-Alanine	Phosphoenolpyruvate	IDP	Phosphoenolpyruvate	ADP	Fructose bisphosphate
Lobster (*Homarus vulgaris*)	Abdominal flexor	1.4	150	0.15	0.15 (1.4)	0.07 (1.0)	0.15 (3.40)	0.12	0.11	0.02	0.5	0.04
Horse-shoe crab (*Limulus polyplemus*)	Leg	1.6	145	0.56	0.06 (1.2)	0.03 (1.0)	0.60 (2.80)	6.6	0.06	—	—	—
Oyster (*Ostrea edulis*)	Phasic adductor	1.5	3.2	0.06	0.50 (1.5)	0.05 (0.95)	3.0 (2.75)	0.13	0.11	0.02	0.77	0.08
Scallop (*Pecten maximus*)	Phasic adductor	0.1	60	—	0.05 (1.2)	0.03 (0.95)	0.18 (2.40)	—	—	0.05	0.73	0.01
Sea anemone (*Metridium senile*)	Basilar	1.0	7.6	0.10	0.1 (1.2)	0.07 (1.04)	1.0 (2.42)	0.10	0.08	—	—	—
Frog (*Rana temporaria*)	Sartorius	1.8	407	0.24	0.05 (0.95)	0.05 (1.0)	0.05 (1.0)	1.0	0.08	0.03	0.50	0.04

Table 1). The difference in properties of oyster and scallop muscle pyruvate kinase is particularly noteworthy, since it is considered that these properties of the enzyme from the oyster are related to its ability to produce extra ATP from the succinate pathway during prolonged anaerobiosis. It is known that the scallop cannot survive even short periods of anaerobiosis.

Effects of substrates. The plots of activity against the concentration of phosphoenolpyruvate for the enzyme from marine invertebrate muscles are sigmoidal (plots not given; see Table 2 for h values). The plot for the enzyme from frog sartorius muscle was hyperbolic (h was 0.95 and the double-reciprocal plot was linear; plots not given). The concentration of phosphoenolpyruvate required for half-maximal activity of the enzyme from the oyster was 0.5mM, whereas that for the enzyme from other muscles ranged from 0.05 to 0.15mM.

The plots of pyruvate kinase activity against ADP concentration (at 2mM-phosphoenolpyruvate) were hyperbolic and double-reciprocal plots were linear for all muscles investigated. The K_m values for ADP (determined from double-reciprocal plots) showed a considerable variation between the enzymes of different muscles: they were 0.06, 0.10, 0.15, 0.56 and 0.24mM for the enzymes from the oyster, the sea anemone, lobster, horse-shoe crab and frog respectively. In addition, pyruvate kinases from the oyster adductor and sea-anemone basilar muscles exhibited substrate inhibition at high concentrations of ADP (2mM; results not given).

Effects of K^+, Mg^{2+} and Mn^{2+}. The effects of K^+ were very similar for pyruvate kinase from all the muscles studied, i.e. the maximal activity was obtained at about 80mM-K^+ and half-maximal activity was reached at about 10mM (results not given).

Both Mg^{2+} and Mn^{2+} could act as cofactors for the enzyme from all the muscles investigated. However, the maximal activity obtained with Mn^{2+} was invariably lower than that with Mg^{2+}. Maximal activity of pyruvate kinase of the different muscles occurs at total Mg^{2+} concentration between 6 and 12mM (at 2mM-ADP and 1mM-phosphoenolpyruvate). Higher concentrations of Mg^{2+} resulted in slight inhibition of the activity.

Inhibition by alanine. Pyruvate kinase from all the marine-invertebrate muscles studied was inhibited by alanine. However, the enzyme from frog sartorius muscle was not inhibited by alanine. Alanine decreases the $V_{max.}$ and increases the K_m of pyruvate kinase for phosphoenolpyruvate. The inhibition by 1mM-alanine was completely removed by 0.2mM-fructose bisphosphate for all the pyruvate kinases studied. However, inhibition by 5mM-alanine was only partially reversed by 0.2mM-fructose bisphosphate for the enzyme from sea anemone and lobster. The concentration of alanine required for inhibition of pyruvate kinase varied considerably between the enzyme from different muscles. Half-maximal inhibition was obtained at concentrations of alanine ranging from 0.4 to 9mM (oyster and horse-shoe-crab muscles; see Fig. 1). In addition, a plot of percentage inhibition against alanine concentration for the enzyme from horse-shoe-crab muscle was markedly more sigmoid ($h = 2.0$) than that for enzyme from oyster muscle $h = 1.1$).

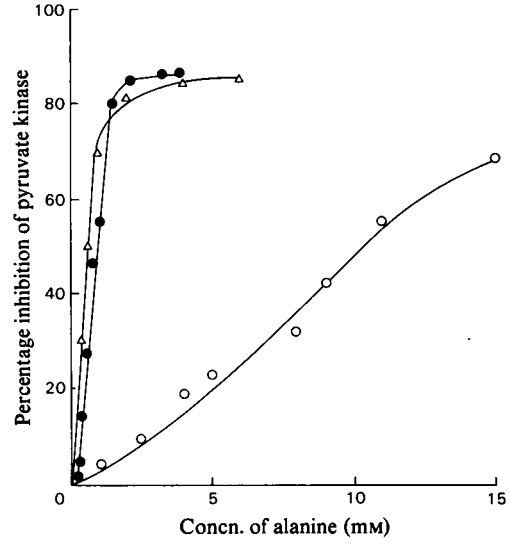

Fig. 1. *Percentage inhibition of pyruvate kinase from some marine invertebrate muscles by alanine*
For experimental details see the text. ○, Scallop adductor muscle; ●, oyster adductor muscle; △, horse-shoe crab leg muscle. In all cases the concentration of ADP was 2mM. The effects were investigated at phosphoenolypyruvate concentrations and pH values of 0.15mM and 6.9, 2mM and 8.2 and 1mM and 7.3 for scallop adductor, oyster adductor and leg muscle of horse-shoe crab respectively. The maximum activities of pyruvate kinase were 48.7, 3.8 and 147.0μmol/min per g at 25°C respectively.

Effect of fructose bisphosphate and other hexose phosphates. Pyruvate kinase from all the marine-invertebrate muscles studied or from frog sartorius muscle was activated by fructose bisphosphate. Fructose bisphosphate decreased the sigmoidicity of the plots of enzyme activity, when present, against the concentration of phosphoenolpyruvate so that the apparent K_m for this substrate was lowered. The plot of percentage activation against fructose bisphosphate concentration for the lobster muscle enzyme is hyperbolic, whereas that for the enzyme from the oyster is markedly sigmoidal ($h = 2.0$) (Figs. 2*a* and 2*b*). The range of concentrations of

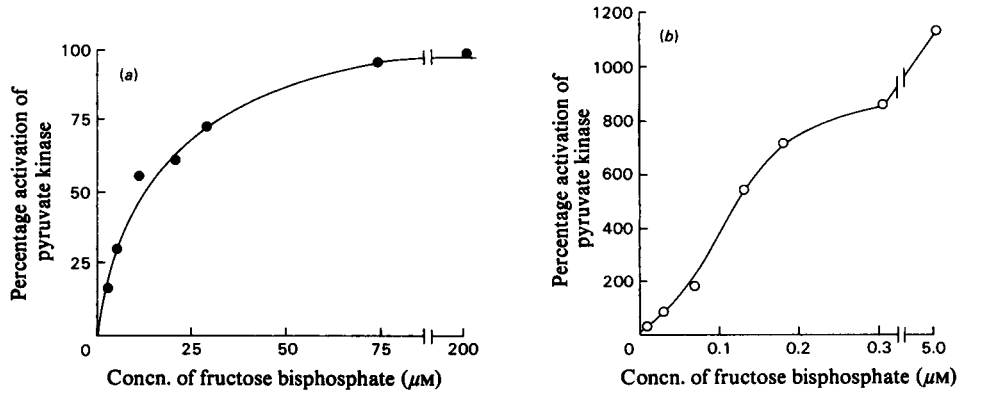

Fig. 2. *Effect of fructose bisphosphate on pyruvate kinase activity from (a) lobster abdominal muscle and (b) oyster adductor muscle*

The concentration of phosphoenolpyruvate was 0.1 and 0.2mM for the experiments with the enzymes from lobster and oyster respectively. The maximum activities of pyruvate kinase were 57.2 and 2.7μmol/min per g wet wt. at 25°C for lobster and oyster adductor muscles respectively.

fructose bisphosphate required for 50% activation of the pyruvate kinases investigated was very low (0.13 and 1.0μM for the enzyme from oyster adductor and lobster abdominal muscles respectively).

The substantial activation of oyster (*Ostrea*) muscle pyruvate kinase by low concentrations of fructose bisphosphate (see Fig. 2*b*) may be related to the pronounced sigmoidicity of response of this enzyme to its substrate (phosphoenolpyruvate), since this activator decreases the sigmoidicity. Furthermore, the enzyme from oyster appears to be more sensitive to alanine inhibition than the enzyme from other muscles (see above).

The compounds fructose 1-phosphate, fructose 6-phosphate, glyceral dehyde3-phosphate and glycerol 3-phosphate (at concentrations of 0.1mM) had no effect on the activity of pyruvate kinase. Glucose 1,6-bisphosphate (0.6mM) activated the enzyme from lobster muscle by 12%, which compares with activation of 95% by fructose bisphosphate.

It has been shown electrophoretically that for pyruvate kinase from muscles of the oyster, scallop and frog only one form of the enzyme is present in these muscles (P. R. Dando, personal communication; P. R. Dando & V. A. Zammit, unpublished work). It is therefore unlikely that any of the results given above can be interpreted on the basis of different properties of two isoenzymes.

Properties of phosphoenolpyruvate carboxykinase from different muscles

pH optima. The optimal pH values for phosphoenolpyruvate carboxykinase activity (at saturation concentrations of substrates) are very similar for the enzymes from different muscles (the range is 7.0–7.1; see Table 1). The activities are decreased sharply (by about 50%) by an increase in pH from 7.2 to 7.5, except for the enzyme from the lobster adductor muscle, which shows a broad pH optimum (the activity is decreased by 25% when the pH is raised from 7.1 to 7.8; see Table 1). A similar dependence on pH for phosphoenolpyruvate carboxykinase from adductor muscle of *Mytilus* to that of the oyster was observed by de Zwaan & van Marrewijk (1973), whereas Mustafa & Hochachka (1973*a*) found a much lower and sharper pH optimum for phosphoenolpyruvate carboxykinase of the oyster (*Crassostrea*) muscle (the optimum pH was 6.0 with Mn^{2+} or pH 5.1 with Zn^{2+} as cofactor). In the present study it was observed that the pH optimum for oyster (*Ostrea*) phosphoenolpyruvate carboxykinase was shifted from 7.0 to pH 7.6 by L-alanine (1mM), whereas fructose bisphosphate had no effect.

Effects of substrates. Double-reciprocal plots of the activity against concentrations of phosphoenolpyruvate for phosphoenolpyruvate carboxykinase from the muscles studied (plots not given) are linear, except that for the enzyme from lobster muscle: the latter is concave upwards, indicating positive cooperativity (plots not given). The K_m values for phosphoenolpyruvate (at an optimal concentration of IDP, i.e. 1mM) for the enzymes from these muscles vary considerably (0.1 and 6.6mM for sea-anemone and horse-shoe crab respectively; see Table 2).

Double-reciprocal plots of the activity against the concentration of IDP for phosphoenolpyruvate carboxykinase from all the muscles studied are linear (plots not given). The K_m values for IDP for the enzymes from these muscles are very similar (0.06

Table 3. *Effects of Mg^{2+} and Mn^{2+} concentrations on the activity of phosphoenolpyruvate carboxykinase from muscles of horse-shoe crab, lobster, oyster and sea anemone*

The assays were performed at the respective pH optima of the individual enzymes (see the text and Table 1). Systematic names are given in Table 2.

	Phosphoenolpyruvate carboxykinase activities (μmol/min per g fresh wt. at 25°C)							
	Horse-shoe crab		Lobster		Oyster		Sea anemone	
Concn. of metal ion (mM)	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mg^{2+} + 0.01 mM-Mn^{2+}	Mg^{2+}	Mg^{2+} + 0.01 mM-Mn^{2+}
0	0.01	0.01	0.01	0.01	0.01	0.05	0.01	0.2
0.5	1.6	1.2	0.2	0.3	0.01	0.10	0.2	0.6
1.0	2.1	2.3	0.3	0.8	0.01	0.25	0.2	0.7
2.0	2.2	2.7	0.7	1.2	0.01	0.54	0.2	0.8
3.0	1.8	2.5	0.9	2.0	0.02	0.54	0.2	0.9
5.0	—	—	1.0	2.5	0.02	0.32	0.1	0.8
10.0	—	—	1.0	3.2	—	—	—	—

and 0.11 mM for crab and lobster respectively; see Table 2).

Effects of metal ions. The activities of phosphoenolpyruvate carboxykinase from the muscles of horse-shoe crab, lobster and frog were stimulated by either Mn^{2+} or Mg^{2+}, although the activity with Mg^{2+} was markedly lower than that with Mn^{2+} at any given metal ion concentration (see Table 3). The enzymes from oyster and sea anemone were almost completely inactive in the presence of Mg^{2+} alone, but addition of a very low concentration of Mn^{2+} (0.01 mM), in addition to Mg^{2+}, increased the activity considerably (see Table 3). This effect was not observed with the enzyme from the other muscles investigated. The significance of this requirement of the enzyme from oyster and sea anemone for Mn^{2+} is not known, but this effect has been observed with the enzyme from rat liver (Foster *et al.*, 1967). Hochachka & Mustafa (1972) and Hochachka & Somero (1973) have suggested that the specificity of phosphoenolpyruvate carboxykinase for a metal ion cofactor that is different from that required by pyruvate kinase could provide the basis for a regulatory mechanism. They suggested that Zn^{2+} is the physiological cofactor for oyster (*Crassostrea*) muscle phosphoenolpyruvate carboxykinase. In the present study, low concentrations of Zn^{2+} were found to inhibit phosphoenolpyruvate carboxykinase activity of oyster muscle (results not given). In addition, although sea-anemone and oyster (*Ostrea*) phosphoenolpyruvate carboxykinase show a specificity for Mn^{2+}, this ion can also serve as a cofactor for pyruvate kinase in these muscles. Therefore the basis for the regulatory mechanism suggested by Hochachka & Mustafa (1972) is not established for the enzymes of *Ostrea* muscle.

Inhibition by ITP and its reversal by alanine. ITP inhibited the activities of phosphoenolpyruvate carboxykinase from oyster and sea anemone competitively with respect to phosphoenolpyruvate (results not given). The K_i values (as determined from secondary plots, results not given) were 0.1 and 0.7 mM for the enzyme from the two animals respectively. The inhibition of oyster muscle phosphoenolpyruvate carboxykinase by ITP is reversed by concentrations of alanine above 2 mM. Mustafa & Hochachka (1973*b*) observed ITP inhibition of phosphoenolpyruvate carboxykinase from oyster (*Crassostrea*) adductor muscle; the inhibition was of a mixed competitive type with respect to phosphoenolpyruvate. In the present study, inhibition of oyster (*Ostrea*) phosphoenolpyruvate carboxykinase by ITP was competitive. Mixed kinetics occur when insufficient Mn^{2+} is added to the assay medium, when chelation effects may complicate the observed kinetics. (Preliminary experiments had established that GTP gave equivalent effects to those of ITP when present at the same concentrations. Therefore, because of the high cost of GTP, ITP was used for subsequent kinetic experiments.)

Control of activities of pyruvate kinase and phosphoenolpyruvate carboxykinase in oyster and sea-anemone muscles

The pH optima for the pyruvate kinase and phosphoenolpyruvate carboxykinase from the oyster (*Ostrea*) suggest that a decrease in pH from 7.6 to 7.0 within the muscle could inhibit the activity of pyruvate kinase and increase that of phosphoenolpyruvate carboxykinase. This would produce a concerted mechanism of control in which glycolytic residues would be directed towards succinate formation. Although no intracellular pH measurement in muscles of marine invertebrates have been reported, it is well established that the pH of body fluids of molluscs decreases after a period of anaero-

biosis [see von Brand (1946) for a review]. (An initial fall in pH could result from the retention of respiratory CO_2, initially, on isolation of the animal from the sea.) However, a regulatory mechanism based solely on different pH optima of the two enzymes in oyster may be an oversimplification. Thus the pH optimum of the oyster enzyme is sensitive to the presence of fructose bisphosphate and any extrapolation of the properties of the enzymes found *in vitro* to the situation *in vivo* would have to take into account the concentration of the fructose bisphosphate in the vicinity of the enzyme, as well as the intracellular pH (see below). Therefore it is possible that the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase may not be regulated solely by changes in pH in the oyster; this may be particularly so for the enzymes from the other animals. Thus the pH profiles of the enzymes from other animals are different from that of the enzymes from the oyster.

Pyruvate kinase from all the animals investigated is activated by fructose bisphosphate. However, the greatest activation by fructose bisphosphate is observed for the enzyme from facultative anaerobes (see Table 2 and Zammit *et al.*, 1978). The role of the feed-forward activation of pyruvate kinase by fructose bisphosphate in the concerted regulation of glycolysis in muscle has been emphasized (Zammit *et al.*, 1978). However, the role of this effect may be somewhat different in muscles in which the succinate pathway is important for energy production during periods of anaerobiosis. An increase in the concentration of fructose bisphosphate, due to activation of phosphofructokinase at the onset of anaerobiosis, would activate pyruvate kinase, leading to an increase in the concentration of alanine in the early stages of anaerobiosis (see de Zwaan & van Marrewijk, 1973). This latter change would have two effects: it would inhibit the activity of pyruvate kinase by reversing the activation due to fructose bisphosphate and stimulate the activity of phosphoenolpyruvate carboxykinase by reversing the ITP inhibition (see Fig. 3). Consequently, glycolytic residues would be directed towards formation of succinate rather than lactate, octopine or alanine. At the termination of anaerobiosis, oxidation of pyruvate via the citric acid cycle would result in a decrease in concentration of alanine so that the activity of phosphoenolpyruvate carboxykinase would be decreased as the concentration of ITP increased. The present study suggests that alanine concentrations in the range of 2–5mM may be important for the regulation of both the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase in oyster muscle.

From the above discussion it is evident that the properties of pyruvate kinase from the muscles of all the marine invertebrates are qualitatively similar, except for the effects of pH on the enzyme from oyster muscle. This suggests that this latter property is of particular importance in the regulation of phosphoenolpyruvate metabolism in facultative anaerobes.

Of the phosphoenolpyruvate carboxykinases studied in the present work, only the enzyme from the basilar muscle of sea anemone (*Metridium*) has similar properties to that from the oyster; i.e. it is inhibited by ITP (although the K_i is 7-fold greater than that for the enzyme from the oyster) and this inhibition is relieved by alanine. The similarity of the properties of phosphoenolpyruvate carboxykinase from oyster and the sea anemone suggests that phosphoenolpyruvate carboxykinase in coelenterate muscle is also involved in the succinate pathway.

The properties of phosphoenolpyruvate carboxykinase from muscles of the other animals studied (i.e. arthropods and vertebrates) are different from those of the enzyme from the oyster and sea anemone. Thus

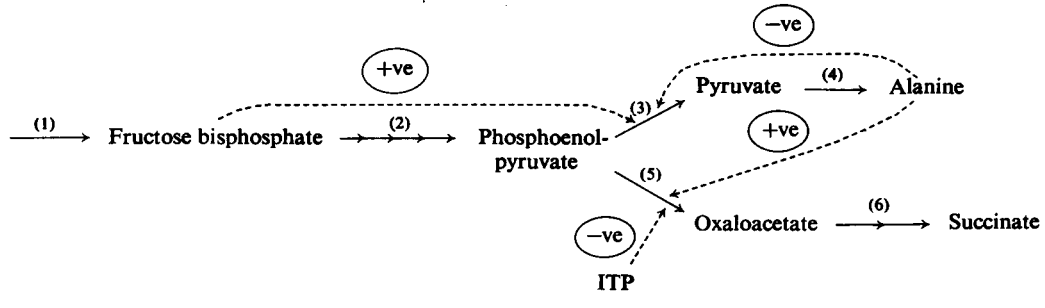

Fig. 3. *A theory of control of the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase in muscles of the oyster and sea anemone*

(1) The initial stages of glycolysis; (2) the reactions of glycolysis interconverting fructose bisphosphate and phosphoenolpyruvate; (3) pyruvate kinase; (4) pyruvate–glutamate transaminase; (5) phosphoenolpyruvate carboxykinase; (6) reactions which convert oxaloacetate into succinate in some marine invertebrate muscles. Broken lines indicate regulatory effects; +ve indicates stimulation; −ve indicates inhibition.

the activities of the enzyme from muscles of the lobster, horse-shoe crab and frog are not inhibited by ITP, they are unaffected by alanine, and for the enzymes from the horse-shoe crab and frog the K_m values for phosphoenolpyruvate are considerably higher than those for the enzyme from oyster and sea anemone (Table 2); the K_m values are also very much higher (about two orders of magnitude) than the concentration of phosphoenolpyruvate in the muscle. Furthermore, in these muscles, the maximal activities of pyruvate kinase are much higher than those of phosphoenolpyruvate carboxykinase (see Zammit & Newsholme, 1976; Zammit *et al.*, 1978). Therefore it is suggested that, in muscles other than those of the oyster and the sea anemone, phosphoenolpyruvate carboxykinase does not function as part of the succinate pathway (see also Zammit *et al.*, 1978). It is suggested that, in these muscles, the enzyme catalyses the conversion of oxaloacetate into phosphoenolpyruvate (rather than the reverse direction). This reaction is part of the pathway by which some amino acids are oxidized or converted into alanine in muscle (see Goldstein & Newsholme, 1976). In crustaceans, the enzyme may also be involved in gluconeogenesis from oxaloacetate in muscle (Behrisch, 1972); this suggestion is supported by the fact that in crustacean muscle there is a positive correlation between the activities of fructose bisphosphatase and phosphoenolpyruvate carboxykinase (Zammit, 1974).

Comparison of data obtained in vitro and in vivo

Although a theory of control of the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase can be formulated from the properties of the enzymes obtained *in vitro*, the physiological significance of the fructose bisphosphate and alanine effects may be questioned when the concentrations that are effective *in vitro* are compared with those observed *in vivo*. Thus the K_i for alanine inhibition of *Ostrea* pyruvate kinase is 0.4mM, whereas the alanine concentration in marine molluscan tissues is much higher [e.g. 22mM in *Mytilus* adductor muscle (Bricteaux-Gregoire *et al.*, 1964; de Zwaan & van Marrewijk, 1973) and 30mM in *Crassostrea* (Lynch & Wood, 1966)], although these high values for alanine concentration were not obtained for resting fully aerobic muscles and thus may represent an upper range of alanine concentration. Nonetheless, it is generally accepted that the concentration of free amino acids in tissues of marine invertebrates is maintained very high for osmotic purposes. The apparent K_a for fructose bisphosphate activation of pyruvate kinase from oyster muscle is 0.13μM, whereas the fructose bisphosphate concentration in oyster muscle, which has been measured in the present work after freeze-clamping the muscle, is approx. 80μM. However, these values of K_i for alanine and K_a for fructose bisphosphate are obtained separately, whereas it has been shown that the degree of activation by fructose bisphosphate is modified by the presence of a high concentration of alanine (see Fig. 1). Furthermore, it is possible that a considerable proportion of the fructose bisphosphate may be bound to protein *in vivo* (Sols & Marco, 1970). Thus changes in the concentrations of both alanine and fructose bisphosphate in the muscle of the oyster may regulate the activity of pyruvate kinase as indicated by the properties of the enzyme *in vitro*.

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