Biochimica et Biophysica Acta, 309 (1973) 296–306

BBA 66900

KINETIC AND MOLECULAR CHARACTERISTICS OF ALLOSTERIC PYRUVATE KINASE FROM MUSCLE TISSUE OF THE SEA MUSSEL *MYTILUS EDULIS* L

DIRK A. HOLWERDA AND ALBERTUS DE ZWAAN
Laboratorium voor Scheikundige Dierfysiologie, 40 Jan van Galenstraat, Utrecht (The Netherlands)

(Received November 23rd, 1972)

SUMMARY

1. At low phosphopyruvate concentrations, fructose 1,6-diphosphate enhances the activity of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from the adductor muscle of sea mussels. At pH 7.6, [phosphopyruvate] = 0.2 mM and [ADP] = 5 mM, half-maximal stimulation is reached with [Fru-1,6-P_2] = 2 μM; at pH 6.7 it is reached with [Fru-1,6-P_2] = 15 μM.

2. Glucose 1,6-diphosphate enhances enzyme activity but less so than Fru-1,6-P_2. Cyclic AMP stimulates to a even lesser extent.

3. The reaction rate is inhibited when the concentration of the co-substrate ADP is substantially higher than phosphopyruvate. The Lineweaver–Burk plots for ADP at fixed concentrations of phosphopyruvate show no cooperative effects, whilst similar plots for phosphopyruvate at fixed concentrations of ADP all show positive cooperativity.

4. Alanine strongly inhibits enzyme activity. At pH 6.7, [phosphopyruvate] = 0.2 mM and [ADP] = 2 mM, 50% inhibition is reached with [alanine] = 0.1 mM; at pH 7.6 it is reached at [alanine] = 1 mM. 0.1 mM Fru-1,6-P_2 counteracts this inhibition giving a hyperbolic substrate-saturation curve.

5. ATP inhibition is strongly pH-dependant. At pH 6.7, [phosphopyruvate] = 0.2 mM and [ADP] = 2 mM, 50% inhibition is reached at [ATP] = 0.62 mM. At pH 7.6 there is a little influence of ATP. Fru-1,6-P_2 also cancels this inhibition. Other trinucleotides and AMP inhibit to a less extent.

6. The molecular mass of the enzyme was found to be 220 000 $\pm$ 10%. On electrofocusing, one main peak is found with pI 6.70. Three smaller peaks have pI values of 6.44, 6.59 and 6.83.

INTRODUCTION

In a previous communication[1] we reported that pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from the posterior adductor muscle of the sea mussel *Mytilus edulis* has allosteric properties.

In common with allosteric pyruvate kinases from other sources[2–4], fructose 1,6-diphosphate has a strong stimulating effect on enzyme activity. However, the effect of pH change proved to be contrary to the effect on pyruvate kinase from yeast[5], rat liver[6,7] or human erythrocytes[4].

We now report kinetic characteristics with regard to the influence of ADP, ATP, fructose 1,6-diphosphate, alanine, succinate and some other metabolites on enzyme activity, and molecular characteristics such as molecular mass and electrophoretic behaviour.

MATERIALS AND METHODS

Chemicals

ADP and glycerol p.a. were purchased from E. Merck AG (Darmstadt, W. Germany), further biochemicals and enzymes were from Boehringer GmbH (Mannheim, W. Germany). Other chemicals were of analytical grade. Sephadex G-150 and DEAE-Sephadex A-50 were obtained from Pharmacia AB (Uppsala, Sweden). Mussels (*M. edulis* L.) were supplied by the Institute of Mussel Research (Texel, The Netherlands).

Enzyme preparation

The enzyme preparation used was the same as described in the previous paper[1]. There was no loss of activity when the enzyme was stored in solution containing glycerol (30%, v/v) at 5 °C for several weeks. No influence of glycerol on kinetics was found.

Enzyme activity

Pyruvate kinase activity was measured according to Bücher and Pfleiderer[8]. In the reaction medium the following concentrations were not varied: $MgSO_4$ (8.3 mM), KCl (67 mM), NADH (0.12 mM), lactate dehydrogenase (36 I.U.). Temperature was 25 °C. Other factors were variable and are indicated in each experiment. The enzyme was pre-incubated for 15 min at 25 °C. Change in absorbance at 340 nm was measured with a Zeiss PMQ II spectrophotometer coupled with a Vitatron UR 400 recorder. The reaction was started by adding the substrate phosphopyruvate. The activity is expressed as μmoles NADH $\cdot$ min^{-1}; the specific activity as the activity per mg protein. Protein was determined using the Lowry method.

Molecular mass

The molecular mass was measured according to Andrews[9] with a Sephadex G-150 column (1.2 cm $\times$ 100 cm) in 0.1 M Tris–HCl buffer, pH 7.6. For calibration, enzymes with known molecular masses were used. These are mentioned under the figure.

Electrofocusing

"Electrofocusing" was carried out to determine possible electrophoretic multiplicity and isoelectric points. A 440-ml LKB electrofocusing column was used with a gradient of glycerol (0–60%, w/v) in water, and pH ranges 3–10 and 5–8. Runs were performed at 4 °C for 48 h.

RESULTS AND DISCUSSION

Fructose 1,6-diphosphate

The influence of increasing concentrations of fructose 1,6-diphosphate (Fru-1,6-P_2) on the initial reaction velocity at low phosphopyruvate concentration and at two pH values is shown in Fig. 1. At both pH values, near-maximal stimulation

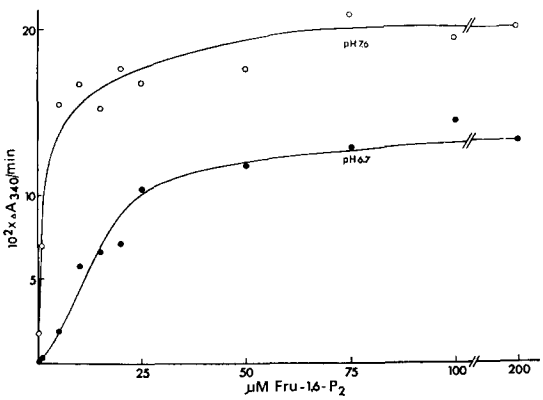

Fig. 1. The influence of the concentration of fructose 1,6-diphosphate on the reaction rate of the pyruvate kinase reaction at various pH values. [Phosphopyruvate] = 0.2 mM; [ADP] = 2 mM; buffer: 0.1 M Tris–maleate.

is reached at [Fru-1,6-P_2] = 50 μM, but there is a great difference in the Fru-1,6-P_2 concentration of half-maximal stimulation ($K_{\frac{1}{2}}$). At pH 7.6 $K_{\frac{1}{2}}$ is 2 μM, at pH 6.7 it is 15 μM. The latter value is within the range in which the concentration of Fru-1,6-P_2 in rat liver fluctuates (5–20 μM)[10] but we do not know in which range this intermediate fluctuates in the muscle during anaerobiosis, nor do we know to what extent the pH will drop.

From the curves it can be seen that at higher pH the effector site is readily accessible for Fru-1,6-P_2, while at lower pH there is a cooperative effect of the activator. The lower maximal stimulation in the latter case is thought to be a genuine pH effect rather than a matter of conformation of the enzyme.

TABLE I

THE EFFECT OF CYCLIC AMP, GLUCOSE 1,6-DIPHOSPHATE AND FRUCTOSE 1,6-DIPHOSPHATE ON PYRUVATE KINASE ACTIVITY

[Phosphopyruvate] = 0.2 mM; buffer: 0.1 M imidazole–HCl, pH 7.6.

Addition	*Enzyme activity (%)*	
	[ADP] = 0.5 mM	*[ADP] = 5 mM*
Control	100	100
0.1 mM Glc-1,6-P_2	150	180
0.3 mM Glc-1,6-P_2	170	250
4 mM cyclic AMP	120	130
0.1 mM Fru-1,6-P_2	180	370

Glucose 1,6-diphosphate, cyclic AMP

Other stimulating factors are listed in Table I. It has been found[11] with hepatic pyruvate kinase that the influence of glucose 1,6-diphosphate (Glc-1,6-P_2) on the enzyme activity is of the same nature as the action of Fru-1,6-P_2. The effect of these two activators was also quantitatively the same. In our case, as in the case with erythrocyte pyruvate kinase[11] the effect of Glc-1,6-P_2 is less pronounced.

Stimulation by cyclic AMP has also been found with pyruvate kinase from loach embryos[12]. Haeckel *et al.*[5] reported inhibition with yeast pyruvate kinase.

Adenosine diphosphate

In Fig. 2A, Lineweaver–Burk plots are shown. When the ADP concentration is substantially higher than the concentration of phosphopyruvate there is considerable inhibition of reaction velocity. It is important to note that the ratio of ADP to phosphopyruvate which leads to inhibition is not constant but decreases with increasing concentration of phosphopyruvate. No positive cooperative action of ADP is found. Similar results have been reported by Tanaka *et al.*[13] with rat liver pyruvate kinase. It was found that the inhibition was not caused by ATP. With the erythrocyte enzyme[4] there might also be inhibition at high levels of the co-substrate, but this is not fully clear. From Fig. 2B it appears that the cooperative action of phosphopyruvate exists at all ADP concentrations. In the presence of 0.1 mM Fru-1,6-P_2 inhibition by large concentrations of ADP does not occur (Fig. 3).

Alanine

Strong inhibition is exerted by alanine (Fig. 4). Whereas V is not much influenced, the apparent K_m is increased from 0.60 mM to 3.0 mM and the Hill coefficient from 1.3 to 1.75 (Fig. 5), indicating that alanine is an allosteric inhibitor. In the presence of 0.1 mM Fru-1,6-P_2 the inhibition is reversed and the saturation curve becomes a hyperbole ($n_H = 1.0$; apparent $K_m = 0.09$ mM).

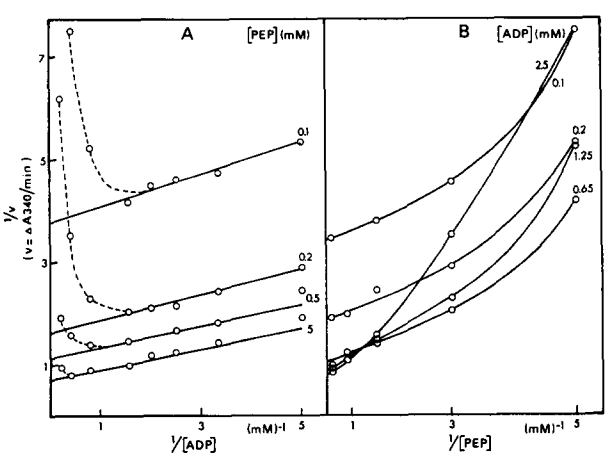

Fig. 2. (A) Lineweaver-Burk plots of the reaction rate of the pyruvate kinase reaction against ADP concentration at various phosphopyruvate concentrations. Buffer: 0.1 M imidazole–HCl pH 7.6 (B) Variation of enzyme activity with phosphopyruvate at various ADP concentrations.

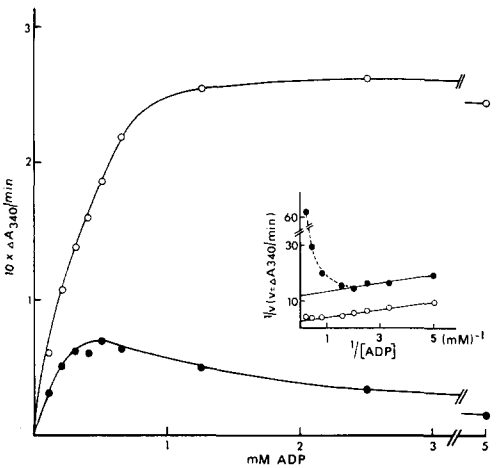

Fig. 3. The effect of ADP concentration on pyruvate kinase activity in the absence (●—●) and presence (○—○) of 0.1 mM Fru-1,6-P_2. [Phosphopyruvate] = 0.2 mM; buffer: 0.1 M imidazole–HCl, pH 6.8.

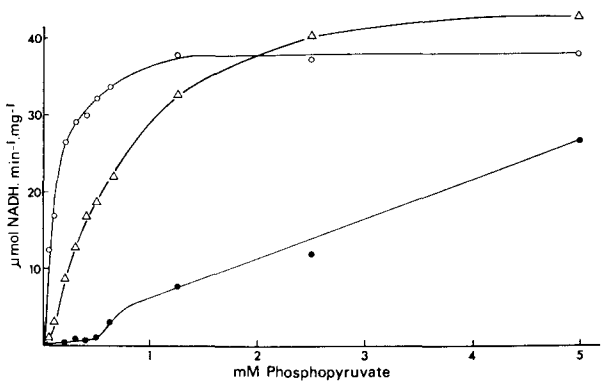

Fig. 4. Reaction rate of the pyruvate kinase reaction against concentration of phosphopyruvate. [ADP] = 2 mM; buffer: 0.1 M Tris–HCl, pH 7.6. ●—●, with 2 mM alanine; ○—○, with 2 mM alanine and 0.1 mM fructose 1,6-diphosphate; △—△, control. At [Phosphopyruvate] = 15 mM (not shown in the figure) the velocities without and with Fru-1,6-P_2 in the presence of 2 mM alanine were identical and used as V in the Hill plot (Fig. 5).

The inhibition by alanine, which is also known with rat liver pyruvate kinase[14,15], is relevant in the case of the mussel enzyme as this amino acid is the first to emerge and, with succinate, the main end-product of glycolysis when the mussel lies dry[16]. Together with lowering of pH by accumulation of organic acids, an increase of alanine concentration would suppress the mussel pyruvate kinase activity, thus favouring the carboxylation of phosphopyruvate, provided that the Fru-1,6-P_2 concentration is low enough under these conditions.

Fig. 6 shows the effect of increasing concentrations of alanine on enzyme activity at pH 7.6 and 6.7. At the higher pH value, when the enzyme is in the activated form, the affinity of the enzyme for alanine is low. At the lower pH value the inhibition curve has a normal shape, indicating that the enzyme exists in a form

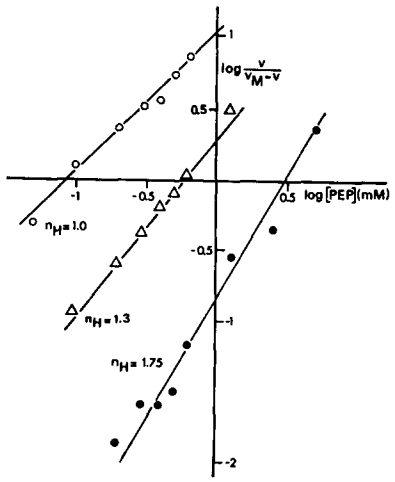

Fig. 5. Hill plot of the reaction rate against phosphopyruvate concentration. ●—●, with 2 mM alanine; ○—○, with 2 mM alanine and 0.1 mM fructose 1,6-diphosphate; △—△, control. Conditions as for Fig. 4.

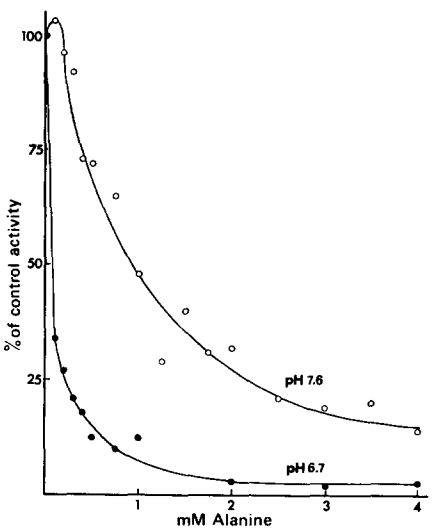

Fig. 6. Effect of alanine concentration on the reaction rate of the pyruvate kinase reaction at various pH values. [Phosphopyruvate] = 0.2 mM; [ADP] = 2 mM; buffer: 0.1 M Tris–HCl.

readily accessible for the ligand. At this pH, 50% inhibition is already reached with 0.1 mM alanine.

Adenosine triphosphate and other nucleotides

The influence of ATP is strongly pH-dependant. At pH 7.6 there is no major change in V and in apparent K_m with 2 mM ATP in the reaction medium (Fig. 7). The Hill plot shows that, in the presence of ATP, the cooperative effect towards phosphopyruvate increases: the Hill coefficient becomes 2.0. The same has been reported[17] for pyruvate kinase type II from rat kidney cortex. At the lower pH value the enzyme activity decreases rapidly with increasing concentrations of ATP,

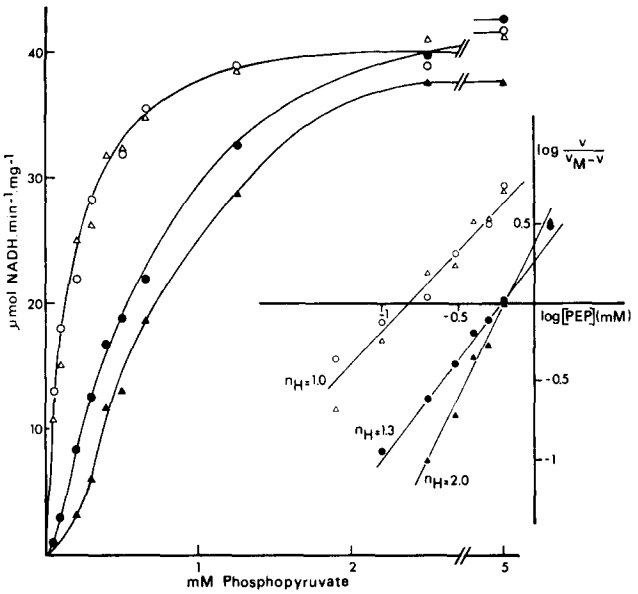

Fig. 7. The influence of ATP on the reaction rate of the pyruvate kinase reaction against phosphopyruvate concentration: Michaelis–Menten plots and Hill plots. [ADP] = 2 mM; buffer: 0.1 M Tris–HCl, pH 7.6. ●—●, control; ○—○, *plus* 0.1 mM Fru-1,6-P_2; ▲—▲, *plus* 2 mM ATP; △—△, *plus* 2 mM ATP and 0.1 mM Fru-1,6-P_2. For the Hill plot, the velocities at [Phosphopyruvate] = 5 mM were taken as V.

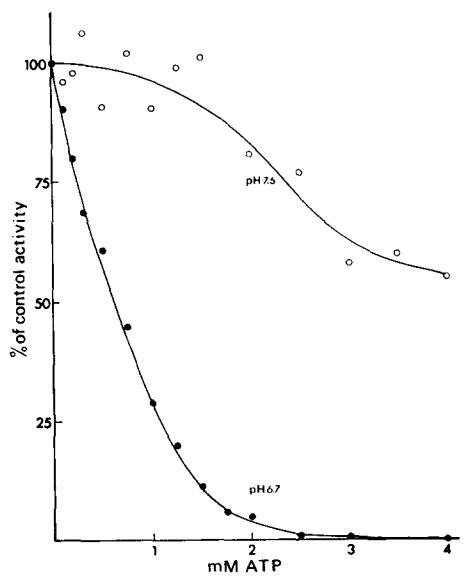

Fig. 8. Effect of ATP concentration on the reaction rate of the pyruvate kinase reaction at various pH values. [Phosphopyruvate] = 0.5 mM; [ADP] = 2 mM; buffer: 0.1 M Tris–HCl.

leading to a saturation curve of normal shape (Fig. 8). The curves have much resemblance to those obtained with rat liver[7] and erythrocyte[4] pyruvate kinase, with an opposite pH dependancy.

Strong coordination of Mg^{2+} by ATP might be responsible for the inhibition. In that case further addition of Mg^{2+} would be expected to counteract the action of ATP. In Table II however it is seen that the inhibition by ATP does not depend on the Mg^{2+} concentration. This means that ATP inhibition is at least partly caused by a direct action on the enzyme (conformation).

In Table III the influence of other nucleotides on the enzyme activity is given. It is seen that ATP is by far the most potent inhibitor under the conditions used.

TABLE II

THE EFFECT OF Mg^{2+} CONCENTRATION ON THE INHIBITION OF PYRUVATE KINASE BY ATP
[Phosphopyruvate] = 0.5 mM; [ADP] = 2 mM; buffer: 0.1 M imidazole–HCl, pH 7.6.

Addition	*Enzyme activity ($\Delta A_{340\ nm}$/min)*
Control (8.3 mM Mg^{2+})	0.124
+ 4 mM ATP	0.063
+ 4 mM ATP + 8 mM Mg^{2+}	0.070
+ 8 mM Mg^{2+}	0.118

TABLE III

THE EFFECT OF NUCLEOTIDES (4 mM) ON PYRUVATE KINASE ACTIVITY
[Phosphopyruvate] = 0.5 mM; [ADP] = 5 mM; buffer: 0.1 M Tris–HCl, pH 6.8.

Addition	*Enzyme activity (%)*
Control	100
ATP	<1
GTP	30
ITP	50
AMP	80

Miscellaneous factors

The following intermediates and other compounds have only little influence on the reaction velocity (conditions as in Table III): aspartic acid and succinate both 4 mM, fructose 1-phosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, glyceraldehyde 2-phosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate–all at 0.1 mM.

Molecular characteristics

The molecular mass has been stated (Fig. 9) as 220 000 ± 10%. Blume *et al.*[18] recently listed molecular masses of pyruvate kinases from several sources. The non-allosteric types from muscle tissue appear to have a higher molecular mass (240 000–250 000) than the allosteric types from yeast[19], liver and erythrocytes (around 200 000). The effect of urea on the molecular mass and the activity of possible subunits is under investigation.

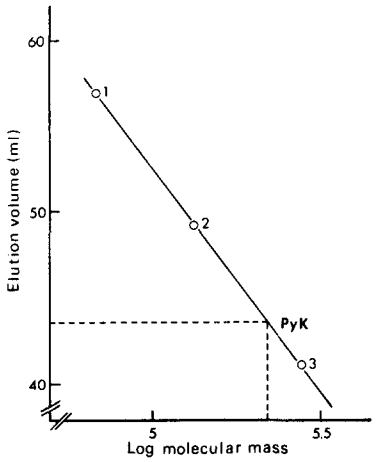

Fig. 9. Elution volume from the Sephadex G-150 column of marker enzymes and of mussel pyruvate kinase against molecular mass. (1) Malate dehydrogenase; (2) glucose 6-phosphate dehydrogenase; (3) xanthine oxidase; PyK, mussel pyruvate kinase.

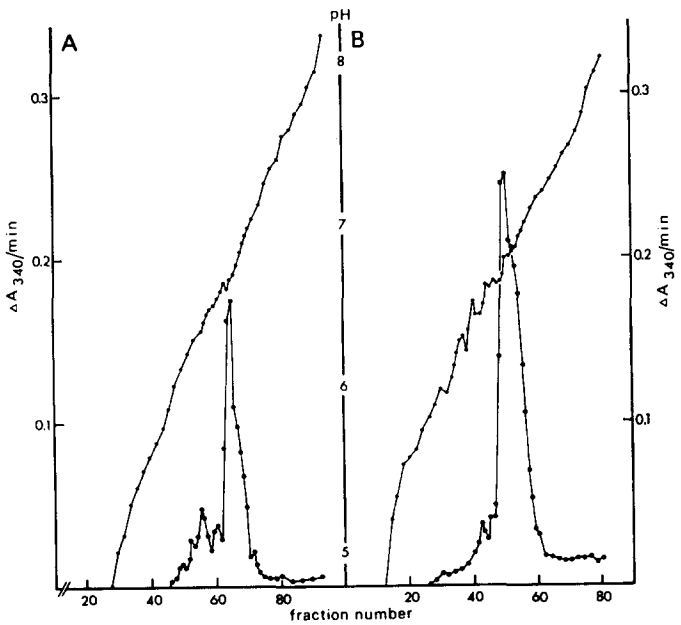

Fig. 10. Elution pattern of mussel pyruvate kinase from the electrofocusing column. (A) Enzyme fraction with specific activity 4 (μmoles NADH·min^{-1}·mg^{-1} protein). (B) Enzyme fraction with specific activity 21. 0.75 vol% Ampholine carrier Ampholyte pH 5–8; other conditions as in the text.

Two fractions of pyruvate kinase of different purity—specific activities 4 and 21, respectively— have been subjected to "electrofocusing". In a preliminary experiment, using a pH gradient 3–10, it was established that all pyruvate kinase activity settled within a narrow range between pH 6 and 7. In the experiment shown in

Fig. 10 a pH gradient 5–8 was used. With both enzyme fractions applied, an identical elution pattern was obtained. The fraction with specific activity 4 has one main peak at pH 6.65, and at least three smaller peaks at pH values of 6.39, 6.54 and 6.80 (Fig. 10A). The fraction with specific activity 21 has one main peak at pH 6.75, and at least three smaller peaks at pH values of 6.49, 6.64 and 6.86 (Fig. 10B). We think that the various peaks are closely related to each other and might be aggregates of identical subunits or monomers with different aggregation numbers. In contrast with our results for the sea mussel, oyster pyruvate kinase[20] shows a pattern with two main peaks at pH values of 5.6 and 6.5 and because of this marked difference in pI it is likely that two distinct pyruvate kinases are present.

An allosteric pyruvate kinase in muscle tissue of the sea mussel may be of physiological significance in the regulation of glycolysis during anoxia. The mussel has to be able to withstand anaerobic conditions due to its habitat, the intertidal zone. The major end-products of anaerobic glycolysis are alanine, succinate and some glutamate instead of lactate[16]. Alanine is the initial major end-product, but during prolonged periods of anoxia the conversion of phosphopyruvate to alanine is gradually prevented in favour of an increased production of succinate and glutamate. The formation of succinate takes place by carboxylation of phosphopyruvate to oxalacetate followed by some reverse steps of the Krebs cycle.

Initial accumulation and the gradual decrease of pH caused by the formation of organic acids might explain why phosphopyruvate becomes available for succinate production, because these conditions suppress pyruvate kinase activity.

The main factors governing the fate of phosphopyruvate seem to be the pH and the concentration of the substrate itself and of the effectors fructose 1,6-diphosphate, alanine and ATP. For *Arenicola* it has been determined[21] that at low tide, when oxygen supply falls of, the pH would fall to a level of about 6.6. Other data are not yet available.

A precise evaluation has to await the quantitative determination of several intermediates and end-products, and off the change off pH during anaerobic metabolism.

REFERENCES

1 Zwaan, A. de and Holwerda, D. A. (1972) *Biochim. Biophys. Acta* 276, 430–433
2 Hess, B., Haeckel, R. and Brand, K. (1966) *Biochem. Biophys. Res. Commun.* 24, 824–831
3 Taylor, C. B. and Bailey, E. (1967) *Biochem. J.* 102, 32c
4 Staal, G. E. J., Koster, J. F. ,Kamp, H., van Milligen-Boersma, L. and Veeger, C. (1971) *Biochim. Biophys. Acta* 227, 86–96
5 Haeckel, R., Hess, B., Lauterborn, W. and Wüster, K.-H. (1968) *Z. Physiol. Chem.* 349, 699–714
6 Bailey, E., Stirpe, F. and Taylor, C. B. (1968) *Biochem. J.* 108, 427–436
7 Rozengurt, E., Jiménez de Asua, L. and Carminatti, H. (1969) *J. Biol. Chem.* 244, 3142–3147
8 Bücher, Th. and Pfleiderer, G. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 435–436, Academic Press, New York
9 Andrews, P. (1964) *Biochem. J.* 91, 222–233
10 Williamson, J. R., Jakob, A. and Scholz, R. (1971) *Metabolism* 20, 13–26
11 Koster, J. F., Slee, R. G., Staal, G. E. J. and Berkel, Th. J. C. van (1972) *Biochim. Biophys. Acta* 258, 763–768
12 Milman, L. S. and Yurowitzki, Y. G. (1967) *Biochim. Biophys. Acta* 146, 301–304
13 Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967) *J. Biochem. Tokyo* 62, 71–91

14 Seubert, W., Henning, H. V., Schoner, W. and L'age, M. (1968) *Adv. Enzyme Reg.* 6, 153–187
15 Llorente, P., Marco, R. and Sols, A. (1970) *Eur. J. Biochem.* 13, 45–54
16 Zwaan, A. de and Marrewijk, W. J. A. van (1973) *Comp. Biochem. Physiol.*, 44B, 429–439
17 Costa, L., Jiménez de Asua, L., Rozengurt, E., Bade, E. G. and Carminatti, H. (1972) *Biochim. Biophys. Acta* 289, 128–136
18 Blume, K. G., Hoffbauer, R. W., Busch, D., Arnold, H. and Löhr, G. W. (1971) *Biochim. Biophys. Acta* 227, 364–372
19 Bischofberger, H., Hess, B., Röschlau, P., Wieker, H.-J. and Zimmermann-Telschow, H. (1970) *Z. Physiol. Chem.* 351, 401–408
20 Mustafa, T. and Hochachka, P. W. (1971) *J. Biol. Chem.* 246, 3196–3203
21 Mangum, C. P. and Shick, J. M. (1972) *Comp. Biochem. Physiol.* 42A, 693–697