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# Effects of Pressure and Temperature on the Catalytic and Regulatory Properties of Muscle Pyruvate Kinase from an Off-Shore Benthic Fish

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SYNOPSIS. Muscle pyruvate kinase from an abyssal *Coryphaenoides* species occurs as a single electrophoretic form with an isoelectric point of about pH 6.0. Maximum catalytic rates are dramatically reduced by pressure. For catalysis at 3°C, the volume change of activation, $\triangle V^*$, is about 44 cm³/mole (calculated between 14.7 and 8000 psi). The value of $\triangle V^*$ decreases at higher temperatures but is pH independent. The activation energy for rattail muscle pyruvate kinase at 14.7 psi is about 13 Kcal/mole and doubles at 12,000 psi. $Mg^{2+}$ saturation kinetics involve positive site-site interactions. Hill plots yield $n$ values of about 2.4 and $K_a$ values of about 2 mM (at 3°C), and these constants are pressure independent. The $K_m$ values for ADP increase slightly with pressure. PEP saturation curves are complex: at high PEP concentrations, the $n$ values are about 2-2.5, while at low PEP levels, values for the Hill constant are about 1.0. The Hill constant for PEP is not affected by pressure, but the apparent $K_m$ increases somewhat with pressure. FDP dramatically activates rattail muscle pyruvate kinase (500% activation with 0.1 mM FDP) by (1) reducing the $K_{m_{PEP}}$, (2) increasing the maximum velocity, and (3) overriding negative ATP modulation of the enzyme. The latter control feature is strictly dependent upon pressure and is not observed at low pressure. In the presence of FDP, the $K_m$ for PEP decreases at high pressures, in this way counteracting the inhibitory effects of pressure. Under low concentrations of substrates, pyruvate kinase activity is probably determined by its kinetic properties and not by energy-volume relationships.

## INTRODUCTION

Pyruvate kinase (PK) (E.C.2.7.1.40) catalyzes the production of pyruvate from phosphoenolpyruvate (PEP) by a transphosphorylation to ADP. The enzyme, which requires $Mg^{2+}$ and $K^+$, so strongly favors pyruvate and ATP formation that it is considered a physiologically irreversible step in glycolysis. In tissue such as liver, which can synthesize glucose from pyruvate, the PK irreversible step is bypassed by the successive action of two enzymes—pyruvate carboxylase and PEP carboxykinase. This pair of enzymes generates PEP for further metabolism to glucose. As in the F6P $\leftrightharpoons$ FDP interconversion, it is clear that simultaneous function of the enzymes involved in pyruvate and PEP production could lead to a futile cycling of carbon at this point in metabolism. (*See* Llorente *et al.*, 1970, for a recent paper dealing with this problem.) In contrast, in tissues such as mammalian muscle, gluconeogenesis does not occur and there is no requirement for a PK by-pass; instead, here we find a requirement for sudden and large changes in PK activity and in glycolytic flux accompanying muscle contraction. These different requirements for control of the PK reaction have been met by the elaboration during evolution of tissue specific isozymic forms of PK (Tanaka *et al.*, 1967; Susor and Rutter, 1968; Mustafa and Hochachka, 1971). Thus, mammalian muscle PK shows a high affinity for PEP and a low $K_i$ for ATP (Reynard *et al.*, 1961), whereas the liver PK has a higher $K_i$ for ATP and a much reduced affinity for PEP, which furthermore is strongly modulated by FDP (Tanaka *et al.*, 1967). Fish muscle PK appears to display properties common to both the liver and muscle type PK of mammals (Somero and Hochachka, 1968). The enzyme is tightly regulated by FDP-feedforward activation and ATP-feedback inhibition. In the trout en-

zyme, the apparent enzyme-substrate affinity for PEP increases when the temperature drops, thus compensating for changes in the kinetic energy of the reactants; but, the control properties of trout PK seem to be temperature independent (Somero and Hochachka, 1968; Somero, 1969). In marine bottom or midwater organisms, such effects of temperature on the kinetic properties of PK would be complicated by pressure. Hence, in this context, we turned our attention to the effects of pressure and temperature on the properties of muscle PK from a *Coryphaenoides* species common in the benthic regions around the Galápagos Islands.

## MATERIALS AND METHODS

*Preparation of rattail muscle PK.* Upon capture of the rattail fish (*see* Phleger and Soutar, 1971), epaxial muscle devoid of red fibers was quickly excised and the enzyme prepared according to Somero and Hochachka (1968). Muscle was homogenized with four volumes of 0.01 M Tris-HCl buffer, pH 7.5, containing 2 mM EDTA. Centrifugation at 30,000 X *g* for 30 min was followed by salt fractionation at 4°C between 40 and 75% with solid $(NH_4)_2SO_4$. The pellet was resuspended in minimal quantities of the homogenization buffer and dialyzed against the same buffer for at least 12 hr at 5°C. Unless otherwise specified, this dialyzed preparation was used as the source of PK in all experiments reported in this paper.

*Assay of PK.* The spectrophotometric assay of Bücher and Pfleiderer (1955), which couples the PK catalyzed production of pyruvate to the lactate dehydrogenase (LDH) catalyzed reduction of pyruvate to lactate, was employed. The oxidation of NADH by LDH was measured as a decrease in $E_{340}$ using a Unicam SP1800 ultraviolet recording spectrophotometer. The standard assay consisted of 50 mM Tris-HCl buffer, pH 7.5 (temperature adjusted), 1 mM phosphoenol pyruvate (PEP), 0.8 mM adenosine diphosphate (ADP), 8 mM $MgSO_4$, 80 mM KCl, 0.1 mM NADH, and excess of non-dialyzed beef heart LDH in a total of 5 ml. The quantities of NADH and LDH used did not limit the rate of reaction at any pressure tested. In studies of control of catalysis, varying amounts of adenosine triphosphate (ATP) and fructose diphosphate (FDP) were added to the reaction mixture. All chemicals were provided by Sigma Chemical Company, St. Louis, Missouri.

Morita (1967) provides a useful list of various pieces of equipment used to maintain moderate hydrostatic pressures. However, there are no readily available instruments which can meet the demands of all pressure studies. The unit used in this study was designed with the co-operation of Mr. David Anderson and Mr. Gene McCulloch, Dept. of Zoology, University of British Columbia, and was built by the American Instrument Company (Aminco, Silver Springs, Maryland). A simplified diagram is shown in Figure 1. The specifications met by this design were these: (1) hydrostatic pressures of up to 20,000 psi; (2) temperature control between 0° and 40°C; (3) time lapse between enzyme addition and recording of activity less than 15 sec; and (4) cell adaptable for use in an ultraviolet spectrophotometer (sapphire windows, UV grade, were used). Although our apparatus met these specifications adequately, some mixing between the cell contents and the pressure fluid did occur. It was estimated to be less than 1% of the total cell volume. The pressure fluid was reagent grade mineral oil, and did not affect enzyme reaction rates.

The reaction mixture was introduced by pouring it through the top opening (*see* Fig. 1). Following proper temperature equilibration, the reaction was initiated by the addition of a small amount of the enzyme solution. After the reaction mixture was stirred with a small glass rod, the thumb screw was tightened "finger-tight," and the pressure was raised by use of a hand-operated hydraulic pump (Enerpac Test Systems, Butler, Wisconsin). The time required for such a procedure was less than 10 sec, or approximately one-tenth

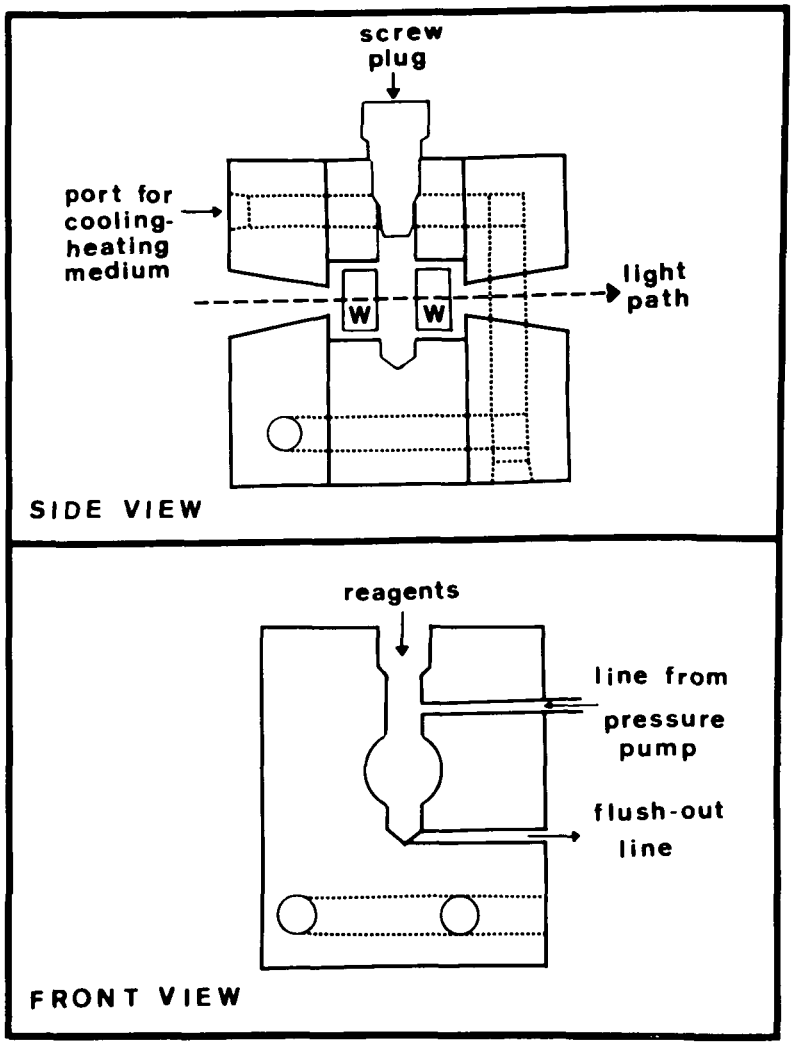



FIG. 1. A simplified diagram of side (upper panel) and front (lower panel) views of the pressure cell employed in these studies. The total volume of the cell is about 5 ml. Windows (labelled W) to the cell were sapphire, UV grade. Temperature control was maintained by circulating water from a Lauda constant temperature bath and circulator. During operation, the cell is closed after addition of the reaction mixture by screwing in the screw plug (upper panel) "finger-tight." The pressure pump can be connected by either of the two lateral channels leading into the pressure cell (lower panel). The lower channel is basically designed to ease in flushing out the cell contents after each assay.

of the total time the reaction was linear. Pressure-release was almost instantaneous, so that experiments could be carried out to examine the rebound-ability of a given enzyme. Cuvette temperatures were maintained by the use of a Lauda K-2/R refrigerated water bath connected to a water jacket surrounding the pressure cuvette.

*Electrophoresis and electrofocus of rat-tail muscle PK.* The epaxial muscle enzyme was investigated electrophoretically and by electrofocus techniques to determine whether unusual enzyme forms that differed from those seen in other fishes were maintained. Electrophoresis was performed according to the procedures given by Susor and Rutter (1968), and electrofocus was carried out according to Haglund

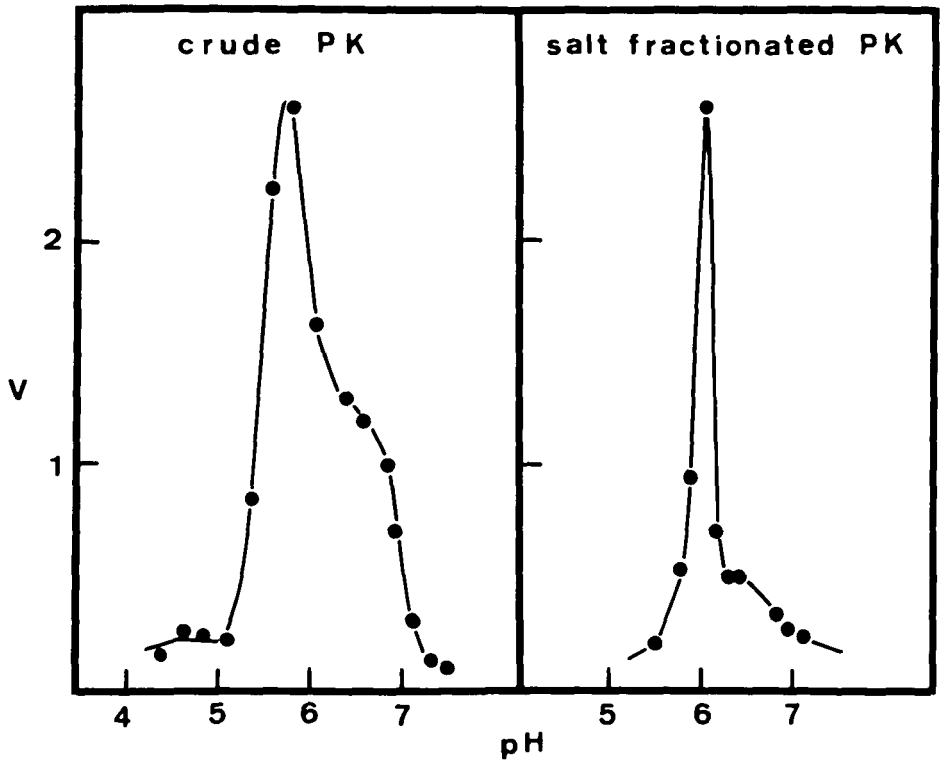



FIG. 2. Two isoelectrofocusing runs of rattail pyruvate kinase. In one experiment (left panel), a high speed supernatant solution was loaded onto the column; the right panel shows the results of electrofocusing an ammonium sulfate fractionated pyruvate kinase. Electrofocusing conditions: 48 hours, 400 volts, 3°C, 110 ml total volume. Fractions were collected in an LKB fraction collector and assayed for activity in the presence of 0.25 mM FDP. The pH of each fraction was determined with a Radiometer pH meter. The isoelectric point for the PK of both preparations is about pH 5.8-pH 6.0.

(1967). The muscle enzyme was run at pH 3-10 (LKB-8141) at 400 volts for 48 hr. The temperature of the apparatus was maintained at 3°C for the entire experiment. All fractions collected were assayed for PK activity in the presence of $2.5 \times 10^{-4}$ M FDP at pH 7.5.

## RESULTS AND DISCUSSION

*Electrophoretic properties of* Coryphaenoides *pyruvate kinase.* Since PK can occur in different isozymic forms (Somero and Hochachka, 1968; Susor and Rutter, 1968), it was important to determine the number of pyruvate kinases present in muscle of *Coryphaenoides.* On cellulose acetate electrophoresis, using conditions described by Susor and Rutter (1968), rattail muscle PK occurs as a single cathodal migrating component. Isoelectric focusing of crude and partially purified preparations of PK also indicate a single major component with enzymatic activity. Under our conditions, the isoelectric point is about pH 6.0 (Fig. 2).

*The pH profile of rattail muscle PK.* The reaction catalyzed by PK is strongly decelerated by pressure at all pH values examined. At 14.7 psi, the pH optimum for rattail muscle PK (at saturating PEP and ADP concentrations) is about pH 7-7.5; the activity drops sharply as the pH becomes more alkaline. The same basic response to pressure occurs between pH 7 and 9, but under pressure, the enzyme displays a slight shift in the pH profile, with activity decreasing steadily from pH 7.0 to 9.0 (Fig. 3). When one of the substrates

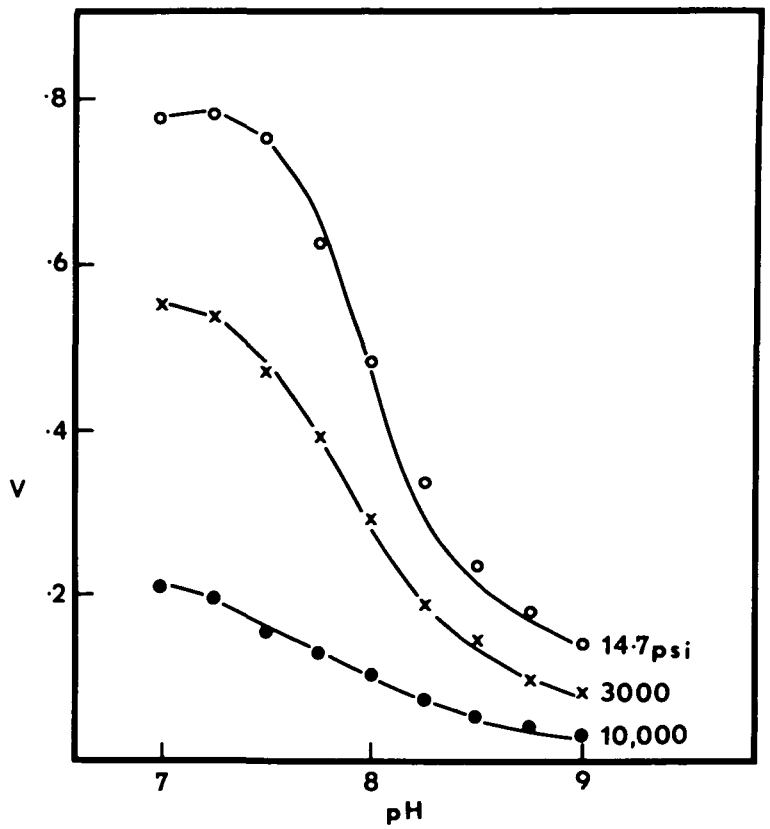



FIG. 3. The pH profile for rattail muscle pyruvate kinase catalysis under conditions of saturating PEP, ADP, $Mg^{2+}$, and $K^+$ at three different pressures and at 3°C. Conditions of assay are given in Materials and Methods. Reaction velocity (v) is the change in optical density at 340 mμ per unit time.

(ADP) is limiting, this effect of pressure upon the pH profile is slightly accentuated, but the effect of pressure is still essentially the same between pH 7.0 and pH 9.5 (Mustafa and Moon, unpublished data). Because of these effects of pH, most of the kinetic experiments to be reported were performed at pH 7.5, this being a compromise between probably *in vivo* pH values in fishes and the pH optimum for the enzyme at 14.7 psi.

*Interacting effects of pressure and temperature on maximum catalytic activity.* The effects of various pressures on rattail muscle PK at saturating PEP and ADP concentrations at various temperatures are shown in Figure 4. Qualitatively, it is clear that the enzyme is less sensitive to pressure deceleration at higher temperature, but at all temperatures, catalytic activity is completely inhibited at 20,000 psi. Of the enzymes we have examined, PK is by far the most pressure sensitive; at 3°C and 5000 psi (conditions well within the biological range for this species of benthic fish), the activity of PK is reduced to less than half the rates at 14.7 psi. Calculations of $\Delta V^*$ from the plots shown in Figure 3 indicate that large increases (about 40-45 $cm^3$/mole) in $\Delta V^*$ accompany the PK reaction. Up to pressures of about 10,000 psi, the value of $\Delta V^*$ itself is dependent upon the temperature as shown in Figure 4 (inset). At higher pressures, the decay curves probably are parallel (Fig. 4). The value of $\Delta V^*$ is independent of pH between pH 7.0 and pH 9.0. The large changes in $\Delta V^*$ associated with the PK catalyzed conversion of PEP to pyruvate probably reflect substantial conformational

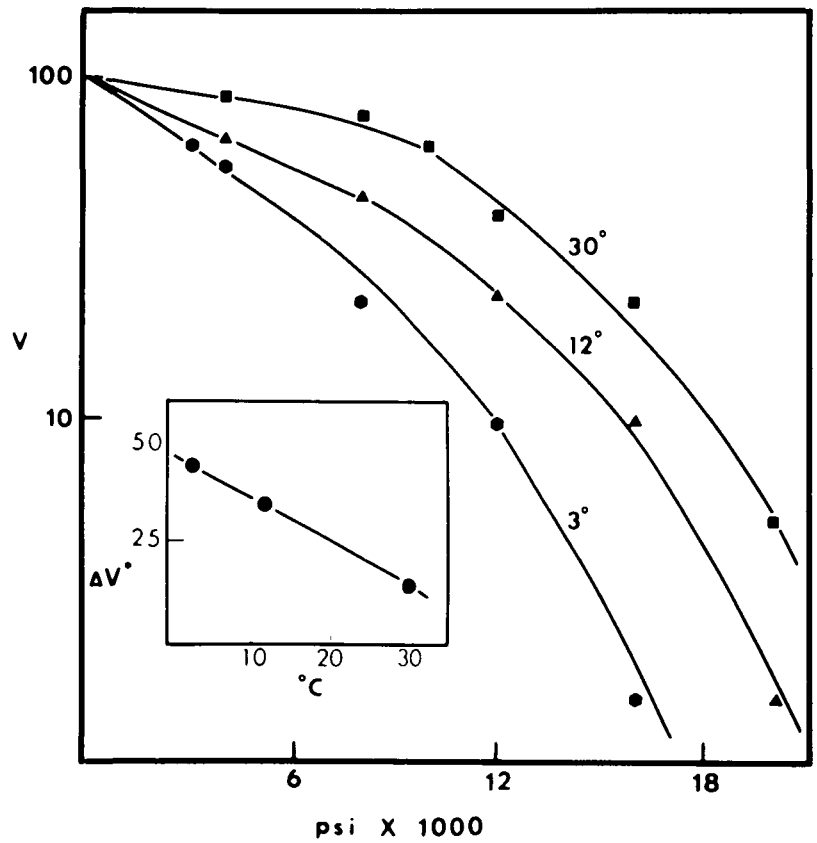



FIG. 4. Semilog plots of reaction velocity (v) versus pressure for maximum catalysis by rattail muscle pyruvate kinase at three different temperatures. Reaction velocity is standardized at each temperature to 100. A plot of the volume change of activation, $\Delta V^*$, as a function of temperature is shown in the inset. The values for $\Delta V^*$ are calculated for catalysis between 14.7 psi and 8000 psi.

changes in the protein catalyst (*see* Johnson and Eyring, 1970; Brandts, 1967).

*Reversibility of pressure-retarding effects on rattail muscle PK.* The effects of pressure on the maximum velocity catalyzed by PK are largely reversible at all temperatures and all pressures examined (Table 1). Two aspects of the recovery of control-levels of PK activity are of interest. (1) Release of pressure leads to an instantaneous increase in catalytic velocity. Such reversibility is commonly noted in the literature on pressure inactivation and denaturation of enzymes (Brandts, 1967; Morita and Becker, 1970). (2) During the first 15-30 seconds following decompression, the catalytic activities of PK are in fact higher than are control (1 atmosphere) rates (Table 1). These data suggest that conver-

TABLE 1. *Reversibility of pressure retardation of rattail muscle pyruvate kinase catalysis.**

| | Catalytic rate as % of rate at 14.7 psi | |
|---|---|---|
| Pressure, psi | Compressed | Decompressed |
| 14.7 | 100 | |
| 2000 | 79 | 100 |
| 4000 | 74 | 118 |
| 6000 | 64 | 121 |
| 8000 | 42 | 125 |
| 10000 | 32 | 100 |
| 12000 | 23 | 97 |
| 14000 | 11 | 89 |
| 16000 | 0 | 79 |

* The protocol in these experiments was as follows: (1) enzyme added at zero time, (2) desired pressure applied within 5 seconds, (3) reaction velocity was followed as a linear function of time for 15 seconds, then (4) the cell was decompressed within a second and the reaction velocity was followed again as a linear function of time for another 15 seconds. Assay conditions as described in Methods and Materials.

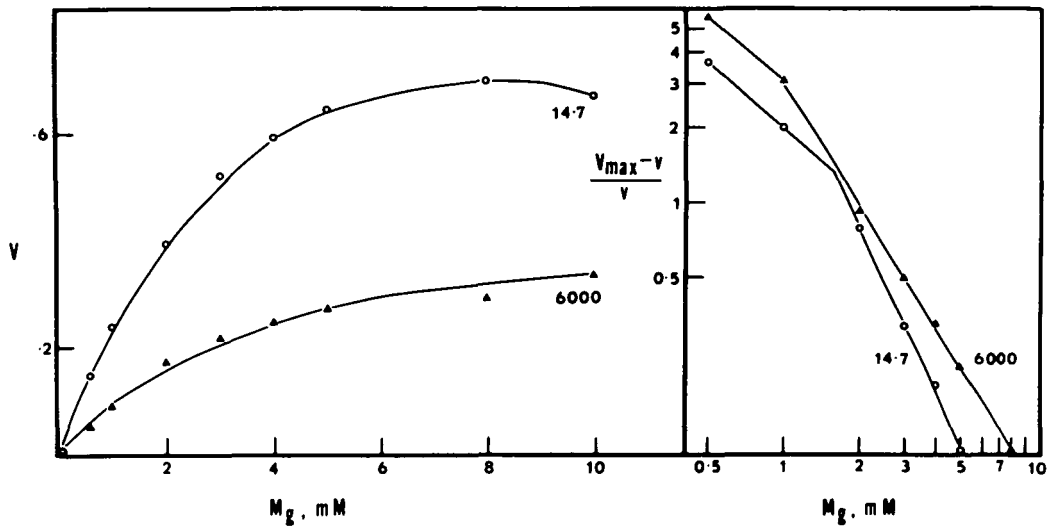



FIG. 5. $Mg^{2+}$ saturation curves for rattail muscle pyruvate kinase at 14.7 and 6000 psi (left panel). Hill plots of the data are shown in the right panel. The $K_a$ for $Mg^{2+}$ is taken as the concentration at which $\frac{V_{max}-v}{v} = 1.0$. Assay conditions are given in Materials and Methods.

sion from a stable, pressure-inhibited enzyme state to a stable depressurized enzyme may require passage through a metastable state which is catalytically unusually active.

*Effects of pressure on the activation energy.* The interacting effects of temperature and pressure are also seen in Arrhenius plots of PK activity at different pressures. As the pressure increases to 16, 000 psi, there is a gradual increase in the calculated activation energy (Table 2).

TABLE 2. *The activation energy for rattail muscle PK at various pressures.**

| Pressure, psi | Activation energy, Kcal/mole |
|---|---|
| 14.7 | 12.9 |
| 4000 | 16.1 |
| 8000 | 24.8 |
| 12000 | 25.9 |

* Calculated between 3° and 12°C for catalysis under saturating PEP and ADP concentrations. The activation energy was calculated from the Arrhenius equation:

$$\mu = 2.3\,R\,\frac{\log K_{T_1} - \log K_{T_0}}{1/T_0 - 1/T_1}$$

where $\mu$ is the activation energy, $R$ is the gas constant with a value of approximately 2, $K_{T_1}$ and $K_{T_0}$ are the rate constants at temperatures $T_1$ and $T_0$ given in °K.

The increase in activation energy with pressure suggests that the catalytic efficiency of the pressurized forms of rattail PK is reduced. These data, taken together with (1), the above noted large $\Delta V^*$ associated with the PK reaction, and with (2) the recovery characteristics, are consistent with an enzyme model displaying considerable conformational flexibility. We were therefore particularly interested in examining catalytic and regulatory properties of the enzyme at different pressures.

*$Mg^{2+}$–enzyme interactions.* As mentioned above, PK shares with other kinases an absolute requirement for a divalent cation, such as $Mg^{2+}$. The $Mg^{2+}$ saturation of the rattail enzyme shows positive site-site co-operative interactions (Fig. 5). The nature of this interaction can be assessed by the Hill equation:

$$\log \frac{v}{V_{max}-v} = n \log [\text{PEP}] - \log k \quad (1)$$

The constant, $n$, in the Hill equation is not an elementary kinetic parameter but is a measure of both the number of $Mg^{2+}$ binding sites and their strength of interaction (Atkinson, 1966). The $n$ value for $Mg^{2+}$ obtained from Hill plots of the data (Fig. 5, right panel) is about 2.4 and is

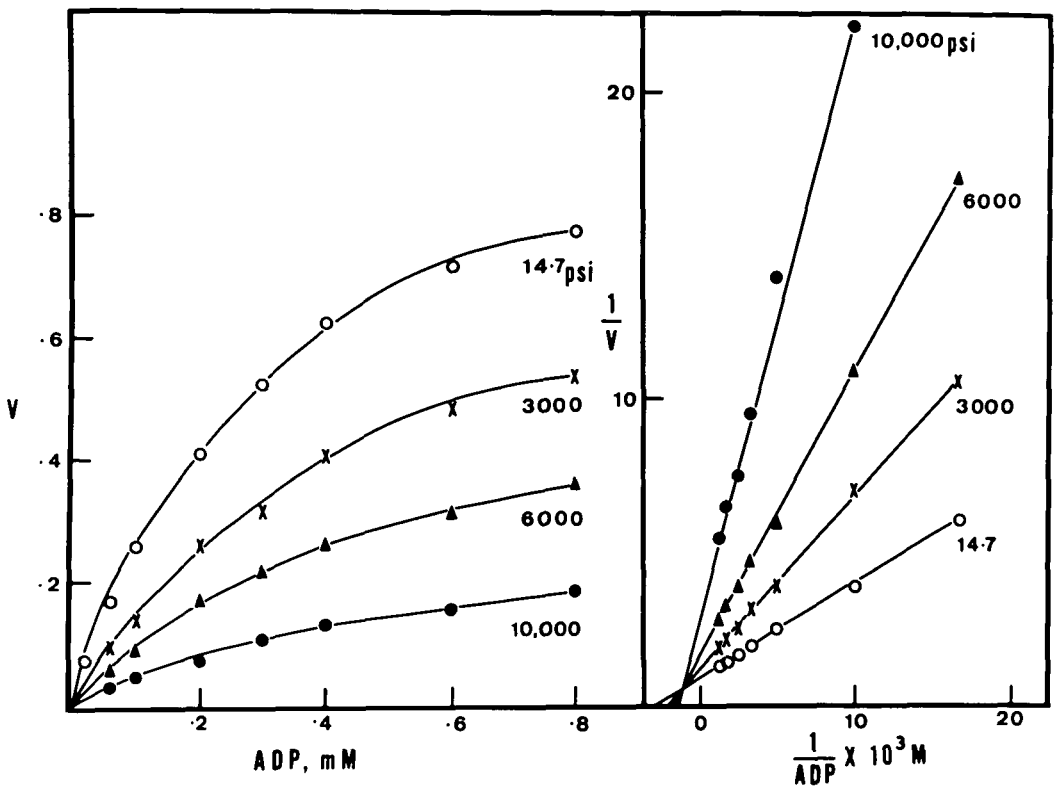



FIG. 6. ADP saturation curves for rattail muscle pyruvate kinase at four different pressures and at 3°C (left panel). Lineweaver-Burk plots of the data are shown in the right panel. Assay conditions are given in Materials and Methods.

unaffected by pressure. The apparent Michaelis constant, $K_a$, (Fig. 5, right panel) is about 2 mM both at 14.7 and 6000 psi. Consequently, the percent pressure inhibition of the activity of PK is comparable at all $Mg^{2+}$ concentrations.

From these data, we can conclude that pressure affects neither the apparent enzyme-$Mg^{2+}$ affinity nor the nature of the positive $Mg^{2+}$ site-site interactions in the rattail pyruvate kinase.

*ADP–enzyme interactions.* ADP saturation curves for the rattail muscle PK at various pressures and at 3°C are shown in Figure 6. The curves in form are Michaelis-Menten hyperbolas. The main effect of pressure is to decrease the $V_{max}$, but as indicated in Lineweaver-Burk plots of the data (Fig. 6, right panel), the $K_{m_{ADP}}$ increases slightly with pressure. $K_{m_{ADP}}$ values of 0.36, 0.50, and 0.57 are obtained at 14.7, 3000, and 6000 psi respectively. In consequence of the slight decrease in the apparent enzyme-ADP affinity, the retarding action of pressure is enhanced at low ADP values. This effect is in contrast to the action of pressure on the $K_a$ for $Mg^{2+}$ but is similar to the effect of pressure on the enzyme-PEP affinity.

*PEP-enzyme interactions.* PEP saturation curves for partially purified rattail muscle PK (Fig. 7) indicate that for this PK, as for other regulatory pyruvate kinases (Llorente *et al.*, 1970), PEP saturation involves positive site-site interactions. Hill plots are complex (Fig. 8, left panel): At high substrate concentrations, $n$ values are in the range of 2-3 with negligible effects of pressure on PEP site-site interactions. At low substrate concentrations, the $n$ value is reduced. Such complex Hill plots have also been reported for

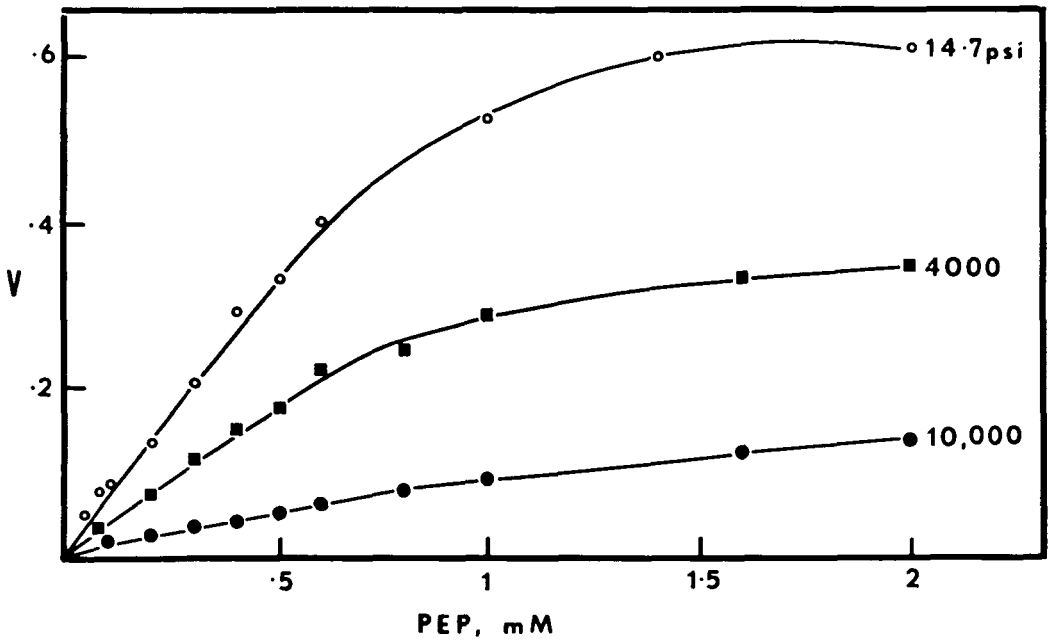



FIG. 7. PEP saturation curves for rattail muscle pyruvate kinase at three different pressures and at 3°C. Assay conditions are given in Materials and Methods.

king crab PK (Somero, 1969) as well as for adipose pyruvate kinases (Pogson, 1968). The apparent $K_{m_{PEP}}$ increases directly with pressure. At 10,000 psi, the apparent $K_m$ value increases from about 0.45 mM to 0.70 mM (Fig. 8, inset). As in the case of pres-

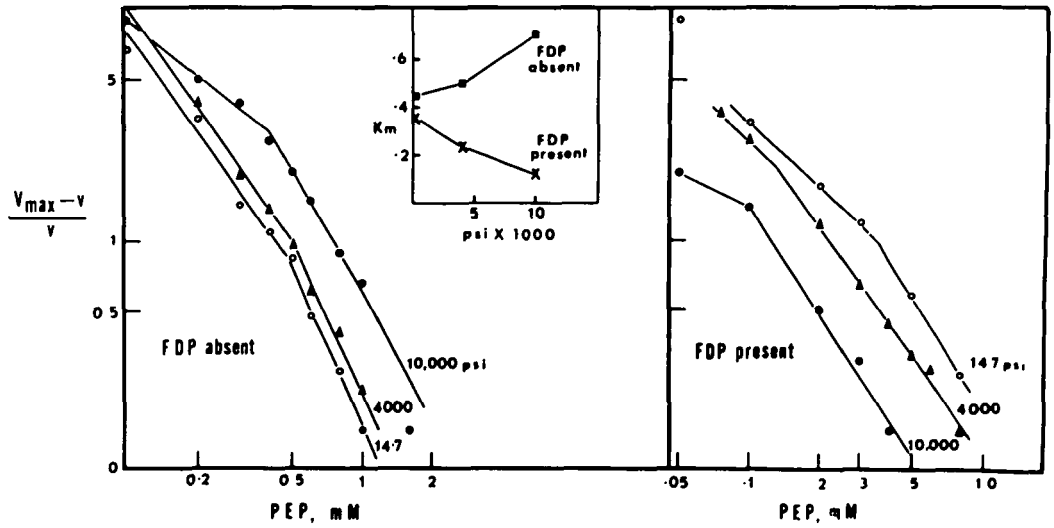



FIG. 8. Hill plots of PEP saturation curves for rattail muscle pyruvate kinase in the absence of FDP (left panel) and in the presence of 10 $\mu$M FDP (right panel). The apparent $K_m$ for PEP is taken as the concentration at which $\frac{V_{max} - v}{v} = 1.0$. The effect of pressure upon the apparent $K_m$ for PEP is shown in the inset. The $K_m$ is given in mM for the enzyme in the presence and absence of FDP.

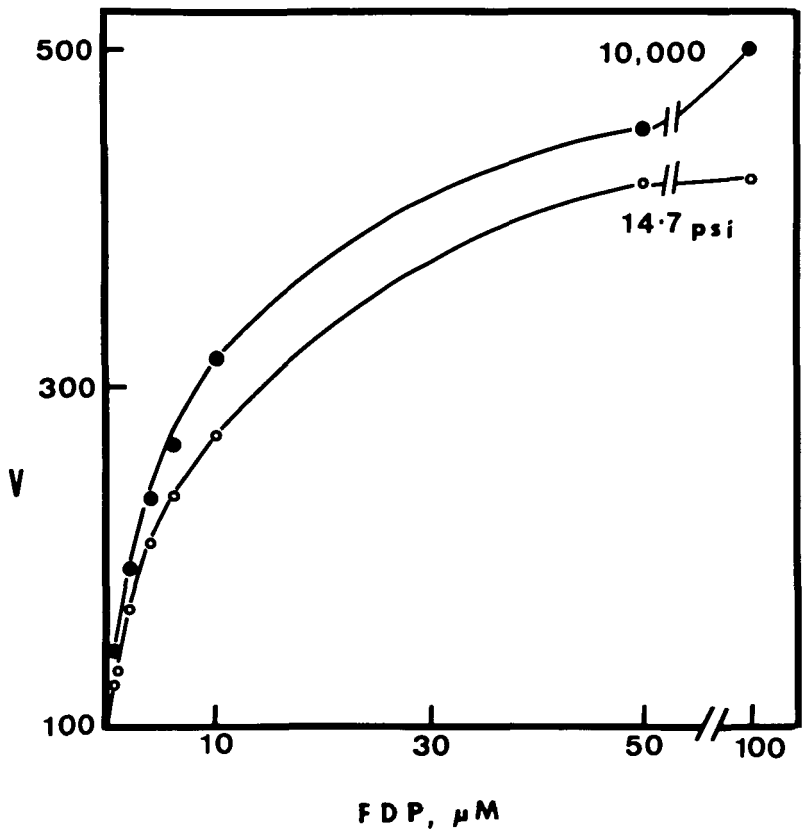



FIG. 9. Effect of FDP upon catalytic rate of rattail muscle pyruvate kinase at two different pressures and 3°C. Assay conditions are as in Materials and Methods except for the low (0.5 mM) levels of PEP. Reaction velocity (v) at both 14.7 and 10,000 psi in the absence of FDP is standardized to 100.

sure effects on the enzyme-ADP affinity, the increase in $K_{m_{PEP}}$ enhances the pressure-retardation of catalysis at low PEP concentrations. As we shall discuss below, these effects of pressure on enzyme-PEP interactions are abolished by FDP.

*FDP regulation of rattail muscle pyruvate kinase.* Rattail muscle pyruvate kinase, like the homologous trout enzyme (Somero and Hochachka, 1968), is strongly feedforward activated by FDP. The effect of pressure on FDP activation of the enzyme is shown in Figure 9. At all FDP values tested, high pressure slightly enhances the degree of FDP activation, but the overall effect is not quantitatively large, and the curves are of the same form. Hence, it is concluded that enzyme affinity for FDP is largely independent of pressure. This is an important result, for it implies that FDP regulation of PK occurs with comparable efficiency at all pressures, irrespective of any possible pressure-directed changes in the $V_{max}$.

Three further aspects of FDP control are pertinent (Fig. 8): (1) FDP decreases the apparent $K_{m_{PEP}}$ and increases the $V_{max}$, consequently leading to greater percent activation at low PEP levels. (2) PEP saturation curves in the presence of FDP are more hyperbolic (with $n$ values of 1 to 1.5) rather than sigmoidal (with $n$ values between 2-3). This conversion of a sigmoidal towards a hyperbolic saturation curve indeed is a reflection of the effect of FDP on the $K_m$ of PEP. (3) In the presence of FDP, the apparent $K_{m_{PEP}}$ is *reduced at high pressures* (Fig. 8, inset).

TABLE 3. *Summary of the values of* $K_m$ *and of* n *for PEP for* Coryphaenoides *muscle pyruvate kinase.*

| Characteristic | 14.7 psi | 4000 psi | 10,000 psi |
|---|---|---|---|
| at high PEP | | | |
| *n* value (no FDP) | 2.2 | 2.2 | 2.2 |
| (with FDP) | 1.6 | 1.5 | 1.5 |
| at low PEP | | | |
| *n* value (no FDP) | 1.4 | 1.2 | 0.9 |
| (with FDP) | 1.0 | 1.0 | 0.5 |
| $K_m$ in absence of FDP in mM | 0.42 | 0.50 | 0.75 |
| $K_m$ in presence of FDP in mM | 0.36 | 0.22 | 0.12 |

At 4000 psi, the $K_m$ is reduced to nearly 1/2 the value at 14.7 psi, and at 10,000 psi, it is about 1/3 the value at 14.7 psi (data summarized in Table 3). This effect of pressure on the $K_{m_{PEP}}$ is precisely opposite to that observed in the absence of FDP. Because of the reduction in the $K_m$ with pressure and the simultaneous FDP activation of the enzyme, at low PEP concentrations, the reaction velocity is determined by these kinetic properties rather than by energy-volume parameters.

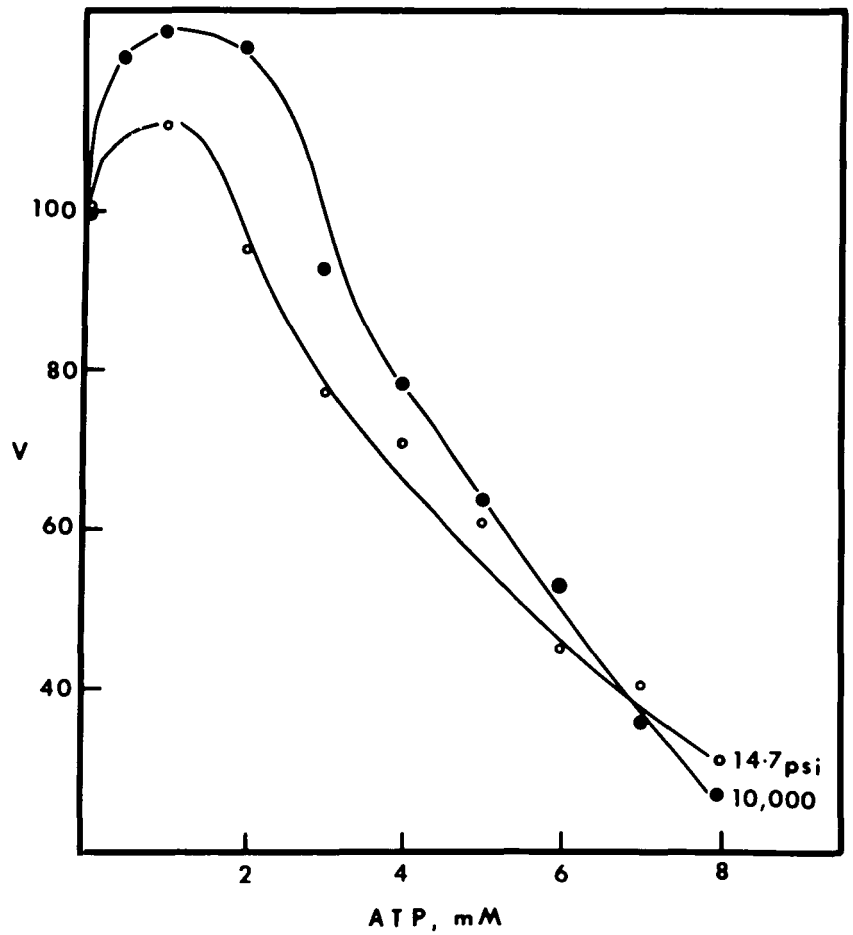



FIG. 10. ATP effects on rattail muscle pyruvate kinase activity at two different pressures and at 3°C. Assay conditions are as given in Materials and Methods.

*ATP-enzyme interactions.* For many unidirectional, energy-yielding reactions, such as that catalyzed by PK, the ratio of ATP/AMP, or the adenylate charge (*see* Atkinson, 1968), is an important control parameter. Thus, under energy-saturating conditions, phosphofructokinase is inhibited by high levels of ATP, while under energy-depleted conditions, it is activated by *high AMP levels,* which are taken to be metabolically equivalent to low ATP levels (Atkinson, 1968). In the case of the rattail PK, high ATP inhibits the enzyme; the apparent $K_m$ is pressure-independent and is in the range of 4 mM ATP (Fig. 10). This value is in agreement with data on other pyruvate kinases (Somero and Hochachka, 1968; Llorente *et al.,* 1970) but, in contrast to mammalian muscle PK, ATP inhibition is non-competitive with PEP. Hence, ATP does not affect the $K_{m_{PEP}}$. Also, in contrast to other PKs, low ATP concentrations activate the rattail enzyme, and this activation is somewhat enhanced by high pressure (Fig. 10). This effect, which has not been observed previously, suggests that for this enzyme, an important signal for both energy-depleted conditions and energy-saturating conditions would appear to be the concentration of ATP itself. In the presence of ATP, there is no change in the nature of PEP site-site interactions; hence, $n$ values for PEP are the same in the presence of ATP (assayed at 3 mM) as in its absence.

*FDP-ATP interactions.* The balance between FDP-feedforward activation and ATP-feedback inhibition of rattail muscle PK is determined by the FDP concentration (Table 4). At low (10 $\mu$M) FDP values and $K_i$ concentrations of ATP, FDP *reverses* ATP inhibition at 14.7 psi (catalytic rates return to the control rates obtained in the absence of both ATP and FDP); at higher pressures, FDP not only reverses, it *overrides,* the ATP inhibition, so that catalytic rates are comparable to those obtained in the presence of FDP alone (Table 4). This latter property, which in the rattail enzyme is *expressed only under pressure,* is commonly observed for mammalian liver PK (Tanaka *et al.,* 1967, Rozengurt *et al.,* 1969), fish PK (Somero and Hochachka, 1968), and molluscan PKs (Mustafa and Hochachka, 1971), when assayed at 1 atmosphere.

TABLE 4. *FDP-ATP interactions in the regulation of pyruvate kinase activity in the rattail. Conditions of assay: 0.5 mM PEP, 0.8 mM ADP, 8 mM $Mg^{++}$, 80 mM $K^+$, excess coupling reagents, and constant amounts of PK. ATP and FDP added as shown below.*

| Conditions | Activity as % of rates at 0.5 mM PEP | | |
|---|---|---|---|
| | 14.7 psi | 3000 psi | 8000 psi |
| 0.01 mM FDP | 169 | 225 | 240 |
| 1.0 mM ATP | 94 | 110 | 125 |
| 0.01 mM FDP<br>1.0 mM ATP | 122 | 274 | 260 |
| 4.0 mM ATP | 60 | 71 | 80 |
| 0.01 mM FDP<br>4.0 mM ATP | 99 | 213 | 210 |

Under conditions of ATP activation of rattail muscle PK (1 mM ATP) the effects of FDP and ATP do not appear to be additive. Comparable activation is observed by FDP alone (*see* Table 4 and Fig. 9).

*Possible irreversible changes in PK upon decompression.* The enzyme preparations used in the above kinetic experiments are stable for many days and probably many weeks. However, we noted an important, apparently irreversible, change in the $K_{m_{PEP}}$ which is a consequence either of decompression (during ascent of the organism after capture) or of partial purification—or both. In crude, high-speed supernatant solutions prepared from freshly captured rattail fish, the $K_{m_{PEP}}$ at 14.7 psi is about 1/10th the value of the partially purified preparations. A second important difference is an increased sensitivity of the $K_{m_{PEP}}$ to pressure: at 6000 psi, the $K_{m_{PEP}}$ increases 2.5-fold and at 10,000 psi by at least 4-fold. Furthermore, the PEP saturation kinetics of these fresh, crude PK preparations are Michaelis-Menten in form at all pressures (Fig. 11) and do not show site-site interactions, in contrast to the saturation curves noted for the partially purified preparations. However, overall

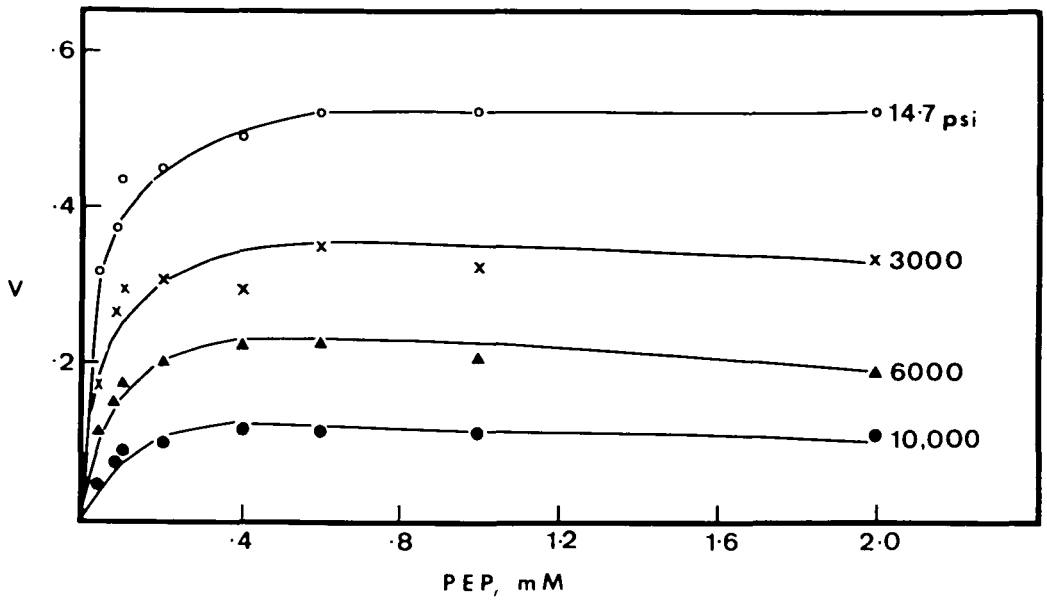



FIG. 11. PEP saturation curves for a crude preparation of rattail muscle pyruvate kinase at four different pressures and at 3°C. A freshly prepared, high speed supernatant solution was used directly as the source of pyruvate kinase activity.

pressure sensitivity of the $V_{max}$ is quite comparable to the salt-fractionated enzyme. These differences between the two kinds of enzyme preparations could be caused by metabolite modulators or cations, which would be present in the high-speed supernatant solutions but which would be removed during partial purification of the enzyme. However, we were unable to find any modulators which would reduce the $K_{m_{PEP}}$ by such a large factor. We therefore raise the possibility that, upon decompression and partial purification, an irreversible conformational change may occur in the rattail muscle PK which alters some of its kinetic properties. Further studies are necessary to settle this matter.

## SUMMARY REMARKS

As pointed out above, for many unidirectional, exergonic reactions such as that catalyzed by PK, the relative concentrations of the adenylates, ATP, ADP, and AMP, constitute an important control parameter (Atkinson, 1968). In the case of rattail muscle PK, low ATP levels can supply a signal for PK activation during energy-depleted conditions, while high ATP levels can supply a signal for PK inhibition during energy-saturating conditions. Such a simple control system, however, would not supply a direct link with other control sites in glycolysis. In contrast, FDP, an end-product of the phosphofructokinase reaction, supplies just such a link for co-ordinating the activities of phosphofructokinase and PK (Fig. 12). Unlike PK, phosphofructokinase is tightly regulated by ATP, ADP, AMP, and $P_i$ (*see* Mansour, 1970). Under energy-depleted conditions when AMP and $P_i$ levels increase (Williamson *et al.*, 1967), AMP and $P_i$ activate phosphofructokinase. This activation leads to increasing FDP and ADP levels which product-activate the enzyme and so autocatalytically increase their own concentrations even further. This process supplies both substrate (ADP) and positive modulator (FDP) for the PK reaction. As FDP levels increase, FDP can feedforward activate PK by (1) reducing the $K_{m_{PEP}}$, (2) increasing $V_{max}$, and (3) reversing or overriding any residual ATP

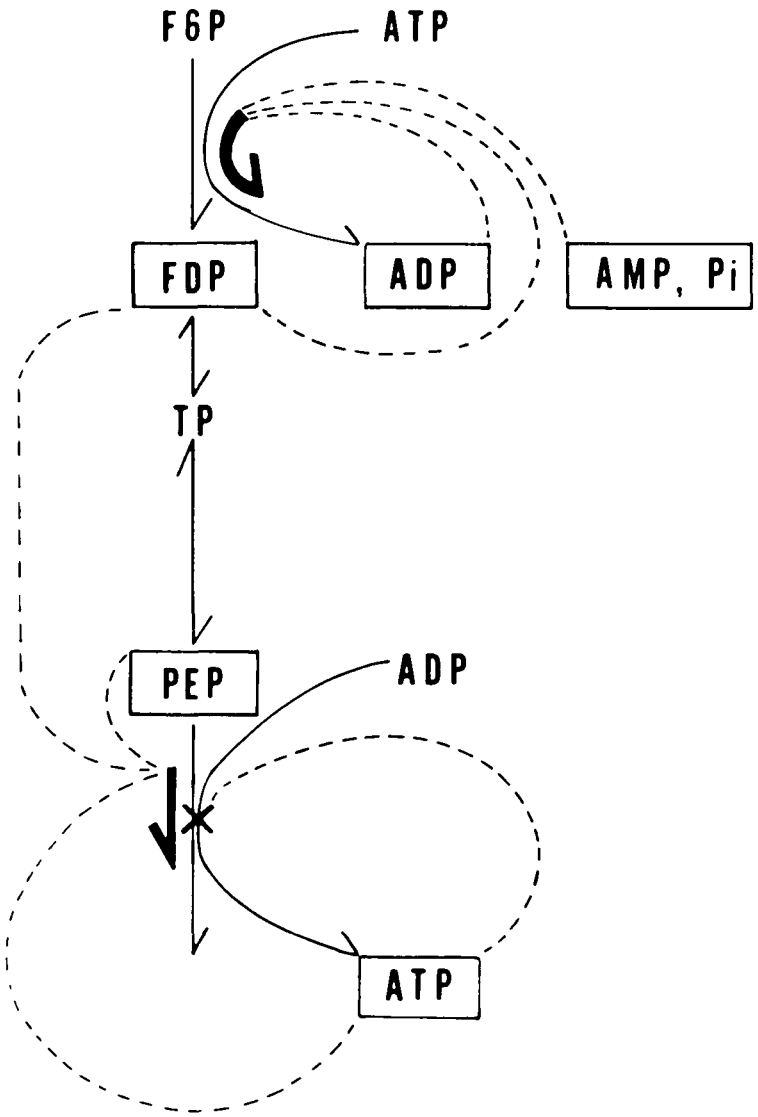
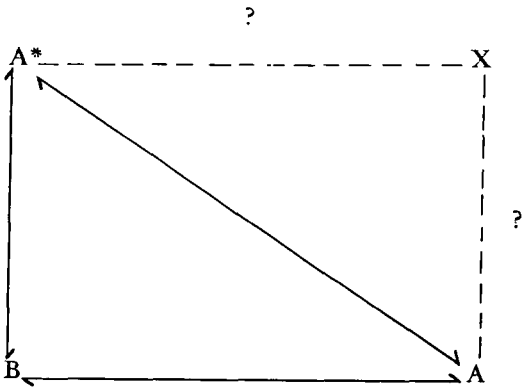



FIG. 12. Postulated control circuitry in the coordination of phosphofructokinase and pyruvate kinase activities in rattail muscle. In this scheme, energy-depleted conditions (high AMP and $P_i$ levels) lead to phosphofructokinase activation; ADP and FDP product activate PFK and so autocatalytically increase their own concentration. This process supplies both substrate (ADP) and positive modular (FDP) for the pyruvate kinase reaction, and could contribute to the general "flare-up" of glycolysis initiated at the PFK step.

inhibition (Fig. 12). From this point of view, FDP activation of PK can be considered as an amplification of the AMP signal of energy-depleted conditions and as part of a general "flare-up" of glycolysis initiated at the PFK step by high AMP and $P_i$ levels. This control circuitry in fish muscle glycolysis appears to be somewhat more complex than that in mammalian muscle, but less complex than that in liver, since a PK-bypass is not required for gluconeogenesis.

In regard to pressure, we can incorporate all the pertinent data on the properties of the rattail muscle PK into a model which assumes that enzyme transitions oc-

cur between at least four (and probably more) states as shown in the following diagram (*see* Susor and Rutter, 1968; Llorente *et al.*, 1970; Kayne and Suelter, 1968, for kinetic and physical-chemical evidence for different conformational states of mammalian pyruvate kinases):




A*: *metastable form (most active)*
A : *normal isolated forms (normally active)*
1. high $K_{m_{(PEP)}}$
2. sigmoidal PEP saturation
3. FDP reverses ATP inhibition

B : *pressurized forms (least active)*
1. high $K_{m_{(PEP)}}$
2. sigmoidal PEP saturation
3. FDP overrides ATP inhibition
4. $K_{m_{(PEP)}}$ stabilized to pressure by FDP
5. pressure-insensitive Michaelis constants for FDP, ATP and $Mg^{2+}$
6. increased activation energy

X : *normal in vivo forms (normally active)*
1. low $K_{m_{(PEP)}}$
2. hyperbolic PEP saturation
3. $K_{n_{(PEP)}}$ most pressure sensitive

In the absence of modulators, the PK reaction is markedly pressure-decelerated, but under low (physiological) concentrations of PEP, ADP, FDP, and $Mg^{2+}$, the catalytic rate can be rendered entirely pressure-independent by means of FDP modulation. Two major mechanisms appear to be involved most critically in FDP modulation of the rattail muscle pyruvate kinase. These involve:

(1) *FDP effects on enzyme-ATP affinity.* Since the balance between FDP-activation and ATP-inhibition is determined by FDP, an important aspect of FDP-control of PK is the modulation of ATP inhibition. *FDP overrides ATP inhibition of rattail PK only when the enzyme is under increased pressures*; at 1 atmosphere, FDP merely reverses ATP inhibition and catalytic rates return to the same levels as occur in the absence of both FDP and ATP.

(2) *FDP effects on enzyme-PEP affinity.* In the presence of even low levels of FDP, the $K_{m_{PEP}}$ is *reduced* by pressure. Since at low substrate levels FDP controls pyruvate kinase activity largely by altering the $K_{m_{PEP}}$, this property *buffers FDP-control of catalysis from any drastic effects of pressure irrepective of any effects of pressure upon the* $V_{max}$.

These catalytic and regulatory properties of the rattail muscle PK would seem to supply an adequate mechanism for avoiding large effects of pressure upon PK catalysis *per se* and upon control of that catalysis. As such, these properties presumably are of functional and selective importance to off-shore benthic organisms, such as the *Coryphaenoides,* which have evolved through much phylogenetic time in an environment of high hydrostatic pressure.

## REFERENCES

Atkinson, D. E. 1966. Regulation of enzyme activity. Annu. Rev. Biochem. 35:85-124.

Atkinson, D. E. 1968. Citrate and the citrate cycle in the regulation of energy metabolism, p. 23-40. *In* T. W. Goodwin [ed.], Metabolic roles of citrate. Academic Press, New York.

Brandts, J. F. 1967. Heat effects on proteins and enzymes, p. 25-72. *In* A. H. Rose [ed.], Thermobiology. Academic Press, New York

Bücher, T., and G. Pfleiderer. 1955. Pyruvate kinase from muscle, p. 435-440. *In* S. P. Colowick and N. O. Kaplan [ed], Methods in enzymology, vol. 1. Academic Press, New York.

Haglund, H. 1967. Isoelectric focusing in neutral and pH gradients—a technique of growing importance for fractionation and characterization of proteins. Sci. Tools 14:17-25.

Johnson, F. H., and H. Eyring. 1970. The kinetic basis of pressure effects in biology and chemistry, p. 1-44. *In* A. M. Zimmerman [ed.], High pressure effects on cellular processes. Academic Press, New York.

Kayne, F. J., and C. H. Suelter. 1968. The temperature-dependent conformational transitions of pyruvate kinase. Biochemistry 7:1678-1684.

Llorente, P., R. Marco, and A. Sols. 1970. Regula-

tion of liver pyruvate kinase and the phosphoenolpyruvate crossroads. Eur. J. Biochem. 13:45-54.

Mansour, T. E. 1970. Kinetic and physical properties of phosphofructokinase. Adv. enzyme regul. 8:37-51.

Morita, R. Y. 1967. Effects of hydrostatic pressure on marine micro organisms. Oceanogr. Mar. Biol. Annu. Rev. 5:187-203.

Morita, R. Y., and R. R. Becker. 1970. Hydrostatic pressure effects on selected biological systems, p. 71-83. *In* A. M. Zimmerman [ed.], High pressure effects on cellular processes. Academic Press, New York.

Mustafa, T., and P. W. Hochachka. 1971. Catalytic and regulatory properties of pyruvate kinases in tissues of a marine bivalve. J. Biol. Chem. 246:3196-3203.

Phleger, C. F., and A. Soutar. 1971. Free vehicles and deep-sea biology. Amer. Zool 11:409-418.

Pogson, C. I. 1968. Adipose-tissue pyruvate kinase. Properties and interconversion of two active forms. Biochem. J. 110:67-77.

Reynard, A. M., L. F. Hass, D. D. Jacobsen, and P. D. Boyer. 1961. The correlation of reaction kinetics and substrate binding with the mechanism of pyruvate kinase. J. Biol. Chem. 236:2277-2283.

Rozengurt, E., L. J. de Asúa, and H. Carminatti. 1969. Some kinetic properties of liver pyruvate kinase (Type L). II. Effect of pH on its allosteric behaviour. J. Biol. Chem. 244:3142-3147.

Somero, G. N. 1969. Pyruvate kinase variants of the Alaskan king crab: Evidence for a temperature-dependent interconversion between two forms having distinct—and adaptive—kinetic properties. Biochem. J. 114:237-241.

Somero, G. N., and P. W. Hochachka. 1968. The effect of temperature on catalytic and regulatory properties of pyruvate kinases of the rainbow trout and the Antarctic fish *Trematomus bernacchii*. Biochem. J. 110:395-400.

Susor, W. A., and W. J. Rutter. 1968. Some distinctive properties of pyruvate kinase purified from rat liver. Biochem. Biophys. Res. Commun. 30:14-20.

Tanaka, T., Y. Harano, F. Sue, and H. Morimura. 1967. Crystallization, characterization, and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. (Tokyo) 62:71-87.

Williamson, J. R., B. E. Herczeg, H. S. Coles, and W. Y. Cheung. 1967. Glycolytic control mechanisms. V. Kinetics of high energy phosphate intermediate changes during electrical discharge and recovery in the main organ of *Electrophorus electricus*. J. Biol. Chem. 242:5119-5124.