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Properties and Mechanism of Action of Creatine Kinase from Ox Smooth Muscle

ANION EFFECTS COMPARED WITH PYRUVATE KINASE

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1. An improved purification procedure for the brain-type creatine kinase from ox smooth muscle is described. 2. Michaelis constants show the characteristic dependence on the concentration of the second substrate: the derived constants are compared with those for the enzyme from ox brain. 3. Inhibition by iodoacetamide gives a biphasic curve and the total extent of the reaction depends on the enzyme concentration. The rate of inhibition at pH 8.6 is not affected by creatine plus MgADP or by a range of simple anions. Addition of creatine plus MgADP plus either NO_3^- or Cl^- ions affords 71.5 and 44% protection respectively. ADP could be replaced by 2-deoxy-ADP but not by $\alpha\beta$-methylene ADP, XDP, IDP, GDP or CDP. Nucleotides that did not protect would not act as substrates. 4. Difference-spectra measurements support the interpretation that addition of NO_3^- ions to the enzyme–creatine–MgADP complex causes further conformational changes in the enzyme accompanying the formation of a stable quaternary enzyme–creatine–NO_3^-–MgADP complex that simulates an intermediate stage in the transphosphorylation reaction. However, the enzyme structure is partially destabilized by quaternary-complex formation. IDP apparently fails to act as a substrate because it cannot induce the necessary conformational change. This behaviour is compared with that of rabbit skeletal-muscle creatine kinase. 5. With pyruvate kinase from rabbit muscle, anions activate in the absence of an activating cation and either inhibit or have no effect in its presence. 6. Both activation and inhibition were competitive with respect to the substrate, phosphoenolpyruvate, and curved double-reciprocal plots were obtained. The results may be interpreted in terms of co-operatively induced conformational changes, and this is supported by difference-spectra measurements. However, the Hill coefficient of 1 was not significantly altered. 7. Inhibition by lactate plus pyruvate is less than additive, indicating that both bind to the same site on the enzyme, whereas that by lactate plus NO_3^- is additive, indicating binding at separate sites. It is inferred that a quaternary enzyme–pyruvate–NO_3^-–MgADP complex could form, but no evidence was obtained to suggest that it possessed special properties comparable with those found with creatine kinase. The implications of these findings for the unidirectional nature of the mechanism of pyruvate kinase is discussed. 8. Lactate or α-hydroxybutyrate could not act instead of pyruvate to form a stable quaternary complex, although both activate the K^+-free enzyme. Only the former inhibits the K^+-activated enzyme. The activating cation both lowers the Michaelis constant for phosphoenolpyruvate and tightens up the specificity of its binding site.

Cytoplasmic creatine kinase (adenosine 5′-triphosphate–creatine phosphotransferase, EC 2.7.3.2) is generally recognized to occur in three forms, which result from the dimerization of two types of subunit. Thus skeletal muscle typically contains an enzyme composed of two M subunits whereas the brain enzyme contains two B subunits. The hybrid (MB) enzyme is frequently associated with heart muscle, although in some animals, such as the rabbit, the muscle enzyme is found, whereas in others, such as the chicken, the brain (BB) enzyme is found (Burger *et al.*, 1964; Eppenberger *et al.*, 1964; Dawson *et al.*, 1965). The 'brain' enzyme also occurs in mammalian smooth muscle (Burger *et al.*, 1964; Focant, 1970). These distinctions are based largely on a comparison of electrophoretic mobilities and it is not known whether or not the 'brain' creatine kinases found in different tissues are identical in other properties and are therefore coded by the same structural gene. Electrophoretic mobility in itself is not a very good criterion because the kinases of different mammals generally have closely similar electrophoretic mobilities, although some species variation does occur. The

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present paper is the outcome of an initial move towards solving this problem (Focant, 1970) by improving the purification procedure for the creatine kinase from ox smooth muscle and describing some of its properties.

One property of particular mechanistic interest found in rabbit skeletal-muscle creatine kinase is the ability of certain anions, such as NO_3^- and Cl^-, to stabilize the dead-end enzyme–substrate complex, creatine–MgADP–enzyme (Milner-White & Watts, 1971). This property has been investigated in the 'brain' enzyme of ox smooth muscle for the purpose of comparing the two enzyme types.

As an adjunct to this work it became of interest to compare the effects of anions on rabbit muscle pyruvate kinase. This led us to investigate whether or not pyruvate kinase could also form a dead-end complex that could be stabilized by anions in a manner analogous to that found with creatine kinase. The effects of various anions were found to be complex and dependent on whether or not the activating cation, K^+, was also present. However, no evidence could be obtained suggesting the formation of a dead-end complex similar to that found with creatine kinase. It is suggested that this may be of significance in connexion with the unidirectional nature of the pyruvate kinase reaction.

Materials and Methods

Materials

ADP, phosphocreatine, NADH, phosphoenolpyruvate, β-hydroxybutyrate and pyruvate kinase (type II, rabbit muscle) were obtained from Sigma (London) Chemical Co. Ltd., London W.6, U.K. ATP and lactate dehydrogenase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Other nucleotides came from Seravac Laboratories (Pty.) Ltd., Feltham, Middx., U.K. Creatine and iodoacetamide were purified as described previously (Milner-White & Watts, 1971). Other salts used were of AnalaR grade or the best obtainable from British Drug Houses Ltd., Poole, Dorset, U.K. Doubly distilled water was used for all solutions.

Pyruvate kinase for the difference-spectra measurements was prepared by the method of Tietz & Ochoa (1958) and traces of impurities were removed by Sephadex-block electrophoresis as described below.

Buffers. All buffers were made 1mM with β-mercaptoethanol unless otherwise stated. For starch-gel and Sephadex-block electrophoresis Tris–citrate buffer, pH8.4, was prepared by dissolving 10.5g of Tris base and 2.1g of citric acid in a final volume of 2 litres. Borate buffer, pH8.6, for the electrode vessels was prepared by dissolving 46.7g of boric acid in a final volume of 2 litres, the pH having been adjusted with 5M-NaOH. Other buffer systems are described in the text.

Purification of ox smooth-muscle creatine kinase

Extraction. The portion of the ox stomach (bonnet) containing the major smooth-muscle mass was obtained from the slaughterhouse immediately the animal was killed and transported in ice to the laboratory. The smooth muscle was dissected out within 1½–2h, rinsed in cold water, weighed and finely minced in a domestic electric mincer (Moulinex). The enzyme was extracted by grinding the minced tissue in a cold mortar with a little acid-washed sand and an equal volume of a cold solution containing 10mM-KCl, 1mM-EDTA and 1mM-β-mercaptoethanol, adjusted to pH7.5 with 1M-NaOH. The homogenate was centrifuged at approx. 13000*g* (MSE High-Speed 18) for 30min at 2°C and the supernatant decanted through glass wool to remove lipid. The pH was then readjusted to 7.4–8.0 with 1M-NaOH.

$(NH_4)_2SO_4$ fractionation. The extract was slowly fractionated at 0°C with finely ground $(NH_4)_2SO_4$ by using a magnetic stirrer and the fraction precipitating between 45–60% saturation collected by centrifugation as described above. The precipitate was dissolved in 2–5ml of the Tris–citrate buffer, pH8.4, and concentrated by dialysis under reduced pressure (Watts & Moreland, 1970) to about 1ml over 24h at 1°C. The jelly-like precipitate, composed mainly of tropomyosin (Focant, 1970), was removed by centrifugation at full speed (approx. 380000*g*) in the MSE High-Speed 18 centrifuge in the 15ml angle rotor for 45min at 4°C.

Sephadex-block electrophoresis. The supernatant from the high-speed centrifugation was then purified batchwise by electrophoresis on a block of Sephadex G-100 [Pharmacia (G.B.) Ltd., London W.13, U.K.] as described by Kumudavalli *et al.* (1970). The sample (1ml) containing 50mg of protein/ml was loaded on to a strip of filter paper (16.5cm × 3.0mm × 2.0mm; MN paper for electrophoresis, Macherey, Nagel and Co., D-516 Duren, Germany) which was inserted across the width and 3cm from the cathode of a Sephadex G-100 block (20cm × 30cm × 0.5cm) in Tris–citrate buffer, pH8.4. The block was connected by the thick filter-paper wicks to electrode vessels containing the borate buffer, pH8.6, and assembled on the refrigerated bed of the Phaerograph (Mini 65) electrophoresis machine (obtainable from Northern Media Supply) set to −3°C. Electrophoresis was carried out for 4h at 200V (constant volts) at 30mA after which the block was divided transversely into 1cm sections each of which was transferred to a sintered-glass Buchner funnel (porosity grade 3) and the Sephadex eluted with 5ml of the Tris–citrate buffer, pH8.4, before filtering under slightly reduced

pressure. Assay of the filtrates for creatine kinase activity showed that the enzyme migrated with the discontinuity front characteristic of this buffer system. The active fractions were reconcentrated by vacuum dialysis as described above and again submitted to electrophoresis under the same conditions. The resulting enzyme preparation gave only one band when analysed by starch-gel electrophoresis (Watts & Moreland, 1970) using a heavy loading. The average specific activity obtained from several preparations was 100 μmol/min per mg of protein and the maximum obtained was 146. The stock enzyme solution was stored at 4°C in the Tris–citrate buffer containing 10mM-β-mercaptoethanol to minimize oxidation of the essential thiol groups. Freezing the enzyme solution caused considerable loss of catalytic activity and even at 4°C activity was steadily lost with a half-life of 1–2 weeks.

The protein concentration was determined by using the specific absorbance of 0.88 ml/mg per cm at 280 nm given for rabbit creatine kinase by Kuby & Noltmann (1962) and for the enzyme from ox brain (Wood, 1963).

Enzyme assays

Creatine kinase activity in the forward direction was followed by the coupled spectrophotometric assay of Tanzer & Gilvarg (1959) in which the product, ADP, is rephosphorylated by phosphoenolpyruvate and the pyruvate formed is reduced to lactate at the expense of NADH that is measured at 340 nm. Assays were carried out in 0.5 ml cuvettes in a Unicam SP.800 spectrophotometer thermostatically maintained at 30°C. The assay mixture contained 25mM-Tris–acetate buffer, pH 8.6, magnesium acetate, 10mM; phosphoenolpyruvate, 3mM; NADH, 0.15mM; potassium acetate, 50mM; pyruvate kinase, 0.01 mg; lactate dehydrogenase, 0.01 mg; and concentrations of ATP and creatine as described in the text. The two coupling enzymes were dialysed before use against a solution of the Tris–acetate buffer, pH 8.6, containing 10mM magnesium acetate, to remove $(NH_4)_2SO_4$. The reaction was started by the addition of ATP and specific activities were calculated as μmol/min per mg of creatine kinase assuming a molar extinction coefficient for NADH of 6220 litre·mol^{-1}·cm^{-1} (Tanzer & Gilvarg, 1959).

Assay for creatine kinase in the reverse reaction was carried out by using the titrimetric procedure and conditions described by Milner-White & Watts (1971).

Pyruvate kinase activity was measured spectrophotometrically at 340 nm and 30°C by coupling to the lactate dehydrogenase reaction as described above. The standard assay mixture contained, per ml: Tris–acetate buffer, pH 8.6, 25mM; ADP, 1mM: potassium acetate, 50mM; lactate dehydrogenase, 0.01 mg; phosphoenolpyruvate, 2mM; magnesium acetate, 3mM; cysteine, 1mM; NADH, 0.15mM; and pyruvate kinase, 2.24 μg, which was used to start the reaction. For the range of anions used (see Fig. 1) the activity of lactate dehydrogenase was not affected by more than 25%. This is not sufficient to affect the reliability of the assay.

In the experiments in which the effect of pyruvate on enzyme activity was being investigated the enzyme activity was measured titrimetrically in a Radiometer pH-stat at pH 9.0 and 30°C with 24.2mM-HCl in the syringe burette.

Difference-spectra measurements

These were made with a Cary 16S spectrophotometer, thermostatically maintained at 30°C, in split path-length cells, the path-length of each half-cell being 4.5 mm. Both cells for test and reference were set up in exactly the same way so that the baseline could be checked before mixing the front and back compartments of test cell. The reference was mixed after scanning to recheck the baseline. Small differences were usually found between the position of the baseline obtained before and after mixing, although repeat runs showed that this did not affect the essential features of the difference spectrum. The baseline differences appeared to result from a change in the light-scattering properties of the enzyme solution when it was diluted by mixing the contents of the two cell compartments. To minimize this effect all solutions were filtered through a Swinnex filter containing a 0.45 μm Millipore disc just before use.

Iodoacetamide-inhibition experiments

These were carried out in the absence of β-mercaptoethanol in a solution containing 25mM-Tris and 7mM-sodium acetate; adjusted to pH 8.6 with NaOH at 30°C, in a total volume of 1.05 ml. The β-mercaptoethanol in the stock creatine kinase solution was removed by filtration through a column (1.5 cm × 30 cm) of Sephadex G-25. The enzyme and any other additions (concentrations are given in the legends to the figures) were equilibrated for 10 min before the reaction was started with 0.05 ml of iodoacetamide in the same buffer solution. Samples (0.1 ml) were transferred immediately and after appropriate time-intervals into 0.9 ml of 5mM-cysteine (free base) solution in ice. The residual creatine kinase activity was determined spectrophotometrically within 2 h as described above.

Results

Creatine kinase

Kinetic properties. Fig. 1 shows the effects on the initial velocity of the creatine kinase reaction of

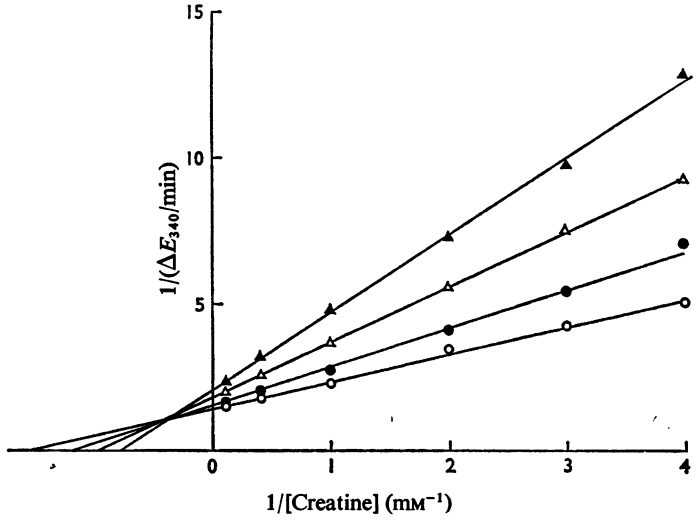

Fig. 1. *Lineweaver–Burk plot showing the effects of various creatine concentrations at several fixed concentrations of MgATP*

The fixed concentrations of MgATP used were: ▲, 0.25mM; △, 0.5mM; ●, 1.0mM, and 2.0mM. ○, The creatine kinase concentration was 0.022mg/ml. Other conditions were as described in the Materials and Methods section.

various concentrations of creatine in the presence of several fixed concentrations of MgATP. A similar result was obtained when, in a separate experiment, the MgATP concentration was varied at several fixed concentrations of creatine. In these experiments the total Mg^{2+} ion concentration was maintained at 10mM to ensure that all the nucleotide substrate was in the form of the Mg complex. The lines through the results, expressed as Lineweaver–Burk plots, were computed with an Olivetti P203 desk computer programmed for linear regression analysis with equal emphasis on all points. The results show that the Michaelis constant for each substrate is dependent on the concentration of the second substrate. Assuming equilibrium kinetics, dissociation constants for creatine, K_a and for $MgATP^{2-}$, K_b, from the free enzyme and from the binary complex of the enzyme with the second substrate, K'_a and K'_b respectively, have been calculated by the graphical method of Florini & Vestling (1957) for a two-substrate system. The derived secondary plots of intercept against each substrate concentration are shown in Fig. 2. The kinetic constants are compared in Table 1 with those for ox brain and ox skeletal-muscle creatine kinase (Jacobs & Kuby, 1970).

Nucleotide specificity. For comparison with the inhibition data obtained with various nucleotides (see below), the ability of various nucleotide diphosphates to act as substrates in the reverse reaction was investigated titrimetrically at pH8.0. The ratio of magnesium acetate to nucleotide was 1:1. At 1.0mM concentration no activity could be detected with 2-deoxy-ADP, β-methylene-ADP, XDP, IDP, GDP or CDP. Raising the concentration to 10mM revealed that 2-deoxy-ADP could act as a substrate, giving about 50% of the activity found with ADP. All the other nucleotides remained inactive.

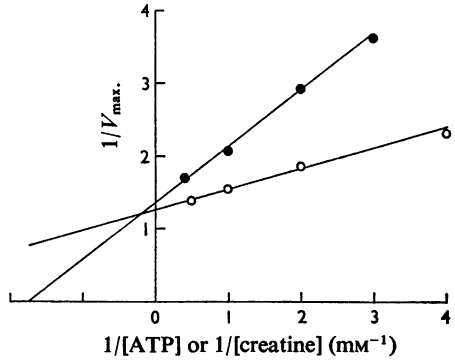

Fig. 2. *Secondary plots of the data from Fig. 1 showing the variation of maximum velocity as a function of substrate concentration*

●, Creatine concentration varied; ○, MgATP concentration varied. The units of $V_{max.}$ are ΔE_{340}/min.

Table 1. *Comparison of the kinetic constants for creatine kinase from ox smooth muscle with those from ox brain and ox skeletal muscle at 30°C*

The results for smooth muscle were obtained at pH8.6 and those for brain and skeletal muscle (Jacobs & Kuby, 1970) at pH8.8. Abbreviation: Cr, creatine.

Kinetic constant	Reaction	Value of constant (mM)		
		Smooth muscle	Brain	Striated muscle
K_a	E+Cr ⇌ Cr-E	2.20	29	53
K_b	E + ATP ⇌ E-ATP	0.75	0.93	0.97
K'_a	E-ATP+Cr ⇌ Cr-E-ATP	0.58	3.7	21
K'_b	Cr-E+ATP ⇌ Cr-E-ATP	0.20	0.13	0.78

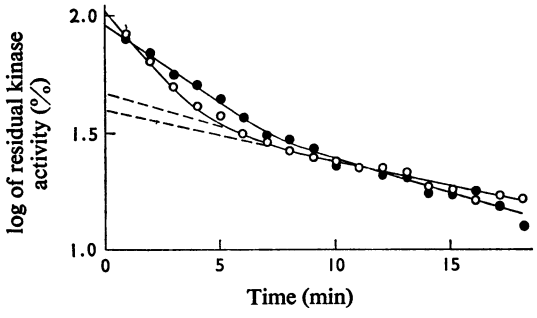

Fig. 3. *Semi-logarithmic plot showing the inhibition of creatine kinase by iodoacetamide in Tris–acetate buffer, pH8.6 and I 0.007, at 30°C*

The reaction mixture contained creatine kinase (0.45mg/ml) and iodoacetamide (●, 0.25mM, or ○, 0.5mM). Other experimental details are given in the Materials and Methods section.

Inhibition by iodoacetamide. The initial velocity of inhibition of ox smooth-muscle creatine kinase in Tris–acetate buffer, pH8.6 and *I* 0.007, containing no β-mercaptoethanol, was proportional to the concentration of iodoacetamide used. However, with 6.7μM enzyme (assuming a mol.wt. of 81000) and 0.5mM-iodoacetamide, not more than 80% inhibition could be obtained. Increasing the enzyme concentration to 22.1μM resulted in an inhibition of about 95%. Semi-logarithmic plots of percentage inhibition against time (Fig. 3) gave typical biphasic curves. Extrapolation of the curve for the secondary reaction to zero time cut the ordinate at approximately 50% inhibition.

Effects of substrates and anions. The effect of creatine plus MgADP was investigated by using 8mM-creatine and either 0.25mM- or 4mM total MgADP (molar ratio Mg^{2+}/ADP = 1:1). In the presence of either 70mM- or 100mM-acetate ions these substrate concentrations were without effect on the progress curve obtained for inhibition by iodoacetamide alone.

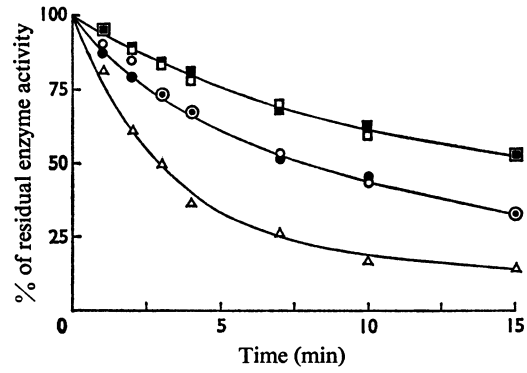

Fig. 4. *Effects of anions on the inhibition of smooth-muscle creatine kinase by iodoacetamide in the absence and presence of the dead-end complex, creatine plus MgADP, at 30°C*

The reaction mixture contained: enzyme, 1.5mg/ml; 0.25mM-iodoacetamide; 7mM-Tris–acetate buffer, pH8.6; and: △, no additive [similar curves were obtained in the presence of 0.1M-sodium acetate, -NaCl or -$NaNO_3$, and in the presence of 8mM-creatine plus either 0.25mM- or 0.4mM-MgADP (ratio 1:1) in the presence and absence of 0.1M-sodium acetate]; ○, 8mM-creatine plus 0.25mM-MgADP plus 100mM-NaCl; ●, 8mM-creatine plus 4mM-MgADP plus 100mM-NaCl; ■, 8mM-creatine plus 0.25mM-MgADP plus 100mM-$NaNO_3$; □, 8mM-creatine plus 4mM-MgADP plus 100mM-$NaNO_3$.

Likewise the addition of a range of concentrations of acetate, Cl^-, NO_3^- and SO_4^{2-} ions alone produced only small and unreproducible effects. However, addition of 100mM-NO_3^- or Cl^- ions in the presence of the dead-end complex resulted in protection against inhibition. The degree of protection obtained was the same with either 0.25mM- or 4mM-ADP in the reaction mixture (Fig. 4), 71.5% by NO_3^- ions and 44% by Cl^- ions relative to the activity with acetate ions.

Nucleotide specificity in the nitrate-stabilized dead-end complex. The ability to protect against inhibition

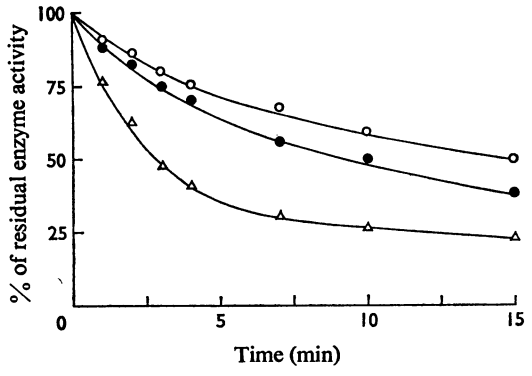

Fig. 5. *Effects of various nucleotides in the NO_3^--ion-stabilized dead-end complex on the rate of inhibition of smooth-muscle creatine kinase by iodoacetamide at 30°C*

The reaction mixture contained: enzyme, 0.55mg/ml; 7mM-Tris–acetate buffer, pH8.6; 0.25mM-iodoacetamide; and: ○, creatine, 8mM; MgADP (ratio 1:1), 1mM; $NaNO_3$, 0.1M; ●, the ADP replaced by 2-deoxy-ADP; △, the ADP replaced by XDP, αβ-methylene-ADP, GDP, IDP or CDP, or without any additives.

by iodoacetamide was used as a measure of the effectiveness with which various nucleoside diphosphates, at 1mM concentration, could form the nitrate-stabilized dead-end complex. Fig. 5 shows that 2-deoxy-ADP was almost as effective as ADP itself and gave 64% protection. With αβ-methylene-ADP, XDP, IDP, GDP or CDP no protection was observed at all and the inhibition curves coincided with that obtained in the absence of substrates.

Difference-spectra measurements. The addition of creatine plus MgADP to ox stomach creatine kinase produces a small difference spectrum with minima at approx. 265, 286 and 296nm. The enzyme appeared to become in some way destabilized because, although the minima at 286 and 296nm did not change appreciably with time, that at 265nm progressively increased (Fig. 6*a*). No explanation was found for this phenomenon, which still occurred after the addition of NO_3^- ions to form the protective creatine–MgADP–enzyme–NO_3 complex (Fig. 6*b*). However, the formation of this complex resulted in a very large increase in the minimum at 296nm, which was accompanied by a marked shift to higher wavelengths. This new feature of the spectrum was relatively stable.

When the effect of MgIDP plus creatine was investigated an initial difference spectrum was obtained but with only a single minimum between 280 and 300nm (Fig. 6*c*). The lower minimum, near to 260nm (Fig. 6*c*) was much more stable than that obtained with

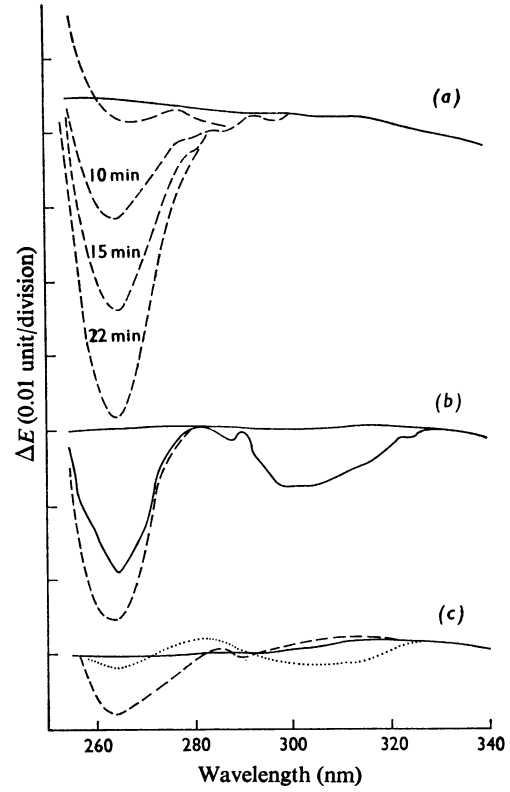

Fig. 6. *Difference-spectra measurements on ox stomach creatine kinase*

Details of procedure are given in the Materials and Methods section. (*a*) Effect of creatine plus MgADP. Into the front compartment of each divided cell was pipetted 1.2ml of a mixture containing 2.25ml of 50mM-Tris–acetate buffer, pH8.5, and 0.5ml of enzyme (10mg/ml). Into the rear compartment was pipetted 1.2ml of a mixture containing 2ml of 80mM-creatine, 0.05ml of 10mM-ADP and 0.2ml of 50mM-magnesium acetate and 0.5ml of the Tris–acetate buffer. ——, Baseline; – – – –, difference spectrum after mixing the contents of the 'test' cell and after the times shown. (*b*) Effect of creatine plus MgADP plus NO_3^- ions. The experiment was carried out as described for (*a*) except that immediately after mixing and running the first difference spectrum (not shown) 0.025ml of 1M-$NaNO_3$ was added to each compartment of both cells and the contents of the 'test' cell were remixed before running the difference spectrum (——). Rerunning this spectrum a few minutes later (– – – –) showed the same progressive hypochromic effect in the 265nm region as was observed in experiment (*a*). (*c*) Effect of creatine plus MgIDP with and without NO_3^- ions. The experiment was carried out as described for (*b*). – – – –, Difference spectrum for creatine plus MgIDP. · · · ·, Difference spectrum for

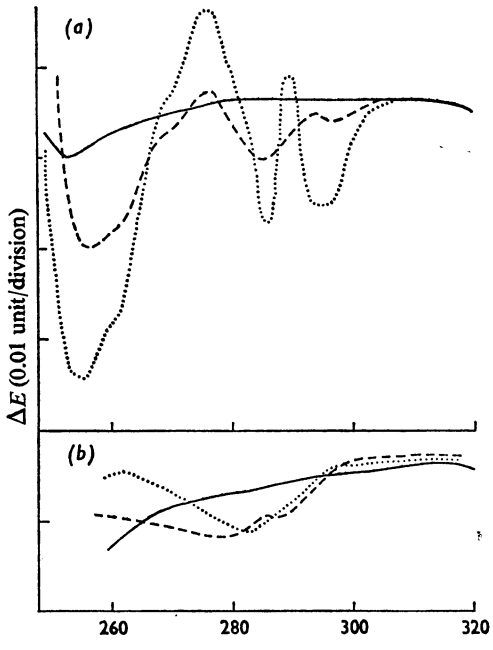

Fig. 7. *Difference-spectra measurements on rabbit muscle creatine kinase*

The experiments were carried out as described in Fig. 6 except that the front compartment of the double-sector cell contained 1.2ml of a mixture containing 0.25ml of enzyme (25mg/ml) and 2.5ml of the Tris–acetate buffer, pH8.5. (*a*) ——, Before mixing; – – – –, difference spectrum for creatine plus MgADP; · · · ·, after addition of $NaNO_3$ to both cells. (*b*) The same experiment as for (*a*) except that ADP was replaced by the same concentration of IDP.

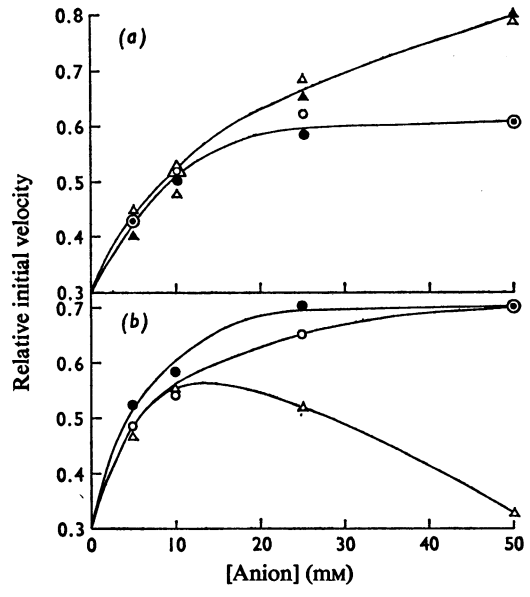

Fig. 8. *Activation of pyruvate kinase by anions in the absence of an activating cation*

The enzyme mixture was pre-dialysed against Tris–acetate buffer, pH8.6, to remove $(NH_4)_2SO_4$. All anions were added as the sodium salts. (*a*), ▲, acetate; △, Cl^-; ○, lactate; ●, NO_3^-; (*b*), ○, ClO_3^-; ●, SO_4^{2-}; △, PO_4^{3-}.

ADP, and repeat scans traced the same curve. The further addition of NO_3^- ions decreased this peak in magnitude but produced a broad shallow trough in the 300–320nm region similar to that found with ADP, although of less magnitude and lacking the fine detail around 288nm.

For comparison, similar experiments were carried out on rabbit muscle creatine kinase. Although the final concentration of rabbit muscle enzyme used was only slightly higher than that used with the ox stomach enzyme, 13μM as against 11.4μM, the difference spectra obtained were much more pronounced (Fig. 7). Addition of creatine plus MgADP gave a difference spectrum that was similar in shape to that obtained with the ox enzyme (Fig. 7*a*), but no instability was observed. On addition of NO_3^- ions the magnitude of all the peaks of the difference spectrum were enhanced but the general shape remained the same. With creatine plus MgIDP a very ill-defined difference spectrum was obtained, with no clear minimum in the 260nm region (Fig. 7*b*). As with the ox enzyme the further addition of NO_3^- ions caused an increase in the absorbance in this region but, in contrast, no great change in the 280–300nm region.

creatine plus MgIDP plus $NaNO_3$. No correction was applied for the approx. 2% dilution caused by adding the $NaNO_3$ solution since test and reference cells would be affected to the same extent. ——, Baseline.

Pyruvate kinase

Effects of anions. When pyruvate kinase is used as supplied by Sigma the $(NH_4)_2SO_4$ present activates the pyruvate kinase. Under these conditions and in the absence of K^+ ions, univalent anions (50mM), added as the sodium salts, such as NO_3^-, DL-lactate, Cl^- and acetate were either without effect or caused slight inhibition. Multivalent anions were markedly inhibitory; 50mM-SO_4^{2-} and 25mM-PO_4^{3-} caused 50% and 40% inhibition respectively.

After the enzyme mixture had been dialysed overnight against the Tris–acetate buffer, pH8.6, all the anions tested were found to activate (Fig. 8) although PO_4^{3-} ions became inhibitory at high concentrations, probably by complexing with the activating Mg^{2+} ion (Fig. 8*b*). Dialysed enzyme was therefore used for all subsequent experiments, acetate being chosen as the buffer anion because in similar experiments on creatine kinase it did not form an inhibitory complex with particular enzyme–substrate combinations.

Attempts to determine the binding constants for individual anions revealed that whereas PO_4^{3-} and DL-α-hydroxybutyrate gave linear double-reciprocal plots, those for DL-lactate and Cl^- were biphasic with an inflexion at about 2.5mM-anion (Fig. 9). By extrapolation of such plots binding constants of 5–6mM were obtained for Cl^-, NO_3^-, lactate and α-hydroxybutyrate ions and 11mM for PO_4^{3-} ions.

Investigation of the dead-end complex, pyruvate–MgADP–enzyme–NO_3^-. The titrimetric assay was used for this investigation because pyruvate is a substrate for the lactate dehydrogenase used in the coupled enzyme assay. Both pyruvate and NO_3^- ions are good inhibitors when added separately to the K^+-activated pyruvate kinase reaction (Table 2). If the anion-stabilized dead-end complex pyruvate–MgADP–enzyme is capable of being formed it might be expected, by analogy with creatine kinase, that addition of both pyruvate and NO_3^- ions to the assay mixture would cause strong inhibition by combining on the enzyme with the MgADP present in the assay mixture. The result of this experiment, at concentrations of anions that fall in the first-order region relative to pyruvate kinase inhibition, is shown in Table 2. The inhibition obtained was no greater than expected from the sum of each anion when added separately.

Investigation of the ability of hydroxy acids to form an anion-stabilized dead-end complex. Consideration of the reaction pathway of the pyruvate kinase reaction leads to the conclusion that if the enzyme is active in the reverse reaction then the true substrate would be enolpyruvate. Experimental evidence in support of this view was presented by Rose (1960). Hence the possibility that lactate and α-hydroxybutyrate might mimic enolpyruvate and form the anion-stabilized dead-end complex was investigated. Table 2 shows that the inhibition of K^+-activated pyruvate kinase by pyruvate plus DL-lactate is considerably less than additive and hence it may be inferred that the hydroxy acid binds to the pyruvate-binding site on the enzyme.

In the absence of an activating cation both DL-lactate and NO_3^- ions separately activate the enzyme and when added together the extent of activation is approximately additive, suggesting that each binds at a separate site on the enzyme but that there is no synergistic effect (Fig. 10). Binding of both anions appears to be competitive with phosphoenolpyruvate so that at infinite substrate concentrations the activation by both anions is eliminated (Fig. 10). Hill plots showed that neither NO_3^- nor lactate has any marked effect on the interaction between the enzyme subunits. A small increase in the Hill coefficient from 1.0 for the native enzyme to 1.3 in the presence of NO_3^- ions was considered barely significant. When these anions were tested with the K^+-activated enzyme the double-reciprocal plots showed that lactate, like pyruvate, causes inhibition that is competitive with phosphoenolpyruvate and, again, when added with NO_3^- ions gives a degree of inhibition that is approxi-

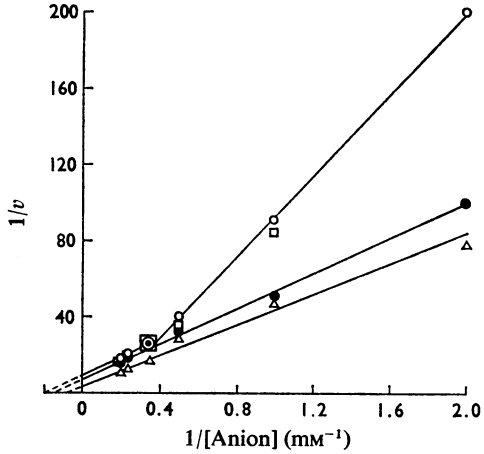

Fig. 9. *Lineweaver–Burk plots showing the effects of some anions on the activity of pyruvate kinase in the absence of an activating cation*

The enzyme mixture was pre-dialysed against Tris–acetate buffer, pH8.6, to remove $(NH_4)_2SO_4$. All anions were added as the sodium salts: △, PO_4^{3-}; ● α-hydroxybutyrate; ○, lactate; □, Cl^-.

Table 2. *Effects of pyruvate, NO_3^- and lactate ions on the activity of K^+-activated pyruvate kinase*

The enzyme was assayed titrimetrically as described in the Materials and Methods section.

Anion	Inhibition (%)
Pyruvate (20mM)	27.5
Nitrate (20mM)	15
Pyruvate (20mM) + nitrate (20mM)	40
Pyruvate (40mM)	50
Nitrate (40mM)	27.5
Lactate (20mM)	7.5
Lactate (40mM)	22.5
Lactate (20mM) + pyruvate (20mM)	25

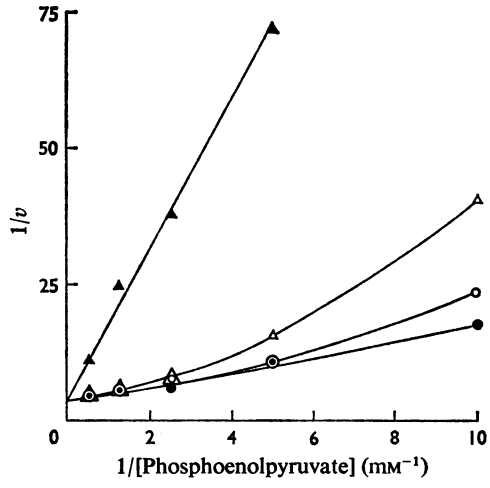

Fig. 10. *Effect of NO_3^- and lactate ions on the Lineweaver–Burk plot for phosphoenolpyruvate with pyruvate kinase in the absence of K^+ ions*

Control curve with no extra anions, ▲; plus 20mM-NO_3^- ions, △; plus 20mM lactate ions, ○; plus 20mM-NO_3^- and 20mM-lactate ions, ●. Other conditions were as described in Fig. 9 and in the Materials and Methods section.

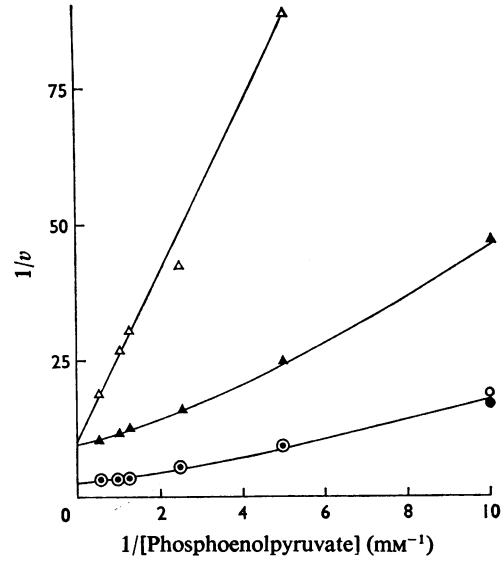

Fig. 11. *Lineweaver–Burk plot for phosphoenolpyruvate showing the effect of α-hydroxybutyrate on pyruvate kinase in the absence and presence of potassium acetate*

In the absence of K^+ ions but without α-hydroxybutyrate, △; plus 20mM-α-hydroxybutyrate, ▲. In the presence of 50mM-K^+ but without α-hydroxybutyrate, ○; plus 20mM-α-hydroxybutyrate, ●. Other conditions were as described in Fig. 9 and in the Materials and Methods section.

mately the sum of those caused by the two anions when added separately.

Addition of 20mM-α-hydroxybutyrate to pyruvate kinase in the absence of an activating cation gives a similar degree of activation to that found with 20mM-lactate (Fig. 11). The plot is also curved concave upwards and activation is competitively prevented by phosphoenolpyruvate, suggesting that both anions have a similar mode of action. However, in the presence of K^+ ions α-hydroxybutyrate, unlike lactate, is not an inhibitor (Fig. 11). The additional effect of NO_3^- was therefore not investigated.

Difference-spectra measurements. The formation of a difference spectrum on addition of K^+ ions to pyruvate kinase, indicating the perturbation of tryptophan residues in the enzyme, has been demonstrated (Hermans *et al.*, 1960; Suelter *et al.*, 1966). This observation was confirmed (Fig. 12*a*). In the absence of K^+ ions a similar difference spectrum is produced by the addition of NO_3^- ions or lactate ions (Figs. 12*b* and 12*c*).

Discussion

Creatine kinase

In a previous study of the enzyme from ox smooth muscle (Focant, 1970) a preparation was obtained that was essentially pure but with a low specific activity (20–40 μmol/min per mg of protein). The isolation procedure used here gave a considerably improved specific activity, the best value obtained being 146 μmol/min per mg, and again the product showed only a single protein band on electrophoresis with a mobility characteristic of a 'brain' enzyme (cf. Watts *et al.*, 1972). Even this improved specific activity is considerably less than the last value of 243 μmol/min per mg reported for the ox brain kinase (Jacobs & Kuby, 1970). However, these workers found that their preparations were extremely unstable and the specific activity declined to 190–200 μmol/min per mg if the enzyme was not stored in liquid N_2. We also found the ox stomach enzyme to be extremely unstable, and freezing to ordinary deep-freeze temperatures (−20°C) was particularly deleterious. Unfortunately the liquid-N_2 technique was not available to us, but it seems probable that the difference in specific activities of the two enzymes could be explained on this basis.

Table 1 compares the kinetic properties of the kinases from ox brain and striated muscle (Jacobs & Kuby, 1970). The brain enzyme shows a much greater

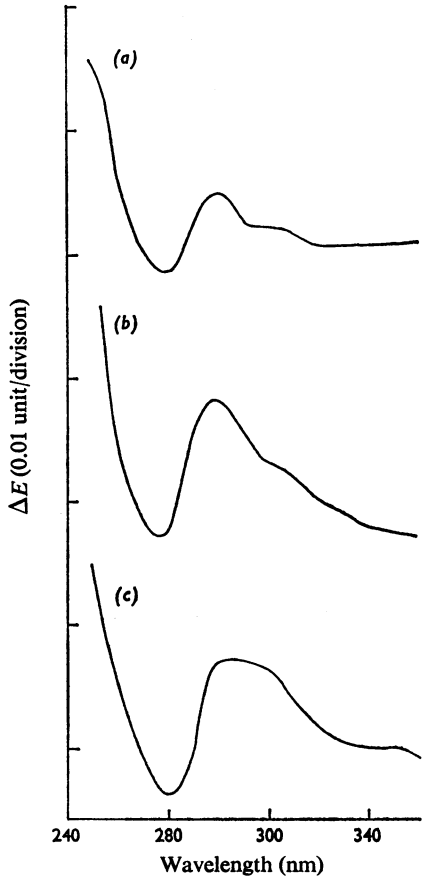

Fig. 12. *Difference spectra of the activation of pyruvate kinase by K^+ ions and anions at 30°C*

Each pair of cells was set up with 1 ml of activating ion, 50 mM, pH 8.6 in one compartment and 1 ml of enzyme in Tris–acetate buffer, pH 8.6 in the second compartment. A positive deflexion is upwards and the traces become horizontal at 340–360 nm. (*a*) Plus potassium acetate; (*b*), plus $NaNO_3$; (*c*), plus sodium lactate. The curves were transcribed from continuous traces obtained with a Cary 16S spectrophotometer.

effect of one substrate on the K_m value for the second substrate. Whereas with the brain enzyme K_a and K_a' and K_b and K_b' both differ by a factor of about seven, for the striated-muscle enzyme the difference is no more than twofold. The present results (Table 1) show that the smooth-muscle enzyme is intermediate in character, with the two sets of constants differing by a factor of nearly four. Further, both K_a and K_a' are very much lower than those of either the brain or the striated-muscle enzymes. Thus unless a disproportionate influence can be attributed to the effects of enzyme instability it would seem that the brain-type enzymes of ox brain and smooth muscle can be distinguished by kinetic measurements. This would support the finding of quite marked differences in the amino acid compositions of the two enzymes (Focant, 1970), particularly with regard to the contents of proline, glycine, alanine and valine.

The nucleotide-substrate specificities of the previous creatine kinases investigated have been found to be fairly broad. With the rabbit brain enzyme, for example, relative activities with ADP, dADP, IDP, UDP and dGDP are 100, 82, 17, 6 and 5 (Eppenberger *et al.*, 1967). The high specificity of the ox stomach enzyme for adenine was, by contrast, quite surprising, in particular the lack of activity with IDP. Fig. 6 indicates that this is clearly not because IDP fails to bind to the enzyme but more probably because it is incapable of inducing the appropriate conformational state for transphosphorylation to occur. Comparison of the difference spectrum (Fig. 6) with that for the rabbit muscle enzyme (Fig. 7), for which IDP is a fairly good substrate (James & Morrison, 1966), suggests that changes in the 280–300 nm region are indicative of the important conformational change (Roustan *et al.*, 1968; Milner-White, 1971). This is more readily apparent when ADP is substrate, although with the rabbit enzyme the whole of the difference spectrum is amplified much more on forming the anion-stabilized dead-end complex than is found with the ox enzyme. Although the general shapes of the difference spectra of the two enzymes in the presence of $MgADP+NO_3^-$ are similar that from the ox muscle shows a general shift to higher wavelengths with minima at 264, 288 and 300 nm as compared with 256, 286 and 295 nm for the rabbit enzyme. The hyperchromic effects around 260 and 295 nm are clearly complex and it would seem that the parts of the chromophoric systems are affected to different extents in the two enzymes. The very large effect at 286 nm in the rabbit enzyme contrasts with the quite small minimum produced by the dead-end complex at 288 nm in the ox enzyme. A further unusual feature of the ox kinase is that the hyperchromic changes around 300 nm extend to about 325 nm. In non-conjugated proteins, such as the phosphagen kinases are believed to be, tryptophan absorbs at the longest wavelengths with an upper limit around 315 nm (Edelhoch, 1967). Although proteins with absorption spectra extending beyond this value have been recorded (Greaser & Gergely, 1973) problems of interpretation arise from the increasing possibility of complications from light-scattering in this region. An instability in the ox enzyme associated with the hyperchromic effect at 264 nm is shown in Fig. 6. This appears not to involve changes at higher wavelengths but it emphasizes that any changes above 315 nm should be interpreted with caution. It can be con-

cluded that while the general spectral changes that accompany formation of the dead-end complex are similar in the two enzymes there is considerable variation reflecting species variation and tissue of origin.

The biphasic nature of the progress curve for the inhibition by iodoacetamide (Fig. 3) appears to be a characteristic feature of brain-type enzymes (Hooton, 1968; Dawson *et al.*, 1967), and the finding that each phase represents about half the enzyme activity (Fig. 3) is in accord with the proposal (Kumudavalli *et al.*, 1970) that alkylation of the essential thiol group in one subunit causes a conformational change that modifies the reactivity of the thiol group on the other subunit. Another related feature of ox brain creatine kinase is that the extent of inhibition is dependent on the enzyme concentration (Atherton *et al.*, 1970). The smooth-muscle enzyme was found to behave in exactly the same way. The maximum inhibition obtained with 6.7 μM-enzyme was 80%, but this increased to 95% when the enzyme concentration was raised to 22.1 μM. The cause of this phenomenon, other than that it appears to reflect some form of protein–protein interaction, is not understood.

Protection of the essential thiol groups against inactivation by iodoacetamide only by the anion-stabilized dead-end complex of the active substrate combination is in accord with the difference-spectra data. It also provides further evidence for the view that this four-component complex is remarkably stable because it closely resembles an intermediate state in the transphosphorylation reaction in which the transferable phosphoryl group is held in the sp^3d planar configuration (Milner-White & Watts, 1971). Here both NO_3^- and Cl^- ions are less effective than with the rabbit muscle enzyme, where the protection was 98% and 80% respectively, even though the MgADP concentration is clearly saturating. As was found with the dogfish muscle enzyme (Simonarson & Watts, 1972), within the limits set by a precise transphosphorylation reaction there is still room for considerable species variability. It is notable that $\alpha\beta$-methylene-ADP is unable to form the stable dead-end complex. In accord with the evidence that a precise steric orientation is required between metal–nucleotide and guanidine substrates (Reed & Cohn, 1972), substitution of a methylene bridge for the oxygen bridge between the α- and β-phosphoryl groups of ADP is sufficient completely to destroy the ability for complex formation.

Pyruvate kinase

Pyruvate kinase is a more complex enzyme than creatine kinase in that the rate of catalysis is enhanced by K^+ ions (Fig. 11). The K^+-activated pyruvate kinase more closely resembles creatine kinase, for which most simple anions either have no effect or are inhibitory (Milner-White & Watts, 1971). In contrast, when the K^+ ions are omitted all the anions tesetd activate pyruvate kinase, although with varying degrees of effectiveness (Figs. 8 and 9).

In the absence of an activating cation the activation by lactate is competitive with the substrate, phosphoenolpyruvate (Fig. 10). The obvious similarity in structure evokes the inference that both bind to the same site on the enzyme, and this is supported by the finding that lactate is a competitive inhibitor for phosphoenolpyruvate in the K^+-activated enzyme. If both lactate and substrate bind at the same site on the enzyme then activation can only be reasonably explained on the basis that the binding of one molecule of lactate to the tetrameric enzyme results in a co-operatively induced conformational change in all the enzyme subunits. The accompanying enhancement of catalytic activity may then be explained by the marked lowering of the Michaelis constant for phosphoenolpyruvate (at least sevenfold; Fig. 10) in those subunits to which the lactate is not binding. The concave-upwards nature of the Lineweaver–Burk plot obtained in the presence of lactate (Fig. 10) is in accord with an allosteric explanation. Similarly, the difference spectra (Fig. 12) indicate that appropriate modifications occur to the enzyme structure, possibly involving changes in the environment of a tryptophan residue (Suelter *et al.*, 1966), even though the Hill coefficient is barely altered. Activation by K^+ ions, on the other hand, appears both to lower the Michaelis constant for phosphoenolpyruvate and to facilitate the catalytic process, causing $V_{max.}$ to be enhanced by a factor of four (Fig. 11). This tightening-up of the substrate-binding site would explain why α-hydroxybutyrate, being less like pyruvate than lactate, will activate the enzyme in the absence of K^+ ions but not inhibit in the presence of K^+ ions (Fig. 11).

Surprisingly, NO_3^- ions, although quantitatively less effective, behave exactly like lactate ions with regard to activation, inhibition and ability to induce a conformation change in the enzyme. The saturating concentration for both lactate and NO_3^- for the K^+-free enzyme is about 20 mM (Fig. 8), but when both are added together at that concentration activation to is some extent additive, particularly at low phosphoenolpyruvate concentrations. Hence it would seem that these two anions could bind close together although not at the same site on the enzyme.

By comparison with creatine kinase the NO_3^- ion might be expected to bind to the site on the enzyme that accommodates the transferable phosphoryl group. This is compatible with the data, but pyruvate kinase is notably different from creatine kinase in that no evidence was obtained to indicate the occurrence of an anion-stabilized enzyme–pyruvate–MgADP dead-end complex. No enhancement of either activation in the absence of K^+ ions or inhibition in the

presence of K^+ ions, other than could be accounted for in terms of simple additivity, occurred when both pyruvate and NO_3^- were added together (Table 2). This supports the finding of Rose (1960) that the true substrate is enolpyruvate rather than pyruvate, the formation of which is only induced by the presence on the enzyme of MgATP. It would be interesting to know if MgADP plus NO_3^- promotes enolization even though a stable dead-end complex does not form. Attempts to mimic enolpyruvate with lactate and α-hydroxybutyrate were also unsuccessful even though lactate binds to the enzyme in both the absence and the presence of K^+ ions.

Pyruvate kinase, like creatine kinase, is thought to function by a direct phosphoryl-transfer reaction (Reynard *et al.*, 1961). However, the failure to form an anion-stabilized dead-end complex, even though all the components appear to bind simultaneously to the enzyme, provides support for the view that the negligible rate of phosphoryl transfer from ATP is because the enzyme is incapable of lining-up the substrates and inducing the correct molecular configuration in the very precise way that has been found to be an essential feature of the activity of creatine kinase (Reed & Cohn, 1972; Watts, 1973).

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References

Atherton, R. S., Laws, J. F. & Thomson, A. R. (1970) *Biochem. J.* **118**, 903–904

Burger, A., Richterich, R. & Aebi, H. (1964) *Biochem. Z.* **339**, 305–314

Dawson, D. M., Eppenberger, H. M. & Kaplan, N. O. (1967) *Biochem. Biophys. Res. Commun.* **21**, 346–353

Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954

Eppenberger, H. M., Eppenberger, M. E., Richterich, R. & Aebi, H. (1964) *Develop. Biol.* **10**, 1–16

Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967) *J. Biol. Chem.* **242**, 204–209

Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575–578

Focant, B. (1970) *FEBS Lett.* **10**, 57–61

Greaser, M. L. & Gergely, J. (1973) *J. Biol. Chem.* **248**, 2125–2133

Hermans, J. Jr., Donovan, J. W. & Scheraga, H. A. (1960) *J. Biol. Chem.* **235**, 91–93

Hooton, B. T. (1968) *Biochemistry* **7**, 2063–2071

Jacobs, H. K. & Kuby, S. A. (1970) *J. Biol. Chem.* **245**, 3305–3314

James, E. & Morrison, J. F. (1966) *J. Biol. Chem.* **241**, 4758–4770

Kuby, S. A. & Noltmann, E. (1962) *Enzymes* 2nd edn. **6**, 516–596

Kumudavalli, I., Moreland, B. M. & Watts, D. C. (1970) *Biochem. J.* **117**, 513–523

Milner-White, E. J. (1971) Ph.D. Thesis, University of London

Milner-White, E. J. & Watts, D. C. (1971) *Biochem. J.* **122**, 727–740

Reed, G. H. & Cohn, M. (1972) *J. Biol. Chem.* **247**, 3073–3081

Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961) *J. Biol. Chem.* **236**, 2277–2283

Rose, I. A. (1960) *J. Biol. Chem.* **235**, 1170–1177

Roustan, C., Kassab, R., Pradel, L. A. & Thoai, N. V. (1968) *Biochim. Biophys. Acta* **167**, 326–338

Simonarson, B. & Watts, D. C. (1972) *Biochem. J.* **128**, 1241–1253

Suelter, C. H., Singleton, R. Jr., Kayne, F. J., Arrington, S., Glass, J. & Mildvan, A. S. (1966) *Biochemistry* **5**, 131–139

Tanzer, M. C. & Gilvarg, C. (1959) *J. Biol. Chem.* **234**, 3201–3204

Tietz, A. & Ochoa, S. (1958) *Arch. Biochem. Biophys.* **78**, 477–493

Watts, D. C. (1973) *Enzymes* 3rd edn. **8**, 383–455

Watts, D. C. & Moreland, B. M. (1970) in *Experiments in Physiology and Biochemistry* (Kerkut, G. A., ed.), vol. 3, pp. 1–30, Academic Press, New York

Watts, D. C., Focant, B., Moreland, B. M. & Watts, R. L. (1972) *Nature (London) New Biol.* **237**, 51–53

Wood, T. (1963) *Biochem. J.* **87**, 453–462