experimental error, are identical, which is taken to mean that the data are not consistent with a Ter mechanism. It is probable that the reaction is a sequential Bi-type mechanism and the true substrates are phosphoenolpyruvate and the Mg\(^{2+}\) chelate of ADP\(^{3-}\).

Fig. 2 shows a double-reciprocal plot of variable concentrations of MgADP\(^{-}\) against different fixed concentrations of Mg\(_{\text{free}}\), with phosphoenolpyruvate kept constant. Inhibition was observed with increasing concentrations of Mg\(_{\text{free}}\), but the lines cross to the right of the ordinate. This pattern was observed at six different concentrations of phosphoenolpyruvate. Double-reciprocal plots of a variable concentration of MgADP\(^{-}\) against reaction rate at different fixed concentrations of ADP\(^{3-}\) at constant concentrations of phosphoenolpyruvate also showed inhibition with the lines intersecting to the right of the ordinate. Again, a similar pattern was evident at six different concentrations of phosphoenolpyruvate.

These observations indicate that Mg\(_{\text{free}}\) and ADP\(^{3-}\) interact with the enzyme. These and other data are consistent with a rapid-equilibrium random mechanism with the formation of three dead-end complexes. These complexes, enzyme–Mg\(^{2+}\), enzyme–ADP\(^{3-}\) and enzyme–Mg\(^{2+}\)-phosphoenolpyruvate, must be taken into account when deriving an initial rate equation for this mechanism. Such an equation involves terms in Mg\(_{\text{free}}\), MgADP\(^{-}\) and ADP\(^{3-}\) concentrations. Since these species are always present in the reaction mixture and it is only possible to keep the concentration of one species constant at a time, the equation is non-linear when expressed in terms of MgADP\(^{-}\) concentration. The determination of kinetic constants under these conditions is difficult and, at best, only approximate. Replacing the MgADP\(^{-}\) terms with [Mg\(^{2+}\)][ADP\(^{3-}\)]/K (where K is the dissociation constant of MgADP\(^{-}\)) in the equation results in linearity of the latter and simplifies the extraction of the kinetic constants from the data.


Abortive Enzyme–Substrate Ternary Complexes Formed by the Fructose 1,6-Diphosphate-Activated Pyruvate Kinase from the Hepatopancreas of Carcinus maenas

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Recent kinetic studies have shown that the non-allosteric type M pyruvate kinase from rabbit muscle shows substrate inhibition by the nucleotide substrate in the forward (Jonson & Cleland, 1974) and reverse (Giles et al., 1975a) reactions in the presence of a constant Mg\(_{\text{free}}\) concentration. In both cases the inhibition is competitive with respect to the non-nucleotide substrate and can be explained by the formation of an enzyme–nucleotide–nucleotide ternary complex in a rapid-equilibrium random mechanism. The mammalian enzyme can also form an enzyme–ADP–pyruvate dead-end complex (Ainsworth & MacFarlane, 1973).

A kinetic analysis of the fructose 1,6-diphosphate-activated form of the allosteric enzyme from the hepatopancreas of Carcinus maenas (the common shore crab) has been reported (Giles et al., 1975b). The results presented are consistent with a rapid-equilibrium random mechanism with the formation of two dead-end complexes, namely enzyme–ADP–ATP and enzyme–ADP–pyruvate (Scheme 1). One consequence of this mechanism...
Scheme 1. Postulated kinetic mechanism of the fructose 1,6-diphosphate-activated type L pyruvate kinase from the hepatopancreas of Carcinus maenas

E, Pyr-P and Pyr represent enzyme, phosphoenolpyruvate and pyruvate respectively. The rate-limiting step is the interconversion of the central complexes.

is that a saturating concentration of phosphoenolpyruvate should overcome the inhibition caused by pyruvate when ADP is the variable substrate. This was verified experimentally (Fig. 1 of Giles et al., 1975b). It was observed, however, that the apparent $K_m^{ADP}$ was significantly higher (600 $\mu$M) than the value obtained (66 $\mu$M) from initial-rate studies, by extrapolating to an infinite concentration of phosphoenolpyruvate. This suggests that at a high concentration of phosphoenolpyruvate an additional enzyme complex(es) forms. It was also thought likely that the enzyme might form other nucleotide dead-end complexes, in an analogous manner to the rabbit muscle enzyme, since the enzyme forms an enzyme-ADP-ATP mixed-nucleotide dead-end complex. This communication reports the results of experiments conducted to investigate the nature of the complexes formed at high substrate concentrations.

Enzyme purified by method B (Giles et al., 1976) was used and assayed at pH 7.4 and 25°C in the presence of 100 mM-total KF and 8 mM-Mg$_2^+$ as previously described (Giles et al., 1975b). The Mg$_2^+$ buffer dipotassium glycerol 1-phosphate (Boyer, 1969) was used and sufficient Mg$_2^+$ added to give the desired [Mg$_2^+$]; the amount required was calculated by using the known dissociation constants of the chelating species present. The enzyme was converted into the fully activated form by adding a saturating concentration of fructose 1,6-diphosphate (500 $\mu$M). The ATP used was purified by Dowex-1 (Cl$^-$ form) chromatography immediately before use to decrease the ADP content. The residual ADP was assayed by normal enzymic means and allowance made when the effect of ATP on the enzyme was studied.

Experiments indicated that at a constant, low concentration of one substrate (either ADP or phosphoenolpyruvate), increasing the concentration of the other resulted in the rate falling at very high concentrations. A double-reciprocal plot (of reaction rate against substrate concentration) was characteristic of substrate inhibition (Fig. 1).

To investigate the type of substrate inhibition occurring (competitive, non-competitive or uncompetitive) experiments were conducted by varying one substrate in the presence of different fixed concentrations of the other. The concentration range of the variable substrate was chosen such that the experimental points were on the linear part of the curve. In this way no significant substrate inhibition by the variable substrate was measured. The results obtained gave two families of linear double-reciprocal plots, one for each variable substrate, with the lines at the higher concentrations of the fixed substrate crossing the other lines to the right of the ordinate. Slope and intercept replots from both primary plots showed that the intercepts were a linear function, and the slopes a non-linear function, of the reciprocal of the concentration of the constant substrate. This is diagnostic of competitive substrate inhibition, where saturation with the variable substrate can reverse the substrate inhibition caused by the other substrate. Thus both substrates, phosphoenolpyruvate and ADP, exhibit competitive substrate inhibition.

Values for the various kinetic constants were obtained from all the data points by fitting them to the rate equation derived for this kinetic mechanism by using a least-
Fig. 1. Double-reciprocal plot of reaction rate as a function of phosphoenolpyruvate concentration, showing substrate inhibition

Conditions of the assay are given in the text with $K_{\text{total}}^+ = 100 \text{mM}$, $Mg^{2+} = 8 \text{ mM}$ and $ADP_{\text{total}} = 80 \text{ mM}$. The points are the experimental data, and the line is the computer fit to the general rate equation for substrate inhibition. $v$ is expressed as $\mu$mol of pyruvate formed/min per 'enzyme unit'. The 'enzyme unit' is that enzyme activity measured in standard assay conditions, namely phosphoenolpyruvate, ADP and fructose 1,6-diphosphate concentrations of 800, 500 and 500 $\mu$M respectively.

squares regression method (Cleland, 1967). The values obtained for the Michaelis constants and for the dissociation constants of the binary complexes were similar to those already reported (Giles et al., 1975b). The small differences can be explained because the linear portion of the curves, from which the earlier constants were obtained, only approximate to the true asymptote. The values of the inhibitory dissociation constants of ADP and phosphoenolpyruvate were larger than the dissociation constants from their binary complexes by 45- and 80-fold respectively. This much less favourable binding means that it is feasible to study either binding process, depending on the substrate concentrations used.

Formation of the enzyme–ATP–ATP complex was demonstrated in the forward reaction by an analysis of the ATP product-inhibition pattern with ADP as the variable substrate. The concentration range of ADP used was such that linear double-reciprocal plots were obtained. A low phosphoenolpyruvate concentration (39 $\mu$M) was used and ten lines, corresponding to ATP concentrations up to 15 $\mu$M, were obtained. The results showed that the intercepts of the primary plot were a linear function of ATP concentration, confirming the formation of an enzyme–ADP–ATP complex. The slope replot, however, was non-linear and could be satisfactorily fitted by a parabola, the curve predicted if an enzyme–ATP–ATP complex formed. This, in conjunction with the linear intercept replot, also suggests that there is a competitive interaction between the first ADP binding and the second ATP inhibition. Dissociation constants of the ATP complexes were calculated by using the least-squares method and assuming that the proposed mechanism is correct.

That the additional ATP inhibition could be the result of the ATP reversing the fructose 1,6-diphosphate activation of the enzyme was eliminated by addition of fructose 1,6-diphosphate up to 2 $\mu$M. No increase in rate in the presence of 15 $\mu$M-ATP was seen.

The results obtained suggest that three dead-end complexes must be added to Scheme 1; these are enzyme–ADP–ADP, enzyme–phosphoenolpyruvate–phosphoenolpyruvate
and enzyme–ATP–ATP. The three nucleotide complexes formed suggest that there is a second nucleotide-binding site which can bind either ADP or ATP. It follows therefore that two mixed nucleotide complexes, enzyme–ADP–ATP and enzyme–ATP–ADP, should be considered if there is independent binding. This possibility merits further study. Furthermore, no account has been taken of the magnesium-chelation state of the substrates. All the experiments were conducted at 8mM-Mg\textsuperscript{2+} conditions in which 99, 92 and 39% of the ATP, ADP and phosphoenolpyruvate respectively were present as their magnesium chelate forms. It is possible that one of the substrate-binding sites has a specificity for one chelation state, whereas the second binds only another form of substrate. It is of interest that two nucleotide-binding sites have been observed in X-ray-crystallographic Fourier difference maps of cat muscle pyruvate kinase at high nucleotide concentrations (Stammers & Muirhead, 1975).


The Effect of Photoperiod on Endogenous γ-Tocopherol and Plastochromanol in Leaves of Xanthium strumarium L. (Cocklebur)

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Where the flowering behaviour of a plant is under photoperiodic control, floral initiation involves reception in the leaves of the stimulus of changing night length, followed ultimately by the conversion of the apex from the vegetative into the reproductive state. The biochemical sequences involved are still unknown.

Certain reports in the literature suggest that vitamin E (tocopherols) may be involved in this process. Sironval & El Tannir-Lomba (1960) found that α-tocopherol (5,7,8-trimethyltocol) could elicit flowering in strawberries, growing under vegetative conditions. γ-Tocopherol (7,8-dimethyltocol) has been reported to accumulate in the leaves immediately before flower formation (Michniewicz & Kamienska, 1969). In our own laboratory Hughes (1971) obtained strong evidence linking tocopherols and related compounds with the process of flower-bud formation in four varieties of Pismum sativum.

We have followed changes in tocopherols and related compounds in Xanthium strumarium L. under both inducing and non-inducing photoperiods, in order to identify any changes which might be correlated with the flowering response. The flowering physiology of this plant has been extensively studied (Evans, 1969), and exposure of vegetative plants to a single inductive photoperiod (corresponding to a night length greater than 8.5h) is sufficient to cause flowering. Plants maintained under a photoperiod allowing less than 8.5h darkness remain vegetative.

We extracted tocopherols and other isoprenoids from mature leaves with cold acetone. Light petroleum (b.p. 40–60°C) was added to the extract, and acetone and other watersoluble materials were washed from the lipid fraction with water. The lipids were fractionated by chromatography on columns of neutral alumina, followed by two-dimensional t.l.c. on silica gel (Whittle & Pennock, 1967). Quantitative determination of separated tocopherols was then performed directly on the t.l.c. plates by using a Zeiss spectrophotometer.