The regulatory properties of yeast pyruvate kinase

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The kinetics of pyruvate kinase from *Saccharomyces cerevisiae* were studied in assays at pH 6.2 where the relationships between the initial velocities of the catalysed reaction and the concentrations of the substrates ADP, phosphoenolpyruvate and Mg^{2+} are non-hyperbolic. The findings were represented empirically by the exponential model for a regulatory enzyme. The analysis shows that ADP, phosphoenolpyruvate and Mg^{2+} display positive homotropic interaction in their binding behaviour with (calculated) Hill slopes at half-saturation equal to 1.06, 2.35 and 3.11 respectively [Ainsworth (1977) *J. Theor. Biol.* **68**, 391–413]. The direct heterotropic interaction between ADP and phosphoenolpyruvate is small and negative, but the overall interaction between these substrates becomes positive when their positive interactions with Mg^{2+} are taken into account. The heterotropic interactions of the substrates, though smaller in magnitude, are comparable with those revealed by the rabbit muscle enzyme [Ainsworth, Kinderlerer & Gregory (1983) *Biochem. J.* **209**, 401–411], and it is suggested that they have a common origin in charge interactions within the active site.

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) exists in several isoenzymic forms. All forms of the enzyme have an obligatory requirement for Mg^{2+} or Mn^{2+} and a univalent cation, preferably K^+ . Allosteric control of the mammalian L-, R- and M_2 -type enzymes has been well characterized (Seubert & Schoner, 1971; Kayne, 1973; Hall & Cottam, 1978), and it has been shown by Haeckel *et al.* (1968) and Hunsley & Suelter (1969b) respectively that the yeast enzymes from *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* possess similar allosteric properties. All enzymes of the 'allosteric' variety display a sigmoidal relationship between the initial velocity of pyruvate formation and the concentration of phosphoenolpyruvate, are allosterically activated by H^+ and fructose 1,6-bisphosphate and are inhibited by ATP (Wieker & Hess, 1971; Johannes & Hess, 1973; Fishbein *et al.*, 1975; Hall & Cottam, 1978). When activated by fructose 1,6-bisphosphate and K^+ , the *S. cerevisiae* enzyme displays hyperbolic kinetics, and it is possible to determine the catalytic mechanism by the classical technique of measuring the initial velocity of reaction as a function of one substrate concentration with other substrate concentrations kept constant at a number of non-saturating values. It was shown by these means that the *S. cerevisiae* enzyme has a mechanism of the Ordered Ter-Bi type with the

substrates binding in the order phosphoenolpyruvate, ADP and Mg^{2+} , the first two substrates binding in their Mg^{2+} -free forms (Macfarlane & Ainsworth, 1972). The investigation of the non-hyperbolic (allosteric) kinetics of pyruvate kinase by similar systematic regimes presents considerable interpretative difficulties, and only a few examples of this approach are known. However, from one such study, Balinsky *et al.* (1973) suggested that the L-type enzyme from human liver has an ordered mechanism with phosphoenolpyruvate binding before ADP. Again, the yeast enzyme from *S. carlsbergensis* has been investigated by Johannes & Hess (1973) in experiments where the initial velocity of the catalysed reaction was measured as a function of phosphoenolpyruvate concentration, at pH 7, in the presence of different fixed concentrations of either fructose 1,6-bisphosphate or ATP and constant total concentrations of ADP, Mg^{2+} and K^+ species. The results were examined in terms of the MWC model (Monod *et al.*, 1965), but it was concluded that the model required extension by the addition of a hybrid conformational state if the data were to be explained. Finally, Ainsworth *et al.* (1983) and Gregory *et al.* (1983) examined the non-hyperbolic kinetics of rabbit muscle pyruvate kinase, interpreting their results by the exponential model. The description that was obtained demonstrated a consistency of behaviour with the equili-

brium random-order kinetics displayed by the muscle enzyme under different experimental conditions (Ainsworth & Macfarlane, 1973). The purpose of the present paper is to give the results of a systematic investigation of the influence of three substrate concentrations on the initial velocity of reaction catalysed by pyruvate kinase from *S. cerevisiae* and to describe an empirical fit to the results obtained by the exponential model.

Theory

With the assumption that the true substrates of yeast pyruvate kinase are the Mg^{2+} -free forms of ADP and phosphoenolpyruvate and free Mg^{2+} (Macfarlane & Ainsworth, 1972), the data presented below show that each binds non-hyperbolically in the presence of fixed concentrations of the other two. An interpretation of this behaviour is attempted by applying the exponential model for a three-substrate enzyme (Ainsworth *et al.*, 1983), as embodied in the equation:

$$\frac{v}{V_G} = \frac{A\alpha_{abc}}{1 + A\alpha_{abc}} \cdot \frac{B\beta_{abc}}{1 + B\beta_{abc}} \cdot \frac{C\gamma_{abc}}{1 + C\gamma_{abc}} \quad (1)$$

where A stands for ADP, B for phosphoenolpyruvate and C for Mg^{2+} and where their corresponding association constants α_{abc} , β_{abc} , γ_{abc} relate to particular values of the fractional saturation of the enzyme by the three substrates ($p_A = a$, $p_B = b$ and $p_C = c$). Assuming that the substrate-binding sites are allosterically linked:

$$\ln \alpha_{abc} = \ln \alpha_{000} + k_{AA} \cdot p_A + k_{BA} \cdot p_B + k_{CA} \cdot p_C \quad (2)$$

$$\ln \beta_{abc} = \ln \beta_{000} + k_{AB} \cdot p_A + k_{BB} \cdot p_B + k_{CB} \cdot p_C \quad (3)$$

$$\ln \gamma_{abc} = \ln \gamma_{000} + k_{AC} \cdot p_A + k_{BC} \cdot p_B + k_{CC} \cdot p_C \quad (4)$$

In eqn. (2), α_{abc} is equal to the ratio $p_A / [(1 - p_A) \cdot A]$ measured when $p_B = b$ and $p_C = c$, and α_{000} is the value the constant would adopt if all the fractional saturations were zero. k_{XY} is an interaction constant in RT units, where X denotes the ligand whose binding causes a change in the association binding constant for Y. Reciprocal allosteric linkage requires $k_{XY} = k_{YX}$. Eqns. (1)–(4) are employed below to fit initial-velocity data measured as a function of the three substrate concentrations $v = f(A, B, C)$, but it is important to note that the ten constants obtained depend on the assumption that the fractional saturation of the enzyme by H^+ and K^+ remains constant throughout.

The application of eqns. (1)–(4) in the present context is strictly empirical, for eqn. (1) depends on the assumption that the catalytic mechanism is equilibrium random order, an assumption that is directly refuted by previous studies of the activated enzyme (Macfarlane & Ainsworth, 1972). The

exponential model therefore provides only an interim means of examining the allosteric kinetics of pyruvate kinase: in this role it employs constants that can be readily determined and gives them a significance that has descriptive value (Ainsworth, 1977).

Experimental

Materials

Phosphoenolpyruvate (cyclohexylammonium salt), NADH (sodium salt), ADP (free acid) and rabbit muscle lactate dehydrogenase were supplied by Boehringer Corp., Lewes, East Sussex, U.K. Tetrapropylammonium hydroxide was supplied by Eastman-Kodak Co., Rochester, NY, U.S.A. Sephadex G-25 and Blue Sepharose CL-6B were obtained from Pharmacia, Uppsala, Sweden. Cacodylic acid and dithiothreitol were from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were either AnalaR or reagent grade as supplied by BDH Chemicals, Poole, Dorset, U.K., or Fisons Scientific Apparatus, Loughborough, Leics., U.K. Fresh brewer's yeast (*S. cerevisiae*) was obtained from Ward's Brewery, Sheffield, S. Yorks., U.K.

Preparation of pyruvate kinase

The initial purification of pyruvate kinase from brewer's yeast (1 kg) was by plasmolysis in toluene and three $(NH_4)_2SO_4$ fractionations in accordance with the method of Röschlau & Hess (1972). A 300 mg portion of the 1.8 g pellet, obtained by centrifugation of the final $(NH_4)_2SO_4$ solution, was dissolved in 40 ml of buffer I (10 mM-sodium phosphate buffer, pH 6.2, containing 2.5 mM- $MgSO_4$ and 1 mM-dithiothreitol) and freed from $(NH_4)_2SO_4$ by passage through a Sephadex G-25 column (34 cm $\times$ 2 cm) equilibrated with buffer I. The eluate was applied to a Blue Sepharose CL-6B column (30 cm $\times$ 1.5 cm), equilibrated and then eluted with buffer I. A protein fraction (not bound to the column) was eluted in the first 200 ml and discarded. The next 80 ml of effluent was obtained on elution with buffer II (0.1 M-EDTA in buffer I), and was again discarded. Subsequent elution was with buffer III (150 mM-NaCl in buffer II): the effluent was collected in 10 ml fractions and contained the major portion of pyruvate kinase activity. Fractions with specific activity greater than 200 units/mg were pooled. Their combined volume was decreased, with simultaneous precipitation of protein, by dialysis against 3.6 M- $(NH_4)_2SO_4$ containing 1 mM-dithiothreitol, pH 6.2, under reduced pressure (250 mmHg). The resulting suspension was stored at 4°C, with comparable suspensions obtained from further 300 mg portions of the original pellet. Finally, the protein

was isolated from the pooled suspensions by centrifugation, redissolved in aq. 25% (v/v) glycerol, and solid $(\text{NH}_4)_2\text{SO}_4$ was added to increase the saturation to 90% of its solubility in water. The resulting mixture was stored at -18°C . A total of 82 mg of protein with a specific activity of 367 units/mg was recovered, representing 12% of the original activity. For comparison, the specific activity of the preparation obtained by Hunsley & Suelter (1969a), determined with slightly different assay conditions, was 299 units/mg. Protein was monitored by using the Bio-Rad Protein Assay kit, and the specific activity was measured as described below.

The preparation was examined by polyacrylamide-gel electrophoresis. The protein migrated as one major staining band that was associated with all the pyruvate kinase activity (Hunsley & Suelter, 1969a).

Preparation of substrates and enzymes for use

Stock solutions of ADP and phosphoenolpyruvate were prepared less than 24 h before use, adjusted to pH 6.2 by addition of tetrapropylammonium hydroxide (10%, w/v), and if necessary stored at 4°C . The ADP was purchased as the monopotassium salt and its contribution of K^+ to the assay solution was taken into account when the total K^+ concentration was adjusted to 100 mM with KCl. The purity and concentration of the substrates were determined enzymically and by direct absorption measurements as described by Macfarlane (1973) and Ainsworth & Macfarlane (1973). Pyruvate kinase was prepared for use by dissolving a small portion of the $(\text{NH}_4)_2\text{SO}_4$ suspension in 0.1 M-tetrapropylammonium cacodylate buffer, pH 6.2, at 25°C , to give enzyme concentrations in the range 0.16–0.04 mg/ml. Preliminary experiments showed the enzyme to be stable for at least 2 h under these conditions.

Enzyme assays

Reaction mixtures were prepared on the assumption that the true substrates of yeast pyruvate kinase are free Mg^{2+} and the Mg^{2+} -free species of ADP and phosphoenolpyruvate (Macfarlane & Ainsworth, 1972; Macfarlane *et al.*, 1974). The apparent dissociation constants for the binding of Mg^{2+} by ADP and phosphoenolpyruvate have been given by Gregory & Ainsworth (1981).

Pyruvate kinase activity was measured by using the continuous lactate dehydrogenase coupled assay method (Bücher & Pfeleiderer, 1955). Initial velocities (in quadruplicate) were determined in a solution (1 ml) of tetrapropylammonium cacodylate (0.1 M in cacodylate), pH 6.2, containing 100 μmol of KCl in addition to the indicated sub-

strates. The reactant mixtures were incubated for 10 min at 25°C before the addition of 0.15 μmol of NADH and an excess (14 units) of lactate dehydrogenase: reaction was then started by adding 5 μl of a suitably diluted pyruvate kinase solution (see above). The initial velocities (v) reported in the Figures are values corrected to an enzyme specific activity of 367 units/mg of protein at 25°C and are expressed as μmol of NADH oxidized/min per mg of protein at 25°C . The specific activity of the enzyme was determined in an assay mixture (1 ml) containing tetrapropylammonium cacodylate buffer, pH 6.2 (100 μmol), KCl (100 μmol), fructose 1,6-bisphosphate (2.0 μmol), ADP (10 μmol), phosphoenolpyruvate (5 μmol), Mg^{2+} (10 μmol), NADH (0.15 μmol) and lactate dehydrogenase (14 units). The assay was conducted as described above.

Computer analysis of data

The constants of the exponential model were evaluated by the DESCENT program described by Ainsworth *et al.* (1983). Refinement of the values was undertaken by the PAPB program (Kinderlerer *et al.*, 1981), but usually only marginal improvement was obtained. The success or failure of the optimization in both these programs is determined at each stage by change in the product of an absolute and relative error, $A \times R$. A and R are related to the error parameters defined in eqns. (5) and (6) below by the expressions $\text{RSS} = \sqrt{A/n}$ and $\text{MD}\% = 100\sqrt{R/n}$, where n is the number of observations.

Results

The data points illustrated in Figs. 1, 2 and 3 are mean results obtained from two independent sets of measurements, each conducted in duplicate. The points lie on curves calculated from the constants of the exponential-model fit to $v = f(A, B, C)$, given in Table 1, the error parameters of which are defined by:

$$\text{RSS} = [\Sigma(v_{\text{calc.}} - v_{\text{obs.}})^2/n]^{\frac{1}{2}} \quad (5)$$

$$\text{MD}\% = 100\{\Sigma[(v_{\text{calc.}} - v_{\text{obs.}})/v_{\text{calc.}}]^2/n\}^{\frac{1}{2}} \quad (6)$$

The error of the data points can also be expressed by eqns. (5) and (6) when for each datum $v_{\text{calc.}}$ is taken to be the mean velocity and $v_{\text{obs.}}$ the mean velocity plus the mean residual for that value: the result is $\text{RSS} = 4.76$ and $\text{MD}\% = 7.71$. Comparison of these values with those for the fit ($\text{RSS} = 6.98$ and $\text{MD}\% = 17.44$) shows that the calculated velocities fall within the RSS error of the measurements, taken on either side of the mean, but exceed the corresponding value of MD%. This indicates that the fit is less good at low velocities, a feature

Fig. 1. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[Mg^{2+}] = 2 \text{ mM}$

In this and succeeding Figures, initial velocities, v , (represented by symbols) are fitted by curves calculated in accordance with the three-substrate exponential model (eqns. 1–4) with the constants given in Table 1, and are expressed as μmol of NADH oxidized/min per mg of protein at 25°C in the presence of 0.1 M-K^+ , $\text{pH} 6.2$, with other conditions as described in the Experimental section. Phosphoenolpyruvate concentrations (mM) were: □, 1.5; ○, 2; △, 2.5; ■, 3; ●, 4; ▲, 5.

Fig. 2. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [ADP] with [phosphoenolpyruvate] = 5 mM

ADP concentrations (mM) were: □, 0.2; ○, 0.4; △, 0.6; ■, 0.8; ●, 1.2; ▲, 2.0. Other conditions and calculations were as described in the legend to Fig. 1.

that may be observed in the Figures. A similar outcome has been noted in the analysis of data relating to other enzymes (Ainsworth, 1977).

Fig. 3. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with $[ADP] = 2 \text{ mM}$

Mg^{2+} concentrations (mM) were: □, 0.75; ○, 1; △, 1.25; ■, 1.5; ●, 2. Other conditions and calculations were as described in the legend to Fig. 1.

There is no evidence in the Figures that dead-end inhibition by $MgADP$ is present. This is consistent with results obtained at the same pH with the fructose 1,6-bisphosphate-activated enzyme (Macfarlane & Ainsworth, 1972).

Table 1 also gives the results obtained by analysing the data $v = f(A, B, C)$ by the two-substrate model $v = f(X, Y)_Z$, an approach that is inconsistent with the evidence that p_Z does not remain constant when the concentration of Z is fixed, i.e. k_{XZ} , $k_{YZ} \neq 0$. The analysis is useful nonetheless because it indicates directly how the binding of one substrate influences the binding of a second without having to consider what might be the net effect created by summing the direct interaction of one substrate on the other with the indirect interaction of the substrates through their influence on the binding of a third (Ainsworth *et al.*, 1983).

Discussion

The general features of the allosteric interactions of yeast pyruvate kinase are evident from the values of the three-substrate exponential-model constants given in Table 1. All the substrates display positive homotropic interactions in their binding behaviour, but, whereas that shown by ADP is not remarkable, that of phosphoenolpyruvate is large and that of Mg^{2+} larger still. These findings confirm the observations reported by Haeckel *et al.* (1968) and Hunsley & Suelter (1969b) but contrast markedly with the uniformly negative homotropic interactions displayed by the rabbit

Table 1. Values of two-substrate and three-substrate exponential-model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction illustrated in Figs. 1-3

Constants representing equivalent data obtained with the rabbit muscle enzyme are included for comparison (Ainsworth *et al.*, 1983). The values of the constants are given to a precision consistent with the stated errors (eqns. 5 and 6): note that rounding-off would considerably increase the values of the error parameters. Temperature 25°C; pH 6.2; A = ADP; B = phosphoenolpyruvate; C = Mg²⁺. The concentration of K⁺ was 0.1 M.

Constant	Yeast pyruvate kinase				Muscle pyruvate kinase $v = f(A, B, C)$ pH 7.4
	$v = f(A, B)_C$	$v = f(A, C)_B$	$v = f(B, C)_A$	$v = f(A, B, C)$	
$\ln [\alpha_{000} \text{ (mm}^{-1}\text{)}]$	0.171	0.562	—	0.571	0.475
$\ln [\beta_{000} \text{ (mm}^{-1}\text{)}]$	-2.633	—	-2.463	-2.292	3.526
$\ln [\gamma_{000} \text{ (mm}^{-1}\text{)}]$	—	-2.381	-2.062	-2.735	-0.622
k_{AA}	1.139	0.180	—	0.224	-1.758
k_{BB}	2.866	—	2.307	2.300	-0.234
k_{CC}	—	3.001	2.838	2.714	-0.893
k_{AB}	0.415	—	—	-0.234	-1.996
k_{AC}	—	0.853	—	0.877	3.231
k_{BC}	—	—	0.688	0.684	1.172
V_G (μmol/min per mg)	389.122	415.591	433.716	515.131	625.987
RSS	6.551	8.979	6.832	6.983	8.525
MD%	7.568	18.454	22.373	17.444	16.551

Table 2. Values of the fractional saturations of yeast pyruvate kinase by ADP, phosphoenolpyruvate and Mg²⁺ (p_A , p_B and p_C respectively) calculated by applying the three-substrate exponential-model constants (Table 1), derived from $v = f(A, B, C)$ data, to the concentration set (A, B)_C

These values were employed in constructing Fig. 1. Temperature 25°C; pH 6.2; A = ADP; B = phosphoenolpyruvate; C = Mg²⁺. The concentration of K⁺ was 0.1 M.

Fractional saturations of yeast pyruvate kinase
(C = 2.0 mM)

A (mM)	B = 1.5 mM			B = 2.0 mM			B = 2.5 mM			B = 3.0 mM			B = 4.0 mM			B = 5.0 mM		
	p_A	p_B	p_C	p_A	p_B	p_C	p_A	p_B	p_C	p_A	p_B	p_C	p_A	p_B	p_C	p_A	p_B	p_C
0.2	0.326	0.231	0.333	0.330	0.357	0.388	0.338	0.510	0.469	0.346	0.636	0.542	0.354	0.768	0.611	0.356	0.829	0.639
0.4	0.529	0.242	0.458	0.540	0.384	0.539	0.549	0.542	0.622	0.553	0.656	0.671	0.556	0.774	0.713	0.556	0.832	0.731
0.6	0.651	0.253	0.546	0.659	0.399	0.621	0.664	0.551	0.685	0.665	0.659	0.721	0.666	0.775	0.753	0.665	0.832	0.767
0.8	0.726	0.258	0.596	0.731	0.404	0.662	0.733	0.554	0.715	0.733	0.660	0.746	0.733	0.774	0.774	0.732	0.832	0.786
1.2	0.808	0.263	0.645	0.811	0.409	0.700	0.811	0.555	0.745	0.811	0.659	0.771	0.810	0.773	0.795	0.810	0.831	0.806
2.0	0.881	0.266	0.682	0.881	0.410	0.729	0.881	0.554	0.767	0.881	0.658	0.790	0.880	0.772	0.812	0.879	0.829	0.821

Table 3. Values of the fractional saturations of yeast pyruvate kinase by ADP, phosphoenolpyruvate and Mg²⁺ (p_A , p_B and p_C respectively) calculated by applying the three-substrate exponential-model constants (Table 1), derived from $v = f(A, B, C)$ data, to the partial concentration sets (A, C)_B and (B, C)_A

The complete sets of 30 points each, (X, Y)_Z, were obtained by combining each value of X with each value of Y: these were employed in constructing Figs. 2 and 3. Temperature 25°C; pH 6.2; A = ADP; B = phosphoenolpyruvate; C = Mg²⁺. The concentration of K⁺ was 0.1 M.

B = 5.0 mM			Fractional saturations of yeast pyruvate kinase			A = 2.0 mM			Fractional saturations of yeast pyruvate kinase		
A (mM)	C (mM)		p_A	p_B	p_C	B (mM)	C (mM)		p_A	p_B	p_C
0.2	0.75		0.260	0.739	0.124	1.5	0.75		0.822	0.169	0.142
0.4	1.00		0.445	0.749	0.230	2.0	1.00		0.831	0.263	0.232
0.6	1.25		0.595	0.779	0.424	2.5	1.25		0.847	0.415	0.404
0.8	1.50		0.707	0.811	0.644	3.0	1.50		0.868	0.611	0.647
1.2	2.0		0.810	0.831	0.806	4.0	2.0		0.880	0.772	0.812
2.0	2.0		0.879	0.829	0.821	5.0	2.0		0.879	0.829	0.821

muscle enzyme (Ainsworth *et al.*, 1983). The heterotropic interactions between the substrates follow the pattern laid down by the muscle enzyme; ADP and phosphoenolpyruvate interact negatively, but both, individually, show a positive interaction with Mg^{2+} . The values of the heterotropic constants are, however, smaller than those provided by the muscle enzyme, and their net effects, as revealed by the fits to the two-substrate model, are all positive. The net positive interaction between ADP and phosphoenolpyruvate clearly arises through their mutual interactions with Mg^{2+} , and confirms a similar finding by Haeckel *et al.* (1968).

The broad agreement between the present results and those provided in the previous reports by Haeckel *et al.* (1968) and Hunsley & Suelter (1969b) cannot be pursued by a detailed comparison because the earlier experiments employed the total concentrations of ADP, phosphoenolpyruvate and Mg^{2+} as the variables. It has been shown that inhibition occurs when any of these variables is increased (Macfarlane & Ainsworth, 1972; Ainsworth & Phillips, 1976), and, indeed, evidence of inhibition can be found in the earlier experiments themselves. This effect makes it difficult to interpret the significance of the maximum velocities that were used to construct Hill plots of the velocity data and therefore prejudices further analysis.

The two-substrate-model constants provided by the analysis of $v = f(X, Y)_Z$ data sets are much closer in value to the constants of the three-substrate model than was observed with the muscle enzyme. The probable reason for the difference is that the heterotropic interactions of the yeast enzyme are smaller than those of the muscle enzyme. In consequence, it is more likely that p_Z will remain constant as X and Y are varied, and more likely, in turn, that the two-substrate X and Y constants will reflect those derived from the three-substrate analysis. This suggestion is supported by calculations of p_A , p_B and p_C (by the three-substrate model) given in Tables 2 and 3. Thus it can be concluded that varying A and B at constant C affects p_C very considerably because the three-substrate values of k_{AC} and k_{BC} are both positive. By contrast, the corresponding values of the pairs k_{AB} , k_{CB} and k_{BA} , k_{CA} are opposed in sign, and hence p_B and p_A should show smaller variations at constant B and A as A , C and B , C respectively are the varied concentrations. In keeping with this analysis, the fit to $v = f(A, B)_C$ is less close to the three-substrate constants than those provided by $v = f(A, C)_B$ and $v = f(B, C)_A$. Also note from Tables 2 and 3 that the values of p_A , p_B and p_C cover the major portions of the spans from 0 to 1 in spite of the relatively small ranges in A , B and C .

Finally, concerning the three-substrate constants themselves: the similarities in sign of the heterotropic interactions displayed by the rabbit muscle and yeast enzymes suggest that they have a common origin in charge interactions of the substrates within the active site (Ainsworth & Macfarlane, 1973; Ainsworth *et al.*, 1983). If so, the observation that the yeast enzyme interaction constants are smaller than those of the rabbit enzyme may be due, at least in part, to the lower pH of the yeast experiments, the effect of which would be to decrease the average negative charges carried by ADP and phosphoenolpyruvate (Macfarlane & Ainsworth, 1972). The negative homotropic interactions of the muscle enzyme were taken to arise from the repulsions that would occur on binding successive molecules of a given substrate with the same charge; supposing this effect still to exist, it is evident that the structural re-arrangements that give rise to the positive homotropic interactions in the yeast enzyme must require rather more energy than is indicated by the values of the constants. These conclusions, though limited by the empirical nature of the model, do show the value of undertaking systematic examination of the kinetics of regulatory enzymes.

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