The competition plot: a simple test of whether 2 reactions occur at the same active-site

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The competition plot: a simple test of whether two reactions occur at the same active site

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The competition plot is a method for determining whether or not two enzyme-catalysed reactions occur at the same active site. It is a plot of total rate against p, where p varies from 0 to 1 and specifies the concentrations $(1-p)a_0$ and pb_0 of two substrates in terms of reference concentrations a_0 and b_0 chosen so as to give the same rates at $p = 0$ and $p = 1$. If the two substrates react at the same site, the competition plot gives a horizontal straight line, i.e. the total rate is independent of p. Independent reactions at two separate sites give a curve with a maximum; separate reactions with cross-inhibition generate curves with either maxima or minima according to whether the Michaelis constants of the two substrates are smaller or larger than their inhibition constants in the other reactions. Although ambiguous results can sometimes arise, experimental strategies exist for avoiding them, for example working as close as possible to the lower of the two limiting rates. When tested with yeast hexokinase, the plot indicated phosphorylation of glucose and fructose at the same site. Conversely, with a mixture of yeast hexokinase and galactokinase it indicated phosphorylation of glucose and galactose at different sites. In both cases the observed behaviour agreed with the known properties of the enzymes. A slight modification to the definition of this plot allows it to be applied also to enzymes that deviate from Michaelis–Menten kinetics.

INTRODUCTION

When an enzyme preparation catalyses two different reactions, it is often of interest to know whether these can be interpreted as competitive reactions occurring at the same active site, or whether the data are more easily explained in terms of multiple active sites (or multiple enzymes in the case of preliminary studies of unpurified enzymes). Detailed kinetic study of the two reactions may well shed light on this question, especially if the two rates can be measured independently of one another. However, if the existence of multiple sites is the primary question of interest, one may well want to have some information about it very early in the investigation, before the enzymes have been purified, before thorough examination of their kinetics, and before committing a significant amount of perhaps precious material to an investigation that may have little point if the multiple reactions are likely to be due to a mixture of enzymes.

In the past, the question arose most often in attempts to characterize activities of unpurified enzymes with small substrates, such as the investigation of arylsulphatase by Webb and Morrow (1959); however, it applies equally well to the specificity of more complex systems, such as protein kinases, where it is often difficult to obtain enzymes and natural substrates in sufficient quantities for kinetic investigation.

Although mixed-substrate experiments can yield information about multiplicity of sites, textbook accounts (e.g. Segel, 1975; Dixon and Webb, 1979) do not contain the detailed analysis needed for establishing the experimental conditions capable of providing unambiguous results. We describe here a simple plot, which we shall call the 'competition plot', that allows the more obvious cases to be distinguished with very little experimentation.

METHOD

For an enzyme preparation (not necessarily pure) that catalyses two different reactions, define one of the substrates as A, preferably the one with the smaller limiting rate (often called the 'maximum velocity'), if this is known, and the other as B. Select a concentration, a_0, of A that gives a conveniently measurable rate, v_0, in the absence of B. Then determine, using the rate equation for the reaction of B if this is known, or trial and error if not, the concentration, b_0, of B that gives the same rate, v_0, in the absence of A.

Prepare a series of mixtures containing A and B at concentrations $a = (1-p)a_0$ and $b = pb_0$ respectively, determine the total rate for each mixture, and plot this against p. A horizontal straight line in the resulting competition plot, i.e. a constant rate independent of p, indicates that the two reactions occur at the same site. (The justification for this and other assertions in this section is given below in the Theory section.)

The competition plot is illustrated in Figure 1, where competition for one site is contrasted with two other types of behaviour that can arise from fairly simple models: completely independent reactions generate a curve with a maximum; antagonistic reactions, in which each substrate is more effective at inhibiting the other reaction than at promoting its own, generate a curve with a minimum.

If the two reactions occur at the same site, the form of the competition plot is independent of the value of v_0. However, to maximize the chances of observing any departure from the behaviour expected for competitive reactions it is best to make it as high as possible, i.e. as close as possible to the smaller limiting rate.

If the plot gives a curve, this is not in general symmetrical, i.e. the extremum does not occur at $p = 0.5$ unless the two limiting rates are equal, so it is advisable to spread the observations over the whole range from 0 to 1. If only a few observations can be made, one should prefer values of p close to 1 over values close to 0 (assuming A and B to be defined as suggested above, with A having the lower limiting rate).

If the two reactions do not obey Michaelis–Menten kinetics but have Hill coefficients h_A and h_B that are essentially constant over the experimental range, the definitions of the concentrations

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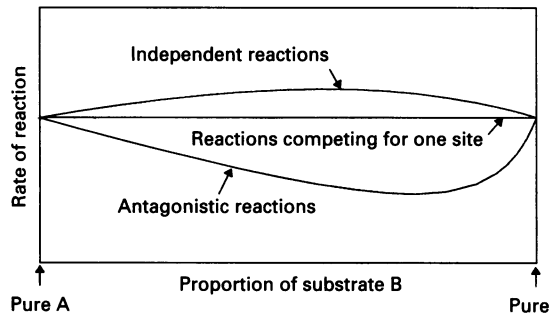

Figure 1 The principal types of competition plot

The total rate of reaction is measured for mixtures of substrates at concentrations $(1-p)a_0$ and pb_0, where the reference concentrations a_0 and b_0 are chosen so that they give equal rates in experiments with only one substrate present, and this total rate is plotted against p. If the two reactions occur at the same site, the rate does not vary with p; if the two reactions occur at two sites with no interaction, the plot gives a curve with a maximum; if each substrate is more effective at inhibiting the other reaction than in reacting in its own, the plot gives a curve with a minimum. Note that when curves are obtained these are not in general symmetrical about $p = 0.5$.

in the mixtures should be expressed as $a = a_0(1-p)^{1/h_A}$ and $b = b_0 p^{1/h_B}$ to give a plot of total rate against p with the same properties as those described.

THEORY

If the two reactions in which A is converted into P and B is converted into Q, both follow Michaelis–Menten kinetics, the rates v_A and v_B for the reactions of A and B in the absence of the other may be expressed in terms of the concentrations a and b of A and B as follows:

$$v_A = \frac{V_A a}{K_{mA} + a} \tag{1}$$

$$v_B = \frac{V_B b}{K_{mB} + b} \tag{2}$$

where V_A and V_B are the two limiting rates, and K_{mA} and K_{mB} are the two Michaelis constants. The point now to be examined is how these equations must be modified if both substrates are simultaneously present.

Model 1: competing reactions at a single site

In the simplest case of competitive reactions (Figure 2a), discussed in textbooks (e.g. Fersht, 1985; Cornish-Bowden, 1979), the competing reactions follow equations algebraically equivalent to those for competitive inhibition, with the inhibition constant replaced by the Michaelis constant of the competing substrate:

$$v_A = \frac{V_A a}{K_{mA}(1 + b/K_{mB}) + a} \tag{3}$$

$$v_B = \frac{V_B b}{K_{mB}(1 + a/K_{mA}) + b} \tag{4}$$

The combined rate v_{tot}, especially pertinent if the assay does not distinguish between the two reactions, is the sum of these two rates:

$$v_{tot} = v_A + v_B = \frac{V_A a/K_{mA} + V_B b/K_{mB}}{1 + a/K_{mA} + b/K_{mB}} \tag{5}$$

Define now a reference concentration a_0 such that the rate v_A has some value v_0 in the absence of B when $a = a_0$, and a reference

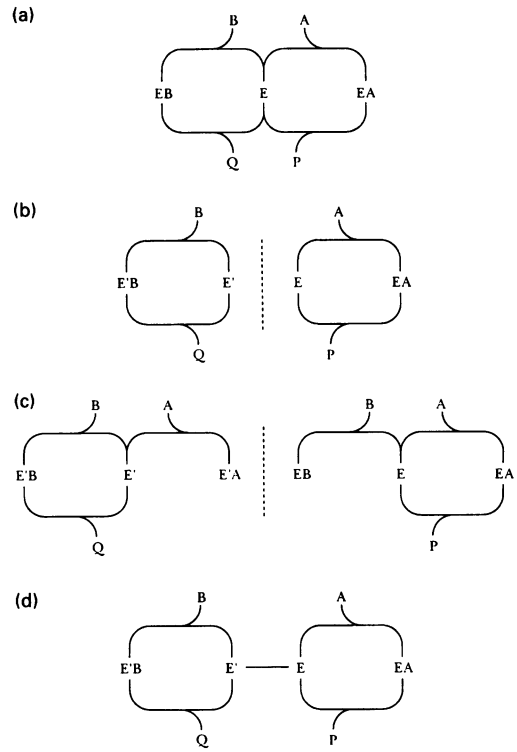

Figure 2 Schemes for two reactions occurring in the same mixture

The following possibilities are shown: (**a**) Model 1, competitive reactions at one site; (**b**) Model 2, two independent reactions; (**c**) Model 3, two reactions, but each substrate can inhibit the other reaction; (**d**) Model 4, two substrates reacting with different conformations of the same site. For simplicity the schemes are written with one substrate and one product in each reaction, but the analysis applies also to reactions with more than one substrate.

concentration b_0 such that the rate v_B has the same value v_0 in the absence of A when $b = b_0$:

$$\frac{V_A a_0}{K_{mA} + a_0} = \frac{V_B b_0}{K_{mB} + b_0} = v_0 \tag{6}$$

If a and b are varied together in such a way that

$$\left.\begin{aligned} a &= (1-p)a_0 \\ b &= pb_0 \end{aligned}\right\} \quad p = 0 \ldots 1 \tag{7}$$

then the total rate as p is varied is:

$$\begin{aligned} v_{tot} &= \frac{V_A(1-p)a_0/K_{mA} + V_B pb_0/K_{mB}}{1 + (1-p)a_0/K_{mA} + pb_0/K_{mB}} \\ &= \frac{V_A(1-p)a_0/K_{mA} + V_B pb_0/K_{mB}}{(1-p)(1 + a_0/K_{mA}) + p(1 + b_0/K_{mB})} \\ &= \frac{v_0[(1-p)(1 + a_0/K_{mA}) + p(1 + b_0/K_{mB})]}{(1-p)(1 + a_0/K_{mA}) + p(1 + b_0/K_{mB})} \\ &= v_0 \end{aligned} \tag{8}$$

The third line of this equation follows from the second by virtue of eqn. (6), i.e. by substituting $V_A a_0/K_{mA}$ with $v_0(1 + a_0/K_{mA})$, and $V_B b_0/K_{mB}$ similarly. If the two substrates compete for the same site, therefore, the rate with mixtures is a constant, independent of the proportions of the two substrates in the mixture.

Model 2: reactions at two fully independent sites

The result of the analysis of Model 1 is useful only if it does not apply equally well to other models. The other extreme that one

can consider is that the two reactions are entirely independent of one another, i.e. that eqns. (1) and (2) apply not only to the pure substrates, but also to mixtures (Figure 2b). In this case eqns. (3) and (4) do not hold, and so eqn. (5) must be replaced by the following equation for the total reaction, with a and b independently variable:

$$v_{tot} = \frac{V_A a}{K_{mA}+a} + \frac{V_B b}{K_{mB}+b} \tag{9}$$

If a and b are varied systematically according to eqns. (7), this becomes as follows:

$$v_{tot} = \frac{(1-p)V_A a_0}{K_{mA}+(1-p)a_0} + \frac{pV_B b_0}{K_{mB}+pb_0} \tag{10}$$

Unlike eqn. (8), eqn. (10) does not define rates independent of p, because when p is close to zero the first term on the right-hand side is virtually independent of p, whereas the second is proportional to p; conversely, when p is close to unity, the first term is proportional to $(1-p)$ and the second is virtually independent of p. It follows that with this model the competition plot of v_{tot} against p is a curve with a maximum at an intermediate value of p (Figure 1).

Model 3: separate catalytic sites with cross-inhibition

In some circumstances the first two models may well provide the most reasonable alternatives, but in others it may be better to regard them as two special cases of a more general model in which the two reactions occur at separate sites, but are not independent of one another because each substrate inhibits the other reaction (Figure 2c). As the two substrates will often be structurally similar, e.g. NADPH and NADH, such cross-inhibition would hardly be surprising. In this case the rate equations are as follows:

$$v_A = \frac{V_A a}{K_{mA}(1+b/K_{iB})+a} \tag{11}$$

$$v_B = \frac{V_B b}{K_{mB}(1+a/K_{iA})+b} \tag{12}$$

In this model, as the two reactions are distinct, there is no reason to expect the two inhibition constants K_{iA} and K_{iB} to be equal to the corresponding Michaelis constants K_{mA} and K_{mB}, and if they were equal it would only be by coincidence. If such a coincidence occurred eqns. (11) and (12) would be indistinguishable from eqns. (3) and (4) and the model would give the same plot of v_{tot} against p as competition at a single site.

Defining p as before, the sum of the two rates given by eqns. (11) and (12) has the following dependence on p:

$$v_{tot} = \frac{V_A(1-p)a_0/K_{mA}}{1+(1-p)a_0/K_{mA}+pb_0/K_{iB}} + \frac{V_B pb_0/K_{mB}}{1+(1-p)a_0/K_{iA}+pb_0/K_{mB}} \tag{13}$$

Differentiating this with respect to p, the slope of a plot of v_{tot} against p is given by:

$$\frac{dv_{tot}}{dp} = -\frac{V_A(1+b_0/K_{iB})a_0/K_{mA}}{[1+(1-p)a_0/K_{mA}+pb_0/K_{iB}]^2} + \frac{V_B(1+a_0/K_{iA})b_0/K_{mB}}{[1+(1-p)a_0/K_{iA}+pb_0/K_{mB}]^2} \tag{14}$$

Setting this to zero gives a quadratic expression in p, and one might be tempted to conclude that a plot of v_{tot} against p could show two stationary points. However, such a conclusion would be mistaken, as one can most simply demonstrate by examining the signs of the slopes at the two experimentally accessible extremes, i.e. at $p = 0$ and $p = 1$:

$$\left(\frac{dv_{tot}}{dp}\right)_{p\to 0} = \frac{v_0\left[a_0\left(\frac{1}{K_{mA}}-\frac{1}{K_{iA}}\right)+b_0\left(\frac{1}{K_{mB}}-\frac{1}{K_{iB}}\right)+a_0b_0\left(\frac{1}{K_{mA}K_{mB}}-\frac{1}{K_{iA}K_{iB}}\right)\right]}{\left(1+\frac{a_0}{K_{mA}}\right)\left(1+\frac{a_0}{K_{iA}}\right)} \tag{15}$$

$$\left(\frac{dv_{tot}}{dp}\right)_{p\to 1} = \frac{v_0\left[a_0\left(\frac{1}{K_{iA}}-\frac{1}{K_{mA}}\right)+b_0\left(\frac{1}{K_{iB}}-\frac{1}{K_{mB}}\right)+a_0b_0\left(\frac{1}{K_{iA}K_{iB}}-\frac{1}{K_{mA}K_{mB}}\right)\right]}{\left(1+\frac{b_0}{K_{mB}}\right)\left(1+\frac{b_0}{K_{iB}}\right)} \tag{16}$$

As the denominators of both expressions must be positive, it is obvious that the signs of the two slopes must be opposite. As, in addition, eqn. (14) defines a continuous function between $p = 0$ and $p = 1$, it follows that there can be either one maximum or one minimum, but not both, and no more than one of either.

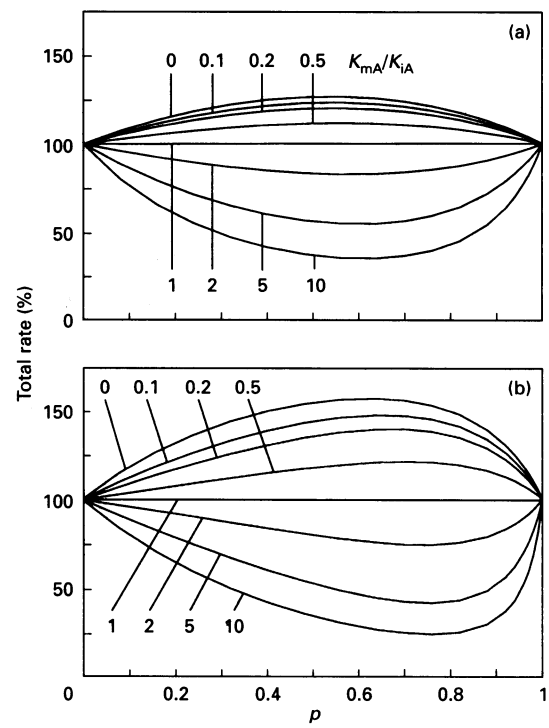

Figure 3 Competition plots for two reactions with mutual inhibition

In (**a**), the curves were calculated for Model 3 using eqn. (13) with $V_B/V_A = 1.5$, $a_0/K_{mA} = 1$, $b_0/K_{mB} = 0.5$, and are labelled with eight different values of $K_{mA}/K_{iA} = K_{mB}/K_{iB}$ from 0 to 10. The curve for $K_{mA}/K_{iA} = K_{mB}/K_{iB} = 0$ is identical with that for independent reactions (Model 2), and the line for $K_{mA}/K_{iA} = K_{mB}/K_{iB} = 1$ is identical with that for competitive reactions at one site (Model 1). In (**b**) the curves are recalculated with $a_0/K_{mA} = 5$, $b_0/K_{mB} = 1.25$.

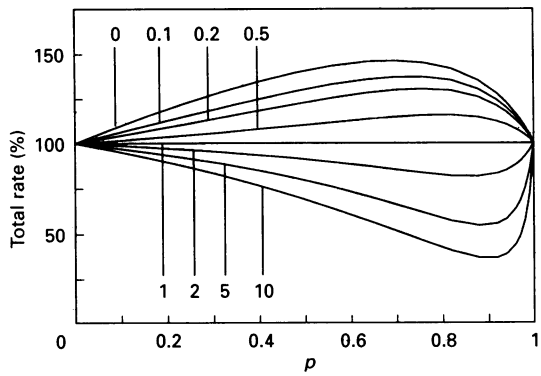

Figure 4 Increased asymmetry with substantially different limiting rates

The curves were calculated for Model 3 using eqn. (13) with $V_B/V_A = 5$, $a_0/K_{mA} = 5$, $b_0/K_{mB} = 0.2$, $K_{mA}/K_{iA} = K_{mB}/K_{iB}$ from 0 to 10, as in Figure 3.

Thus the secondary stationary point must occur outside the range $p = 0$ to $p = 1$. Loosely speaking, though convenient as a mnemonic, one can say that a maximum [positive slope in eqn. (15)] indicates that the two reactants act more as substrates than as inhibitors, whereas a minimum indicates that they act more as inhibitors than as substrates.

Some curves calculated with this model are shown in Figure 3a, where the limiting rates have been assumed to be of similar, but not identical, magnitude ($V_B/V_A = 1.5$), and a_0/K_{mA} has been set to 1. The curves for $K_{mA}/K_{iA} = 1$ and $K_{mA}/K_{iA} = 0$ correspond to competition for one site and independent reactions respectively: although the curvature in the latter case is not very great, with a maximum departure from constancy of about 25%, it is nonetheless large enough to permit these two models to be distinguished in practice without demanding an unrealistic degree of experimental precision. In any case, the sensitivity can be substantially improved by using higher substrate concentrations, as is illustrated in Figure 3b, where all the curves are recalculated for $a_0/K_{mA} = 5$ and deviate from constancy by much larger amounts.

Note that the curves are not symmetrical about $p = 0.5$, but have extrema biased towards saturation of the enzyme with the higher limiting rate. This asymmetry may be quite pronounced, especially in the case of antagonistic reactions, if the limiting rates of the two reactions are very different (Figure 4). To be sure

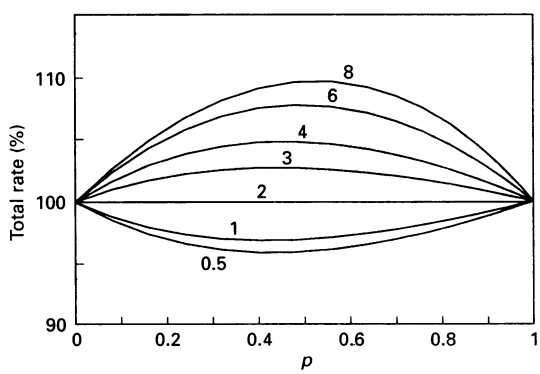

Figure 5 Fortuitous cancellation of terms

When the cross-inhibition model (Model 3) applies, fortuitous cancellation of terms can be eliminated by changing the concentration. The curves were calculated from eqn. (13) with $V_B/V_A = 1.5$, $K_{mA}/K_{iA} = 0.1558$, $K_{mB}/K_{iB} = 3.895$, and are labelled with the values of a_0/K_{mA}, which ranged from 0.5 to 8 (with b_0/K_{mB} ranging correspondingly from 0.2857 to 1.4545). Although fortuitous cancellation may generate a curve indistinguishable from the line for competition at one site (as in the line for $a_0/K_{mA} = 2$), this occurs only at one pair of concentrations.

of detecting any departure from constancy that may exist, therefore, it is not sufficient to limit consideration to intermediate values of p: there may be little variation in rate in the range 0–0.5, but a large variation between 0.9 and 1. If the limiting rates do differ by a factor of 10 or more, there may be considerable practical difficulties for achieving this ideal, because a rate that is virtually saturating for one activity may be so low for the other that it requires such low substrate concentrations that accurate measurement may be difficult or impossible.

If $K_{mA} - K_{iA}$ and $K_{mB} - K_{iB}$ have opposite signs, eqns. (15) and (16) suggest that fortuitous cancellation may occur so that Model 3 generates a constant rate with p even though the reactions are not truly competitive. As Figure 5 shows, this is indeed the case; however, as the fortuitous cancellation only occurs at one a_0, b_0 pair ($a_0/K_{mA} = 2$ in Figure 5), this can be distinguished experimentally from true competition, which gives exactly the same plot under all conditions.

Model 4: competition for different conformations of the same site

In discussing competitive reactions we have tacitly assumed that the two substrates bind not only to the same site, but also to the same conformational state of that site. As this may sometimes be an unacceptable assumption, we need to consider whether the conclusions would be altered if the model were drawn as in Figure 2d, where A and B react with two different states of the enzyme E and E′ that are linked by a reaction with equilibrium constant $K_0 = [E']/[E]$.

For this model the two rates are given by the following equations:

$$v_A = \frac{V_A a}{K'_{mA}(1 + K_0 + K_0 b/K'_{mB}) + a} \quad (17)$$

$$v_B = \frac{V_B b}{K'_{mB}(1 + 1/K_0 + a/K_0 K'_{mA}) + b} \quad (18)$$

where K'_{mA} is the value that K_{mA} would have if E′ did not exist, and K'_{mB} is the value that K_{mB} would have if E did not exist. However, the substitutions $K_{mA} = K'_{mA}(1 + K_0)$ and $K_{mB} = K'_{mB}(1 + 1/K_0)$ transform these equations exactly into eqns. (3) and (4), and consequently eqn. (8) applies as well to this model as to Model 1; thus the competition plot does not distinguish between these models, i.e. it does not provide information about whether substrates that bind at the same site induce different conformations or not.

As this conclusion may appear surprising, it may be useful to examine the mechanism in the light of the method of King and Altman (1956). It is evident that no kappa products exist in the rate equation that contain both a and b, because the two halves of the mechanism are linked only by the reaction between E and E′: any such kappa product would inevitably represent an illegal King–Altman pattern that failed to lead to a unique sink. This explains why eqns. (3) and (4) apply to the mechanisms of Figure 2(a) and 2(b) equally well, with only the definitions of the kinetic parameters in terms of rate constants altered. Consequently it makes no difference to the competition plot whether the two substrates bind to the same or different conformations, provided that they bind in such a way that only one can be bound at one time.

Reactions that deviate from Michaelis–Menten kinetics

In analysing the competition plot we have hitherto assumed that the two competing reactions both obey Michaelis–Menten kinetics, but it would be useful to know whether this assumption is necessary, i.e. to know whether the competition plot will give

the same result for competing reactions that deviate from Michaelis–Menten kinetics. Answering this in a general way is complicated, but one can obtain an idea of the result by assuming that such enzymes display a constant Hill coefficient over the range of the experiment. Although the relationship of this assumption to models of co-operativity with a secure theoretical base is not very obvious, many enzymes obey it surprisingly well in practice, especially if the co-operativity is positive (see e.g. Cornish-Bowden and Koshland, 1975). One may expect it, therefore, to give a reasonable basis for predicting the behaviour of the competition plot when applied to non-Michaelian enzymes.

If one attempts to derive eqn. (8) after replacing a and b in eqns. (3) and (4) by a^{h_A} and b^{h_B} respectively, i.e. assuming Hill coefficients of h_A and h_B respectively, it proves to require the condition $(1-p)^{h_A}+p^{h_B} \equiv 1$, which is true only if $h_A = h_B = 1$, i.e. if the Hill model simplifies to the same Michaelis–Menten kinetics that have been considered already. If both Hill coefficients are greater than one (i.e. if both reactions show positive co-operativity), the curve shows a minimum, and if both are less than one it shows a maximum.

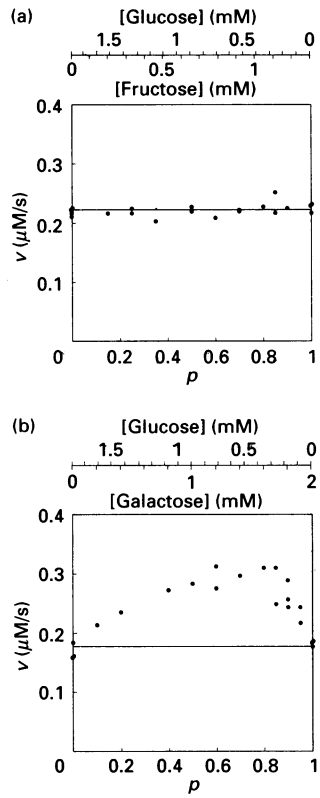

Figure 6 Competition plots showing phosphorylation of hexoses (a) at the same site and (b) at different sites

(**a**) Phosphorylation of glucose and fructose at concentrations $(1-p) \times 1.8$ mM and $p \times 1.3$ mM respectively, catalysed by yeast hexokinase; (**b**) phosphorylation of glucose and galactose at concentrations $(1-p) \times 1.8$ mM and $p \times 2.0$ mM respectively, catalysed by a mixture of yeast hexokinase and galactokinase. In both cases p was varied from 0 to 1. Points that would otherwise be superimposed (all at $p = 0$ or $p = 1$) have been displaced slightly away from one another in a diagonal direction. Phosphorylation of glucose, fructose or galactose was assayed by coupling the production of ADP to oxidation of NADH by means of pyruvate kinase and lactate dehydrogenase in the presence of excess phosphoenolpyruvate (Kornberg and Pricer, 1951). The reaction was monitored at 340 nm in a Pye–Unicam PU 8660 recording spectrophotometer, in a 1 ml mixture containing 50 mM glycylglycine, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ATP, glucose, fructose, 2.5 mM phosphoenolpyruvate, 0.25 mM NADH, pyruvate kinase (60 nkat/ml) and lactate dehydrogenase (60 nkat/ml). Each reaction mixture was preincubated for 4 min at 30 °C to remove any ADP initially present as a contaminant of the ATP, and the reaction was started by addition of hexokinase alone (for glucose and fructose) or a mixture of hexokinase and galactokinase (for glucose and galactose).

This suggests a slight modification of the definition of the competition plot (which has no effect on its use with enzymes obeying Michaelis–Menten kinetics) to allow it to give the same behaviour regardless of the Hill coefficients. Defining the reference concentrations a_0 and b_0 exactly as before, we now define the concentrations in the mixtures as $a = a_0(1-p)^{1/h_A}$ and $b = b_0 p^{1/h_B}$. With these definitions eqn. (8) can be derived by the same logic as before.

MATERIALS AND METHODS

Hexokinase (yeast type C-301, EC 2.7.1.1) and galactokinase (yeast, EC 2.7.1.6) were obtained from Sigma Chimie S.a.r.l., La Verpillière, France; phosphoenolpyruvate, ATP, NADH, EDTA, pyruvate kinase (rabbit muscle, EC 2.7.1.40) and lactate dehydrogenase (rabbit muscle, EC 1.1.1.27) were obtained from Boehringer, Mannheim; glucose, fructose and galactose were obtained from Aldrich-Chimie S.a.r.l., Strasbourg, France.

Details of the assay conditions are given in the legend to Figure 6.

DISCUSSION

A convenient example for testing the predicted behaviour of the competition plot is provided by yeast hexokinase, an enzyme that has been considered for many years to catalyse phosphorylation of glucose and fructose at the same site, a belief supported by numerous kinds of experiment, both kinetic (e.g. DelaFuente et al., 1970; Ricard et al., 1972) and structural (Fletterick et al., 1975). On the other hand, the activity of yeast hexokinase towards galactose is low, and galactokinase has no detectable activity towards glucose (Heinrich, 1964). Accordingly, a competition plot for hexokinase-catalysed phosphorylation of glucose and fructose ought to show no variation in rate, whereas a plot made for phosphorylation of glucose and galactose catalysed by a mixture of these enzymes should show a maximum. As shown in Figure 6, the results were as expected.

Another example is provided by acetohydroxy acid isomeroreductase from spinach chloroplasts: studies of the effect of 2-acetolactate on the kinetic parameters for the reaction with 2-aceto-2-hydroxybutyrate were interpreted by supposing that these two substrates compete for the same enzyme form (Dumas et al., 1992); subsequent investigation (D. Job, personal communication) has shown that the competition plot leads more directly and simply to the same conclusion.

The competition plot thus offers a simple solution to a problem that can appear at first sight difficult or complicated. Although mixed-substrate experiments have long been used for investigating whether multiple activities are best explained in terms of multiple active sites, they have never yielded their full potential, because they have not usually been designed to yield simple behaviour when the simplest plausible model applies. For example, in studying the action of arylsulphatase on *p*-nitrophenyl sulphate and 2-hydroxy-5-nitrophenyl sulphate, Webb and Morrow (1959) used a mixture of the two substrates at concentrations that gave rates differing by a factor of about 2 when examined separately, and found a total rate for the mixture equal to about 60% of the sum of these rates. Although this result was consistent with their interpretation that the action was due to competition for a single site, it could also have been explained in terms of separate sites with cross-inhibition (our Model 3), and their analysis was less simple than analysis of a competition plot would have been. Their design would only have given unambiguous results (total rate equal to the sum of separate rates) in the case of two totally independent reactions in

which neither substrate had any effect on the other reaction: not a very likely model for two such similar substrates.

The competition plot has some similarities with the 'constant-velocity plot' of Whitehead (1984), but it is experimentally less demanding. The constant-velocity plot requires determination of the combinations of two concentrations that generate the same velocity. [In the discussion of Whitehead (1984) these were two effector concentrations, as they also were in the experiments of Mastrantonio et al. (1983) on donkey spleen deoxycytidate aminohydrolase, but the same ideas could be applied to substrates.] For each such velocity the two concentrations are plotted against one another, and the forms of the lines obtained and their intersection points allow one to deduce information about the binding sites.

There is no doubt that the constant-velocity plot is a powerful method that could profitably be used more widely than it has been. However, it is less straightforward to use than the competition plot, where instead of determining combinations of concentrations that do in fact give the same rate one sets up reaction mixtures that ought to give the same rate if the simplest model is valid. The competition plot also has the advantage of being a null method, i.e. it has a predicted effect of zero, so that to the limit of the experimental accuracy even the smallest deviation from constancy provides evidence against the simplest model.

Although the presence of a maximum or a minimum in the competition plot is sufficient to rule out competition for a single site, the converse is not necessarily true, because fortuitous cancellation of terms in more complex models can also lead to a rate independent of the proportions of substrates. However, such fortuitous cancellation is expected only at one particular rate, and thus should be easily recognized if experiments are carried out over as wide a range of rates as possible.

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