Analysis of Hill slopes predicted by the four-ligand exponential model for a regulatory enzyme

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The four-ligand exponential model for a regulatory enzyme is described as it is applied to kinetic studies of yeast pyruvate kinase in which the concentrations of four ligands are systematically varied. The Hill slopes predicted by this model are calculated for the two situations in which the fourth ligand is either a substrate or an effector. It is shown that the individual terms that make up the expression for the Hill slope assist the interpretation of the observed behaviour in terms of the constants employed by the model.

INTRODUCTION

The kinetics of pyruvate kinase from *Saccharomyces cerevisiae* have been systematically studied as a function of the concentrations of both substrates and individual effectors (Morris *et al.*, 1984, 1986; Kinderlerer *et al.*, 1986; Rhodes *et al.*, 1986). The results of these investigations were interpreted by use of the exponential model for a regulatory enzyme (Ainsworth *et al.*, 1983) on the assumption that the various ligands bind independently to the enzyme (Morris *et al.*, 1984). According to this model, the initial rate of pyruvate formation, v , is represented by:

$$\frac{v}{V_G} = p_A \cdot p_B \cdot p_C \cdot [1 + (y-1)p_D] \quad (1)$$

where p_A , p_B and p_C respectively stand for the fractional saturation of the enzyme by ADP, phosphoenolpyruvate (both in their Mg^{2+} -free forms) and free Mg^{2+} . p_D stands for the fractional saturation by the effector, which may be variously H^+ , fructose 1,6-bisphosphate or an obligatory activator such as K^+ or NH_4^+ . (Note that K^+ and NH_4^+ were present at saturating concentrations in the studies where the effect of these ions on the rate of reaction was not being explicitly examined.) V_G represents the initial rate of product formation when the enzyme is completely saturated by substrates, $p_A = p_B = p_C = 1$, in the absence of D. A different rate is measured when the enzyme is also saturated by D, and the change is defined by multiplying V_G by the constant y : inhibition is associated with values $0 < y < 1$ and activation with values $y > 1$. When the activator is obligatory, $y = \infty$, and D behaves as a fourth substrate. The exponential model is applied to eqn. (1) by assuming that the binding constants of the several ligands are defined by equations of the type:

$$\ln w_i = \ln w_0 + k_{wW} \cdot p_W + k_{wX} \cdot p_X + k_{wY} \cdot p_Y + k_{wZ} \cdot p_Z \quad (2)$$

where the association constant w_i {equal to $p_W / [(1-p_W) \cdot W]$ } is defined for the i th solution by the particular values of the fractional saturations obtaining therein. k_{XY} is an interaction constant in RT units, where X denotes the ligand whose binding causes a change in the association constant for Y. Reciprocal allosteric linkage requires $k_{XY} = k_{YX}$.

The analysis of data $v = f(A, B, C, D)$ by eqns. (1) and (2) provides 15 constants, all of which are responsible for the changing relationships that were observed (Morris *et al.*, 1984, 1986; Kinderlerer *et al.*, 1986; Rhodes *et al.*, 1986). It was found possible to comment meaningfully on the measured values of the constants in terms of what is known of the catalytic mechanism, but, in a situation of this complexity, it is difficult to see in more than general terms how individual constants or ligand concentrations exert their influence on the initial velocity. The purpose of the present paper is to show that the analytical expression for the slope of the Hill plot of data $v = f(W)_{X,Y,Z}$ provides an explicit statement of the contributions made by each ligand to the overall measure of interaction. The additional insight that is provided is exemplified by reference to the studies introduced above.

THEORY

The Hill slope of the plot $\ln[p_A/(1-p_A)] = f(\ln A)$ is defined as

$$n_H = \frac{1}{1-p_A} \cdot \frac{d[\ln p_A]}{d[\ln A]} \quad (3)$$

By analogy, the Hill plot can be employed to represent velocity data $v = f(A)_{B,C,D}$ by assuming that the fractional saturations of ligands whose concentrations are kept constant remain unchanged as A is varied, i.e.:

$$p_A = \frac{v}{V_{\max}}$$

where $V_{\max}$ is an apparent maximum velocity measured when A is sufficiently large to make $p_A = 1$. Hence:

$$n_H = \frac{1}{1-v/V_{\max}} \cdot \frac{d[\ln v]}{d[\ln A]} \quad (4)$$

Data for pyruvate kinase can obviously be represented by eqn. (4). Equally, eqn. (4) can be interpreted by the exponential model because both $V_{\max}$ (Kinderlerer *et al.*, 1981) and $d[\ln v]/d[\ln A]$ can be defined by the model equations. It should be noted, however, that $v/V_{\max}$ is generally not equal to p_A (Ainsworth & Gregory, 1978) and hence that the simple analogy with eqn. (3) no longer holds. The same hiatus is present in any application of eqn. (4) where the kinetic data are inconsistent with the

restriction $p_A = v/V_{\max.}$, an objection in principle that has not prevented the widespread empirical use of the Hill slope as a measure of ligand interaction displayed in the kinetic behaviour of allosteric enzymes. Eqn. (4) is therefore used here as the basis of analysis.

From eqn. (1):

$$\frac{d(\ln v)}{d(\ln A)} = \frac{d(\ln p_A)}{d(\ln A)} + \frac{d(\ln p_B)}{d(\ln A)} + \frac{d(\ln p_C)}{d(\ln A)} + \frac{p_D(y-1)}{1+p_D(y-1)} \cdot \frac{d(\ln p_D)}{d(\ln A)} \quad (5)$$

which, for conciseness, is equated term by term with:

$$\frac{d(\ln v)}{d(\ln A)} = d_A^A + d_A^B + d_A^C + G \cdot d_A^D \quad (6)$$

Note that $G = 0$ when $y = 1$ and $G = 1$ when $y = \infty$, the latter relation showing that an obligatory activator is equivalent to a substrate. With the definitions:

$$\frac{dp_X}{d(\ln A)} = \frac{N_X}{D_A} = p_X \cdot d_A^X \quad (7)$$

and

$$\phi_X = p_X(1 - p_X); \quad Z_X = (1 - \phi_X k_{XX})$$

we obtain, by differentiating the several forms of eqn. (2):

$$N_A = \phi_A \{ Z_B Z_C Z_D - [Z_B \phi_C \phi_D k_{CD}^2 + Z_C \phi_B \phi_D k_{BD}^2 + Z_D \phi_B \phi_C k_{BC}^2 + 2\phi_B \phi_C \phi_D k_{BC} k_{CD} k_{DB}] \} \quad (8)$$

$$N_B = \phi_A \phi_B [Z_C Z_D k_{AB} + Z_C \phi_D k_{AD} k_{DB} + Z_D \phi_C k_{AC} k_{CB} + \phi_C \phi_D (k_{AC} k_{CD} k_{DB} + k_{AD} k_{DC} k_{CB} - k_{AB} k_{DC}^2)] \quad (9)$$

$$N_C = f_{BC}(N_B) \quad (10)$$

where f_{BC} is the transformation that interchanges the B and C subscripts of symbols appearing in N_B . Similarly:

$$N_D = f_{BD}(N_B) \quad (11)$$

The four differentials have a common denominator defined by:

$$D_A = Z_A Z_B Z_C Z_D + \phi_A \phi_B \phi_C \phi_D k_{AB}^2 k_{CD}^2 \times (f_0 + f_{BC} + f_{BD}) - [Z_A Z_B \phi_C \phi_D k_{CD}^2 \times (f_0 + f_{BC} + f_{BD} + f_{AC} + f_{AD} + f_{AC, BD}) + 2Z_D \phi_A \phi_B \phi_C k_{AB} k_{BC} k_{CA} (f_0 + f_{AD} + f_{BD} + f_{CD}) + 2\phi_A \phi_B \phi_C \phi_D k_{AB} k_{BC} k_{CD} k_{DA} (f_0 + f_{BC} + f_{CD})] \quad (12)$$

where f_0 is the identity transformation. Eqns. (8)–(12) can be readily changed to represent differentiation with respect to $\ln X$ by appropriate re-definition of the constants k_{XX} and k_{XY} .

Note that when $p_X = 0$ or 1 then $\phi_X = 0$ and $Z_X = 1$. The differentials can therefore be simplified to reproduce the corresponding equations previously given for two and three ligands respectively by Ainsworth & Gregory (1978) and Ainsworth *et al.* (1983), except, that is, for the accidental omission of the term $2\phi_A \phi_B \phi_C k_{AB} k_{BC} k_{CA}$ from the denominator of the three-ligand differentials. (The missing term is symmetrical in A, B, C and its omission does not affect the conclusions that were previously drawn.)

The symmetry evident in eqns. (9) and (12) shows that the transformation f_{AX} leads to:

$$\left(\frac{N_X}{D_A} \right)_{X \neq A} = \left(\frac{N_A}{D_X} \right)_{X \neq A} \quad (13)$$

and hence that linkage is present (Wyman, 1964).

It will be noted that eqns. (5)–(12) depend only on the interaction constants k_{XY} and the fractional saturations. The latter values can either be arbitrarily chosen or, alternatively, can be calculated from given values of the corresponding concentrations, $\ln w_0$ and k_{XY} by the iterative program PAPB described by Kinderlerer *et al.* (1981). The same program provides the value of $V_{\max.}$ by raising the substrate concentration, which acts as the variable of the Hill plot, to an effectively infinite value with the concentrations of the other ligands kept at their original values.

With these parameters to hand, eqns. (5)–(12) provide for the completion of eqn. (4) and the evaluation of the Hill slope. To perform these calculations, eqns. (4)–(12) have been incorporated in a program HILL, examples of whose output are considered below. The program also incorporates a search routine that seeks for the maximum (or minimum) value of the Hill slope as a function of the concentration of the substrate used in the plot. The accuracy of Hill slopes calculated in this manner has been confirmed by a second program NH, which obtains Hill slopes by numerical differentiation of the known relationship $v = f(W)_{X,Y,Z}$.

RESULTS AND DISCUSSION

Tables 1, 2 and 3 give Hill slopes calculated from sets of constants that describe respectively the influence of NH_4^+ , fructose 1,6-bisphosphate and H^+ concentration on the kinetics of yeast pyruvate kinase measured as functions of the concentrations of ADP, phosphoenolpyruvate and Mg^{2+} (Rhodes *et al.*, 1986; Morris *et al.*, 1986; Kinderlerer *et al.*, 1986). Tables 4 and 5 represent arbitrarily chosen constants that reproduce activation and inhibition by a K-system effector (Monod *et al.*, 1965); the first of these examples was considered briefly by Morris *et al.* (1986). In general form, the Tables identify factors in eqns. (4), (5) and (6) that contribute to the Hill slope and give them the values that they would adopt in solutions with defined ligand concentrations or enzyme saturations. A number of points arise from an examination of these Tables.

(1) Linkage

For the calculations with all $p_X = 0.5$, $2dp_X/d(\ln Y) = d\tilde{Y}$. The common proportionality means that the linkage relationship required by eqn. (13) can be observed in the corresponding values of $d\tilde{Y}$ recorded in Table 1, lines 1–4, Table 2, lines 1–3, and Table 3, lines 1–3. When p_X takes different values, linkage still obtains but is evident only after multiplication of each $(d\tilde{Y})_{W,X,Y,Z}$ by p_X . It also follows from this that the relative effects on the Hill slope of linked changes in enzyme saturation by given ligands are inversely proportional to the individual saturations. (For examples see Table 2, lines 4–9.)

(2) Symmetry in ϕ_X

The differentials $d\tilde{Y}$ are symmetrical with respect to changes in p_Z ($Z \neq X, Y$) that provide identical values of ϕ_Z [see eqns. (8)–(12)] but not with respect to corresponding changes in p_X , again because $d\tilde{Y}$ is calculated by division of $dp_X/d(\ln Y)$ by p_X . (For examples see Table 2, lines 4–9.)

Table 1. Analysis of Hill-slope parameters defined by eqns. (1) and (4)-(6) by using constants given by Rhodes *et al.* (1986) for the pyruvate kinase-catalysed reaction with A = ADP, B = phosphoenolpyruvate, C = Mg^{2+} and D = NH_4^+ (fourth substrate)

(M) indicates that the varied ligand concentration defines the maximum Hill slope that can be measured at the given concentrations of the remaining ligands. Units of concentration are mM, and units of affinity constants, w_0 , mm^{-1} . The entries in the Table are rounded-up.

Line	A	B	C	D	p_A	p_B	p_C	p_D	v	$V_{\max.}$	Primary variable	k_{BC}	k_{AD}	k_{BD}	k_{CD}	γ	V_G	$\frac{d(\ln v)}{d(\ln X)}$	G	$(1-v/V_{\max.})^{-1}$	Hill slope
	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	$\ln \delta_0$	k_{AA}	k_{BB}	k_{CC}	k_{DD}	k_{AB}	k_{AC}	k_{BC}										
	1.308	-2.028	-3.439	-4.385	0.517	1.744	1.614	-0.226	-0.444	1.334	0.886		-1.188	0.570	1.906	—	1.000				
1	0.242	1.916	1.766	47.181	0.500	0.500	0.500	0.500	0.063	0.148	A	0.732	-0.004	0.380	-0.035	1.074	1.000	1.730	1.858		
2	0.242	1.916	1.766	47.181	0.500	0.500	0.500	0.500	0.063	0.251	B	-0.004	1.512	1.130	0.715	3.353	1.000	1.332	4.466		
3	0.242	1.916	1.766	47.181	0.500	0.500	0.500	0.500	0.063	0.299	C	0.380	1.130	2.356	1.108	4.974	1.000	1.264	6.289		
4	0.242	1.916	1.766	47.181	0.500	0.500	0.500	0.500	0.063	0.261	D	-0.035	0.715	1.108	1.079	2.867	1.000	1.314	3.768		
5	0.002	2.500	1.000	85.000	0.003	0.644	0.228	0.669	3×10^{-4}	0.201	A	1.000	-7×10^{-4}	0.003	-9E-4	1.002	1.000	1.001	1.003		
6	0.020	2.500	1.000	85.000	0.034	0.640	0.234	0.663	0.003	0.201	A	1.002	-0.007	0.032	-0.009	1.017	1.000	1.017	1.035		
7	0.490	2.500	1.000	85.000	0.635	0.586	0.436	0.583	0.095	0.201	A(M)	0.515	-0.011	0.367	-0.034	0.836	1.000	1.891	1.582		
8	20.000	2.500	1.000	85.000	0.991	0.580	0.612	0.562	0.198	0.201	A	0.009	-7×10^{-4}	0.006	-0.001	0.013	1.000	78.27	1.020		
9	200.00	2.500	1.000	85.000	0.999	0.580	0.616	0.562	0.200	0.201	A	9×10^{-4}	-8×10^{-5}	6×10^{-4}	-2×10^{-4}	0.001	1.000	783.9	0.997		
10	0.500	2.500	0.010	85.000	0.572	0.382	0.002	0.381	2×10^{-4}	0.457	C	6×10^{-4}	0.002	1.004	0.002	1.009	1.000	1.000	1.009		
11	0.500	2.500	0.100	85.000	0.576	0.390	0.020	0.389	0.002	0.457	C	0.006	0.024	1.044	0.024	1.099	1.000	1.003	1.104		
12	0.500	2.500	1.026	85.000	0.645	0.598	0.466	0.595	0.107	0.457	C(M)	0.257	0.788	2.275	0.803	4.124	1.000	1.305	5.385		
13	0.500	2.500	10.000	85.000	0.734	0.772	0.973	0.786	0.434	0.457	C	0.007	0.009	0.029	0.010	0.055	1.000	1.970	1.088		
14	0.500	2.500	100.00	85.000	0.739	0.778	0.997	0.793	0.455	0.457	C	7×10^{-4}	8×10^{-4}	0.003	9×10^{-4}	0.005	1.000	202.8	1.008		

Table 2. Analysis of Hill-slope parameters defined by eqs. (1) and (4)-(6) by using constants given by Morris *et al.* (1986) for the pyruvate kinase-catalysed reaction with A = ADP, B = phosphoenolpyruvate, C = Mg^{2+} and D = fructose 1,6-bisphosphate (an effector)

Other details are as in Table 1 legend.

Line	A	B	C	$10^3 \times D$	p_A	p_B	p_C	p_D	v	$V_{\max.}$	Primary variable	k_{AC}	k_{BC}	k_{AD}	k_{BD}	k_{CD}	γ	V_G	$\frac{d(\ln v)}{d(\ln X)}$	G	$(1-v/V_{\max.})^{-1}$	Hill slope
	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	$\ln \delta_0$	k_{AA}	k_{BB}	k_{CC}	k_{DD}	k_{AB}	k_{AC}	k_{BC}											
	0.951	-0.928	-2.720	2.258	1.302	1.432	2.966	1.340	-0.860	0.263	0.786			1.004	1.017	0.469	0.966	1.000				
1	0.164	0.771	1.613	1.541	0.500	0.500	0.500	0.500	0.123	0.290	A			0.946	-0.023	0.414	0.421	1.329	-0.017	1.735	2.306	
2	0.164	0.771	1.613	1.541	0.500	0.500	0.500	0.500	0.123	0.345	B			-0.023	1.668	1.681	0.925	3.310	-0.017	1.554	5.144	
3	0.164	0.771	1.613	1.541	0.500	0.500	0.500	0.500	0.123	0.371	C			0.414	1.681	4.000	1.504	6.069	-0.017	1.495	9.079	
4	0.117	0.152	2.209	2.314	0.500	0.100	0.500	0.500	0.025	0.061	A			0.945	-0.017	0.434	0.432	1.355	-0.017	1.681	2.279	
5	0.117	0.152	2.209	2.314	0.500	0.100	0.500	0.500	0.025	0.308	B			-0.003	1.203	0.243	0.134	1.440	-0.017	1.086	1.565	
6	0.117	0.152	2.209	2.314	0.500	0.100	0.500	0.500	0.025	0.092	C			0.434	1.213	2.549	0.706	4.184	-0.017	1.366	5.718	
7	0.232	3.915	1.178	1.026	0.500	0.900	0.500	0.500	0.221	0.590	A			0.945	-0.002	0.434	0.432	1.370	-0.017	1.599	2.192	
8	0.232	3.915	1.178	1.026	0.500	0.900	0.500	0.500	0.221	0.290	B			-0.003	0.134	0.243	0.134	0.371	-0.017	4.192	1.554	
9	0.232	3.915	1.178	1.026	0.500	0.900	0.500	0.500	0.221	0.536	C			0.434	0.135	2.549	0.706	3.106	-0.017	1.703	5.291	
10	0.182	0.854	1.690	1.174	0.500	0.500	0.500	0.400	0.123	0.375	C			0.367	1.579	3.833	1.668	5.755	-0.014	1.490	8.577	
11	0.149	0.697	1.539	2.021	0.500	0.500	0.500	0.600	0.122	0.361	C			0.367	1.579	3.833	1.112	5.755	-0.021	1.512	8.707	
12	0.107	1.6×10^{-4}	2.389	2.561	0.500	1×10^{-4}	0.500	0.500	2×10^{-6}	0.294	B			-3×10^{-4}	1.000	2×10^{-4}	1×10^{-4}	1.000	-0.017	1.000	1.000	
13	0.251	37.861	1.097	0.936	0.500	0.990	0.500	0.500	0.243	0.251	B			-3×10^{-4}	0.010	0.020	0.011	0.030	-0.017	34.37	1.043	

Table 3. Analysis of Hill-slope parameters defined by eqns. (1) and (4)–(6) by using constants given by Kinderlerer *et al.* (1986) for the pyruvate kinase-catalysed reaction with A = ADP, B = phosphoenolpyruvate, C = Mg^{2+} and D = H^+ (an effector)

Other details are as in Table 1 legend.

Line	A	B	C	$10^3 \times D$	p_A	p_B	p_C	p_D	v	$V_{\max.}$	Primary variable				dX^A	dX^B	dX^C	dX^D	γ	V_G	G	$(1-v/V_{\max.})^{-1}$	Hill slope
	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	$\ln \delta_0$	k_{AA}	k_{BB}	k_{CC}	k_{DD}	k_{AB}	k_{AC}	k_{BC}	k_{AD}	k_{BD}	k_{CD}									
1	0.377	2.322	2.573	3.084	0.500	0.500	0.500	0.500	0.064	0.200	A	0.661	-0.082	0.317	-0.282	1.163	-0.947	1.471	1.711				
2	0.377	2.322	2.573	3.084	0.500	0.500	0.500	0.500	0.064	0.164	B	-0.082	1.284	0.904	-0.005	2.111	-0.947	1.642	3.468				
3	0.377	2.322	2.573	3.084	0.500	0.500	0.500	0.500	0.064	0.234	C	0.317	0.904	2.582	-0.614	4.385	-0.947	1.377	6.040				
4	0.591	2.322	2.573	3.084	0.642	0.482	0.566	0.440	0.100	0.200	A	0.457	-0.085	0.226	-0.271	0.801	-0.749	2.000	1.603				
5	0.377	2.641	2.573	3.084	0.495	0.580	0.556	0.500	0.082	0.164	B	-0.084	1.020	0.715	0.008	1.643	-0.947	2.000	3.286				
6	0.377	2.322	3.153	3.084	0.525	0.569	0.700	0.452	0.117	0.234	C	0.152	0.383	0.920	-0.336	1.719	-0.787	2.000	3.439				
7	0.385	2.322	2.573	3.084	0.507	0.499	0.503	0.497	0.066	0.200	A(M)	0.652	-0.082	0.314	-0.283	1.149	-0.936	1.488	1.711				
8	0.377	2.364	2.573	3.084	0.499	0.511	0.508	0.500	0.067	0.164	B(M)	-0.082	1.253	0.888	-0.005	2.063	-0.947	1.684	3.474				
9	0.377	2.322	2.566	3.084	0.500	0.499	0.497	0.501	0.063	0.234	C(M)	0.317	0.906	2.599	-0.613	4.405	-0.950	1.371	6.041				
10	2.888	2.934	2.106	3.936	0.900	0.500	0.500	0.500	0.116	0.381	C	0.053	0.932	2.475	-0.520	3.952	-0.947	1.435	5.673				
11	0.476	10.424	1.762	2.681	0.500	0.900	0.500	0.500	0.116	0.357	C	0.364	0.090	2.059	-0.612	3.093	-0.947	1.478	4.574				
12	0.309	1.590	7.870	3.865	0.500	0.500	0.900	0.500	0.116	0.147	C	0.031	0.089	1.409	-0.060	0.318	-0.947	4.665	1.484				
13	0.309	1.590	7.870	3.865	0.500	0.500	0.900	0.500	0.116	0.161	B	-0.182	0.998	0.049	0.189	0.686	-0.947	3.552	2.438				
14	0.481	2.019	3.224	13.344	0.500	0.500	0.500	0.900	0.016	0.057	C	0.197	0.902	2.320	-0.067	3.888	-7.045	1.373	5.341				
15	0.484	3.728	0.001	2.326	0.500	0.500	1×10^{-4}	0.500	1×10^{-6}	0.357	C	2×10^{-8}	7×10^{-8}	1.000	-5×10^{-8}	1.000	-0.947	1.000	1.000				
16	0.295	1.460	67.904	4.066	0.500	0.500	0.990	0.500	0.127	0.130	C	0.002	0.007	0.010	-0.005	0.025	-0.947	42.200	1.039				

Table 4. Analysis of Hill-slope parameters defined by eqns. (1) and (4)–(6) by using arbitrarily chosen constants in which ligand C behaves as an activator

On lines 1–6 (M) indicates a maximum Hill slope, in lines 8–12 a minimum. Concentration units are arbitrary.

Line	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	k_{AA}	k_{BB}	k_{CC}	k_{AB}	k_{AC}	k_{BC}	γ	V_G	$\frac{d(\ln v)}{d(\ln X)}$	G	$(1-v/V_{\max})^{-1}$	Hill slope
	A	B	C	p_A	p_B	p_C	v	$V_{\max.}$	Primary variable X	$d\hat{A}_X$	$d\hat{B}_X$	$d\hat{C}_X$			
1	0.947	0.100	0.080	0.582	0.484	0.174	0.282	0.819	$A(M)$	0.566	0.705	0.564	0	1.524	1.937
2	0.429	0.100	0.200	0.438	0.517	0.360	0.227	0.893	$A(M)$	0.901	0.963	0.638	0	1.339	2.496
3	0.178	0.100	0.400	0.281	0.534	0.538	0.150	0.926	$A(M)$	1.070	0.739	0.365	0	1.193	2.159
4	0.079	0.100	0.800	0.181	0.604	0.728	0.109	0.942	$A(M)$	0.982	0.312	0.102	0	1.131	1.464
5	0.048	0.100	4.000	0.151	0.727	0.945	0.110	0.955	$A(M)$	0.872	0.109	0.009	0	1.129	1.107
6	0.045	0.100	8.000	0.145	0.739	0.972	0.107	0.957	$A(M)$	0.868	0.095	0.004	0	1.126	1.084
7	—	0.100	0.080	Minimum not found	Minimum not found	Minimum not found	—	—	—	—	—	—	—	—	—
8	16.675	0.100	0.200	0.974	0.887	0.541	0.863	0.893	$A(M)$	0.026	0.008	0.006	0	29.70	0.998
9	2.754	0.100	0.400	0.875	0.902	0.708	0.789	0.926	$A(M)$	0.118	0.026	0.013	0	6.748	0.971
10	1.429	0.100	0.800	0.799	0.911	0.832	0.729	0.942	$A(M)$	0.183	0.031	0.010	0	4.394	0.942
11	0.845	0.100	4.000	0.721	0.920	0.962	0.664	0.955	$A(M)$	0.246	0.032	0.002	0	3.276	0.911
12	0.792	0.100	8.000	0.711	0.921	0.981	0.655	0.957	$A(M)$	0.254	0.032	0.001	0	3.169	0.907

Table 5. Analysis of Hill-slope parameters defined by eqns. (1) and (4)–(6) by using arbitrarily chosen constants in which C behaves as an inhibitor when $k_{BC} = -2$ and as an activator when $k_{BC} = +2$

(M) indicates a maximum throughout. Concentration units are arbitrary.

Line	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	k_{AA}	k_{BB}	k_{CC}	k_{AB}	k_{AC}	k_{BC}	γ	V_G	Primary variable	$V_{max.}$	$d\hat{X}$	$d\hat{B}_X$	$d\hat{C}_X$	$\frac{d(\ln v)}{d(\ln X)}$	G	$(1-v/V_{max.})^{-1}$	Hill slope
	A	B	C	p_A	p_B	p_C	v		X	$d\hat{X}$										
1	0.098	1.000	—	0.368	0.715	—	0.263	0.924	A(M)	0.898	0.235	—	0	—	0.235	—	1.133	0	1.398	1.585
2	0.125	1.000	1.000	0.368	0.615	0.226	0.226	0.902	A(M)	1.076	0.457	—0.435	0	—0.435	0.457	—0.435	1.533	0	1.335	2.047
3	0.196	1.000	2.500	0.431	0.539	0.459	0.232	0.869	A(M)	1.152	0.758	—0.442	0	—0.442	0.758	—0.442	1.910	0	1.364	2.607
4	0.300	1.000	5.000	0.505	0.489	0.653	0.247	0.820	A(M)	1.000	0.834	—0.283	0	—0.283	0.834	—0.283	1.834	0	1.430	2.624
5	0.399	1.000	10.000	0.543	0.436	0.807	0.237	0.755	A(M)	0.832	0.752	—0.127	0	—0.127	0.752	—0.127	1.583	0	1.457	2.308
6	0.484	1.000	50.000	0.547	0.365	0.960	0.200	0.654	A(M)	0.722	0.650	—0.019	0	—0.019	0.650	—0.019	1.372	0	1.438	1.974
	$\ln \alpha_0$	$\ln \beta_0$	$\ln \gamma_0$	k_{AA}	k_{BB}	k_{CC}	k_{AB}	k_{AC}	k_{BC}	γ	V_G	Primary variable								
	10 × A	B	C	p_A	p_B	p_C	v	$V_{max.}$	X	$d\hat{X}$										
7	0.975	1.000	—	0.368	0.715	—	0.263	0.924	A(M)	0.898	0.235	—	0	—	0.235	—	1.133	0	1.398	1.585
8	0.703	1.000	0.050	0.323	0.765	0.188	0.247	0.953	A(M)	0.935	0.199	0.247	0	0.247	0.199	0.247	1.134	0	1.349	1.530
9	0.557	1.000	0.100	0.292	0.801	0.332	0.234	0.965	A(M)	0.931	0.157	0.169	0	0.169	0.157	0.169	1.088	0	1.320	1.437
10	0.384	1.000	10.000	0.285	0.936	0.985	0.267	0.989	A(M)	0.774	0.035	0.001	0	0.001	0.035	0.001	0.809	0	1.370	1.109

(3) Sensitivity of Hill slope to constants k_{XX} and k_{XY}

Examination of eqns. (8)–(12) shows that they are maximally sensitive to the values of k_{XX} and k_{XY} when the ϕ terms have their maximum value of 0.25, that is at half-saturation by the ligands. Eqn. (7) shows, however, that $d\bar{x} = N_X/(D_Y \cdot p_X)$ and hence that the peak $d\bar{x}$ contribution to the Hill slope will usually occur at values of $p_Z = 0.5$ and p_X rather less. These effects can be observed in every Table, but are particularly obvious in Table 4, lines 1–6, and Table 5, lines 1–6. In spite of the complication with regard to p_X , it is clear that the preferred standard for a general comparison of the influence of k_{XX} and k_{XY} on the several Hill slopes is that the enzyme should be half-saturated by all its ligands. Comparison of Table 2, lines 1–3, with Table 2, lines 4–6, and Table 2, lines 7–9, and of Table 3, line 3, with Table 3, lines 10–12 and 14, bears out the contention that the differentials $d\bar{x}$ are almost always larger when all the saturations are set to 0.5 than otherwise and hence that this condition secures the maximum sensitivity of the Hill slope to the values of all the interaction constants. As a corollary, it follows that the Hill slope measured with the standard conditions will be close to the maximum that can be obtained with the given constants. Thus Table 3, lines 1–9, demonstrates the effect of changing the concentration of the primary variable, either to obtain $v/V_{\max} = 0.5$ or to find the maximum Hill slope, when the concentrations of the remaining ligands are kept constant at the values that provided half-saturation. The first alternative leads to a decreased Hill slope and the second to a negligible increase, behaviour that can be reproduced exactly by the constants given in Tables 1 and 2. [The maximum Hill slopes recorded in Table 3, lines 7–9, do not correspond to the absolute maximum values that can be achieved; what these might be is not known, but reduction of p_B to 0.001 when A is the primary variable or of p_A to 0.001 when B is varied (other $p_X = 0.5$) decreases the negative interaction arising from k_{AB} and leads to Hill slopes of 1.759 and 3.519 respectively. These values are marginally greater than any recorded in Table 3.]

(4) Influence of constants k_{XX} and k_{XY} on Hill slope

Following the arguments of the previous section, the influence of k_{XX} and k_{XY} on the Hill slope is examined through the evaluation of the differentials when the enzyme is half-saturated by all of its ligands. The calculations are exemplified in Table 1, lines 1–4, Table 2, lines 1–3, and Table 3, lines 1–3. Examination of the Tables shows that $d\bar{x}$ is always positive. Further examination suggests that k_{XX} is usually the major factor in determining the values of $d\bar{x}$, but the correlation is not perfect: for example, in Table 1, line 1, and Table 1, line 4, $d_A^A < d_B^B$ whereas $k_{AA} > k_{DD}$. The disparity confirms the value of determining the Hill parameters as measures that incorporate all the interactive effects on the binding of a given ligand (Rhodes *et al.*, 1986). Finally, with regard to $d\bar{x}$, note that it usually provides the largest contribution to the Hill slope. $d\bar{x}$ can be greater than $d\bar{y}$, though the difference seems to be marginal under standard conditions (Table 1, line 4, and Table 2, line 2). However, when the value of p_X corresponding to the variable X is increased, $d\bar{x}$ can become much larger than $d\bar{y}$, as may be observed by comparing d_B^B and d_B^B in

Table 2, line 2, Table 2, line 8, and Table 2, line 13. The origin of this effect is described by eqn. (7).

The remaining terms $d\bar{y}$, where X, Y are substrates, contribute increments to the Hill slope that usually correlate in sign with k_{XY} ; the single exception (under standard conditions) is d_B^B in Table 3, line 2. It is not possible to predict the magnitude of $d\bar{y}$ *a priori*, again an illustration of the usefulness of the direct calculation in any discussion of interactive effects. The same remarks apply when Y is an effector, but, in this instance, the contribution of $d\bar{x}$ to the Hill slope is moderated by the value of G .

(5) Influence of constants k_{XY} on ligand affinity

If p_X is changed in value at constant values of the remaining p_Z , then the nature of k_{XZ} may be deduced fairly obviously by the change in Z that is required to maintain p_Z constant; for example, if p_X is decreased and Z falls, k_{XZ} is negative. These effects are present throughout the Tables, but are particularly evident in Table 2.

(6) 'Direct' and 'indirect' routes of interaction

Eqns. (8)–(12) show that the differentials whose values determine the Hill slope are themselves dependent on constants k_{XY} and the parameters ϕ_X , derived from the fractional saturations of the protein by its ligands. When the fractional saturations are given fixed values [as recommended in subsection (3)] the relationship between k_{XY} and the Hill slope is 'directly' determined by eqns. (8)–(12), and ϕ_X acts merely to change the sensitivity of the response. However, when it is the ligand concentrations that are fixed, k_{XY} operates additionally through eqn. (2) and binding equations of the form:

$$p_W = \frac{W \cdot w_i}{1 + W \cdot w_i} \quad (14)$$

so as to establish the parameters that enter eqns. (8)–(12). Indeed, examples can be found (described in Tables 4 and 5) where k_{XY} can only influence the Hill slope by this 'indirect' route. Table 5 relates to a three-ligand system in which A and B are substrates and C is an effector with $\gamma = 1$. As a result, $G = 0$ and d_A^C does not affect the Hill slope measured as a function of A . All the k_{XY} constants are set to zero except $k_{AB} = 2.5$ and $k_{BC} = \pm 2.0$. With these conditions, eqns. (8), (9) and (12) lead to:

$$N_A/p_A = (1 - p_A) \cdot (1 - \phi_B \phi_C k_{BC}^2) \quad (15)$$

$$N_B/p_B = \phi_A \cdot (1 - p_B) k_{AB} \quad (16)$$

$$D_A = 1 - \phi_B \phi_C k_{BC}^2 - \phi_A \phi_B k_{AB}^2 \quad (17)$$

and division of the first two equations by the third gives the differentials d_A^A and d_A^B respectively, which together determine the Hill slope.

It is evident from the values of the maximum Hill slopes and the concentrations of A at which they are observed, both recorded in Table 5, that increasing C inhibits the enzyme when k_{BC} is negative, but activates it when k_{BC} is positive. In addition, it will be observed that the Hill slopes are markedly larger when k_{BC} is negative. Examination of eqns. (15)–(17) shows that these differences in behaviour cannot arise directly from the value of k_{BC} because the constant only appears therein as the square. (To emphasize the point, when all $p_X = 0.5$, d_A^A , d_A^B and the Hill slope, 2.7416, remain constant irrespective of whether k_{BC} is $+2$ or -2 .) The differences

can therefore only arise indirectly through changes in the fractional saturations as determined by eqns. (2) and (14), and from the consequent effects discussed in subsection (3). It is noteworthy that p_A and p_B have values that move away from 0.5 as k_{BC} changes from negative to positive; in consequence, d_A^B in particular makes a much smaller contribution to the Hill slope when B is activating.

Too much should not be made of the distinction between 'direct' and 'indirect' interactions because it is evident that the 'indirect' route is always operative, linking together the k_{XY} constants and ϕ_X values at given ligand concentrations. It is interesting, however, to have found examples where the distinction is clear, and the recognition of it creates a qualification to the procedure recommended in subsections (3) and (4).

(7) The value of $V_{\max}$.

$V_{\max}$ is established as the velocity recorded when the variable substrate concentration is raised to a very large value while the original concentrations of the other ligands are maintained. $V_{\max}$ therefore reflects the changed saturation of all the ligands [eqns. (1), (2) and (14)] and hence the relative magnitude of the interactions that can be initiated by a given ligand. This conclusion is confirmed by Table 1, lines 1–4, and Table 2, lines 1–3, which show that, when standard conditions are employed for the comparison, $V_{\max}$ increases with the Hill slope. Similar correlations can be found in the Tables (e.g. Table 3, lines 10–13), but no more than a general trend can be claimed, for departures from expectation do occur (Table 3, line 2).

In the systems examined in Tables 4 and 5, C acts as a K-system effector in the sense that the presence of C in solution does not affect the value of the global maximum velocity, V_G , and $V_{\max} = (p_B)_{A \rightarrow \infty}$. Nevertheless, increasing C reduces $V_{\max}$ when A is the variable and k_{BC} is negative (Table 5, lines 1–6), a result that arises from the progressive reduction in the value of $(p_B)_{A \rightarrow \infty}$ (the reduction of p_B at non-saturating values of A can be observed in the Table). The opposite result appears when k_{BC} is positive (Table 4, lines 1–6, and Table 5, lines 7–10).

In the examples given in the Tables, the maximum Hill slope always appears at $v/V_{\max} < 0.5$, and the minimum appears at $v/V_{\max} > 0.5$. The same result has been found in further unpublished calculations, including examples where the Hill slope was never greater than 1.

(8) The Hill slope

In Table 2, a Hill slope of 9 is recorded for a system described by constants relating to pyruvate kinase, a value greatly in excess of the theoretical limit of 4 for the binding of a single ligand to a tetrameric molecule. The Tables contain many other examples where the Hill slope exceeds 4. It is obvious that the limit is broken because the dependence of the velocity on the concentration of substrates does not follow the simple model on which the Hill equation is based. It can also be noted that Hill slopes greater than 4 have been measured in kinetic studies of pyruvate kinase by Hunsley & Suelter (1969).

(9) Shape of Hill plot

Artificial data have been constructed according to eqns. (1) and (2) with a wide variety of k_{XY} and ligand concentration values and then represented on a Hill plot. Usually the shape of the plot agrees with expectation: the slope decreases or increases monotonically towards unity

at the extreme values of $\ln X$, with the maximum or minimum slope appearing at intervening $\ln X$ values. Table 1, lines 5–14, provides detailed examples of this behaviour, and Table 2, lines 12 and 13, and Table 3, lines 15 and 16, offer confirmation with different sets of k_{XY} constants. The monotonic increase or decrease of the Hill slope towards the extremes in $\ln X$ does not appear to be general, however. Thus Table 4, lines 1–6, shows the influence of a K-system activator (C) on a Hill plot constructed as $f(A)_B$. As C increases, the maximum Hill slope appears at progressively lower values of $\ln A$ and simultaneously decreases in value towards unity. Associated with this change, however, is the progressive enhancement of a minimum in the Hill slope that moves towards lower values of $\ln A$ as C increases (Table 4, lines 7–12). As a result, the Hill plot at the highest C does not present a straight line of unit slope but rather is a shallow sigmoidal curve that moves about the theoretical line.

(10) Inhibition and activation

Two types of inhibition are found in the Tables. In Table 3 the value of y is approximately zero, indicating that D (i.e. H^+) behaves as a non-competitive inhibitor. The factor $G = 0$ when $p_D = 0$ but otherwise takes negative values [eqns. (5) and (6)]. Negative values of d_X^D therefore make positive contributions to the Hill slope in proportion to their magnitude. For this reason, the negative values of k_{AD} and k_{CD} lead to significant positive contributions to the Hill slope whereas k_{BD} (positive) does not (compare the values of d_X^D in Table 3, lines 1–3).

When $y = 1$ and $G = 0$, the influence of the effector on the Hill slope arises only through the interactive effect of its saturation on the other saturations; the result may be an inhibition, as in Table 5, lines 1–6, or an activation as in Table 2, lines 1–3, Table 4, lines 1–6, and Table 5, lines 7–10, the alternatives depending on the signs and magnitudes of k_{XY} in ways already discussed above. The considerations involved can be amplified by examination of Table 4, lines 1–6, an example that was introduced by Morris *et al.* (1986) to demonstrate that the exponential model can generate a 'sigmoidal-to-hyperbolic' transition on adding an activator to the reactant solutions. This type of transition is forbidden to a two-ligand enzyme, when $y = 1$, for then:

$$v/V_G = p_A \quad (18)$$

where p_A is determined by:

$$\ln \alpha_i = \ln \alpha_0 + k_{AA} \cdot p_A + k_{BA} \cdot p_B \quad (19)$$

and a binding equation of the form given by eqn. (14). It is evident from these equations that the curve shape $v = f(A)_B$ is identical when the fractional saturation of the effector B, p_B , is either 0 or 1 (Ainsworth & Gregory, 1978). The restriction is lifted when three ligands are involved. Thus in Table 4, lines 1–6, the positive value of k_{AB} provides, through d_A^B , a contribution to the Hill slope that is maximal in the region of $p_B = 0.5$. However, progressive addition of the effector C causes p_B to increase beyond 0.5 ($k_{BC} = 2$) and hence decreases the positive contribution of d_A^B to the maximum Hill slope, finally to negligible amounts. The reduction of d_A^B can be offset by maintaining the contribution of d_A^A , that is by reducing p_A as indicated in subsections (1)–(4). This pressure, together with the increasing affinity of the enzyme for A ($k_{AB} = k_{BC} = 2$), ensures that the

maximum Hill slope appears at progressively lower values of A as C increases. The overall effect displayed in Table 4, lines 1–6, is therefore an activation accompanied by a decreased sigmoidicity in the relationship $v = f(A)_{B,C}$ (Monod *et al.*, 1965). Table 5, lines 7–10, displays similar effects with different constants.

(11) The factor $(1 - v/V_{\max})^{-1}$

Although it is very difficult to discern systematic influences on $(1 - v/V_{\max})^{-1}$, except when v is arbitrarily fixed and subsection (7) applies, it should not be forgotten that the factor is a very important element in determining the Hill slope. It is therefore fortunate that the interest of the analysis centres more on the differentials $d\tilde{x}$ than on the final Hill slope to which they contribute.

(12) Conclusion

The points raised above illustrate that analysis of the Hill slope facilitates the interpretation of observed behaviour in terms of the interaction constants by

evaluating the net contribution of each changing saturation to the Hill slope and ligand affinity.

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