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Simple graphical methods for use with complex ligand-binding and enzyme mechanisms

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Simple graphical plots in distribution-free space are shown to provide estimates of mechanism and kinetic parameters for complex steady-state processes. The methods were tested using simulated 1:1, 1:2, 2:2, 2:3 and 3:3 functions, and real data from polyol dehydrogenase, NADPH oxidation in the presence of DL-glyceraldehyde, and NADPH oxidation in the presence of an NADPH-binding protein. Estimates of mechanism and parameters agreed closely with those found from non-linear regression by computer.

Enzyme mechanism; Receptor; Ligand binding; Transport; Kinetics; Pharmacokinetics

1. INTRODUCTION

Enzyme-substrate interactions, ligand-receptor interactions, transport processes and pharmacokinetic processes are usually studied under steady-state conditions. Such processes are generally described by a rational polynomial function of the type:

$$v = \frac{a_1S + a_2S^2 + \ldots.a_nS^n}{1 + b_1S + b_2S^2 + \ldots.b_mS^m} \quad (1)$$

where v = the variable under study (e.g. $v/[E_0]$; initial velocity/(enzyme concentration) for an enzyme-catalysed reaction), or the concentration of bound ligand in a ligand binding study); S = the concentration of the varied substrate or free ligand; $a_1 \ldots a_n$ and $b_1 \ldots b_m$ are constants, all other ligand and product concentrations are constant, and there is no receptor or enzyme polymerisation. The values of n and m (i.e. the degree of the function) reflect the number of receptor or enzyme species reacting with the ligand. Normally $n <= m$. If $n = m = 1$ then the equation simplifies to Michaelis-Menten or Scatchard 1:1 functions where $a_1 = V_{max}/K_m$ (or n/K_d) and $b_1 = 1/K_m$ (or $1/K_d$).

For these simple 1:1 functions, data are usually plotted in one of a number of graphical spaces [1,2] or subjected to non-linear regression [3,4]. However, many ligand-receptor processes are complex, and exhibit more than one functionally significant ligand-binding interaction (see e.g. [5]). In these cases the 1:1 function is an oversimplification, and may lead to erroneous interpretations.

The behaviour of complex steady-state mechanisms has been studied in some detail (see e.g. [6–9], and the procedure for their analysis usually involves iterative non-linear regression by computer followed by some technique to distinguish 'goodness of fit' [5,10–12]. However, these techniques and their interpretation may not be accessible to all, and many situations require a simple procedure to distinguish between mechanisms and provide quantitative information on binding or catalytic constants. For simple 1:1 mechanisms the distribution-free 'direct linear' plot [1] or its transforms [13,14] are less dependent on arbitrary assumptions than methods involving

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least squares. Distribution-free plots have been used to compute second-order stability constants of inorganic complexes (see [15]).

The aim of the present study was to develop simple distribution-free graphical methods for:
(i) Determination of the number of receptor or enzyme species reacting with the ligand, by determining the values n and m in eqn. 1, i.e. the degree of the rational polynomial function.
(ii) Determination of the values of the binding or catalytic constants in the mechanism, i.e. the values of $a_1 - a_n$ and $b_1 - b_m$ in eqn 1.

2. METHODS

2.1. *Theoretical*

The general rational polynomial function (eqn 1) may be transformed to give:

$$a_n = \frac{v}{S_n}(1 + b_1S + ..b_mS^m) - (\frac{...a_{n-1}S^{n-1}}{S^n}) \quad (2)$$

$$b_m = \frac{1}{S^m}(\frac{a_1S + ...a_nS^n}{v}) - (\frac{1 + ..m_{m-1}S^{m-1}}{S}) \quad (3)$$

These equations may then be used to derive functions that can be plotted in distribution-free space. Thus, for the 2:2 function:

$$a_1 = \frac{v}{S}(1 + b_1S + b_2S^2) - (\frac{a_2S^2}{S}) \quad (4)$$

$$a_2 = \frac{v}{S^2}(1 + b_1S + b_2S^2) - \frac{a_1S}{S^2} \quad (5)$$

$$b_1 = \frac{1}{Sv}(a_1S + a_2S^2) - \frac{1 + b_2S^2}{S} \quad (6)$$

$$b_2 = \frac{1}{S^2v}(a_1S + a_2S^2) - \frac{1 + b_1S}{S^2} \quad (7)$$

In distribution-free space, a plot of a_1 vs b_1 for the 2:2 function will have x intercept:

$$-(\frac{1}{S} + b_2 - \frac{a_2S}{v}) \quad (8)$$

and y intercept:

$$\frac{v}{S} + vb_2 - a_2S \quad (9)$$

Note that if the function is of degree 1:1, then $a_2 = b_2 = 0$, and the intercepts simplify to $-1/S$ and v/S as in the direct linear plot [1,13].

A plot of a_2 vs b_2 for the 2:2 function will have x intercept:

$$-(\frac{1}{S^2} + \frac{b_1}{S} - \frac{a_1}{Sv}) \quad (10)$$

and y intercept:

$$\frac{v}{S^2} + \frac{vb_1}{S} - \frac{a_1}{S} \quad (11)$$

For the 3:3 function, a plot of a_3 vs b_3 will have x intercept:

$$-(\frac{1}{S^3} + \frac{b_2}{S} + \frac{b_1}{S^2} - \frac{a_1}{S^2v} - \frac{a_2}{Sv}) \quad (12)$$

and y intercept:

$$\frac{v}{S^3} + \frac{vb_2}{S} + \frac{vb_1}{S2} - \frac{a_1}{S^2} - \frac{a_2}{S} \quad (13)$$

Note that where $m = n + 1$, as in the 1:2 function (simple substrate inhibition),

$$b_2 = \frac{1}{S^2}\left(\frac{a_1S}{v} - 1 - b_1S\right) \quad (14)$$

and a plot of a_2 vs b_2 will show that $a_2 = 0$.

2.2. *Procedure*

(i) Obtain accurate binding, transport or initial velocity data over as wide a ligand concentration range as feasible. Statistical aspects of plots in distribution-free space have been covered previously [1,13,14,16].

(ii) Calculate the x and y intercepts from the data (i.e. $-1/S$ and v/S) and plot a_1 vs b_1. Plot each pair of intercepts and draw a line through them into the positive right-hand quadrant, similar to a plot of K_m vs V_{max} [1,14,16]. The median intersection point of these lines gives the positive co-ordinates of a_1 and b_1 from the axes. If the data are a poor fit to the 1:1 function, then there will be a spread of intersections, possibly even two sets of intersections, as the higher parameters are not included in the intercept calculations. However, the values obtained from the medians of the intersections should be sufficiently accurate for a first approximation, and a return to this plot for refinement may be made later.

(iii) Calculate the x and y intercepts for a plot of a_2 vs b_2, using eqns 10 and 11 above, inserting the values for a_1 and b_1 obtained in the first plot. If the function is of degree 1:1, then the intercept values will be either zero or vanishingly small. If the function is of degree 1:2 (simple substrate inhibition), then the lines will intersect at the value of b_2 on the x axis, showing $a_2 = 0$. If the function is of degree 2:2 (or higher), then the median intersection point gives the positive co-ordinates of a_2 and b_2 from the axes.

(iv) Calculate the x and y intercepts for a plot of a_3 vs $b3$, using eqns 12 and 13 above, inserting the values obtained for a_1, a_2, b_1 and b_2 in the previous plots. If the function is of degree 2:2, then the intercept values will be either zero or vanishingly small. If the function is of degree 2:3, then the lines will intersect at the value of b_3 on the x axis, showing $a_3 = 0$. If the function is of degree 3:3, then the median intersection point gives the positive co-ordinates of a_3 and b_3 from the axes.

(v) This process of plotting a_n vs b_n continues until the calculated intercepts are vanishingly small (0–10^{-10}), or intersections in negative quadrants show the higher degree function to be invalid. The general formulas, eqns 2 and 3 above, may be used to derive intercepts for functions higher than 3:3; although in practice this may be rarely necessary, few examples of such high degree functions having been verified.

(vi) If the function obeyed by the data is higher than 1:1, say 1:2 or 2:2, then the values for a_2 and b_2 may be used to calculate the x and y intercepts in a plot of a_1 vs b_1 from eqns 9 and 10, and the values of a_1 and b_1 refined. This could be repeated iteratively either graphically, or using a computer.

2.3 *Data*

Simulated data were calculated using the Lotus 1-2-3 spreadsheet on an IMB PC compatible computer. Initial velocity data for bovine lens polyol dehydrogenase were obtained with xylitol as substrate as described in [10]. The oxidation of NADPH (100 μM) in the presence of DL-glyceraldehyde (100 mM) and potassium phosphate buffer (pH 6.2, 100 mM) was followed at 340 nm as in [17]. NADPH oxidation in the presence of an NADPH-binding protein (bovine lens aldose reductase) was also followed spectrophotometrically as described [17,18].

3. RESULTS AND DISCUSSION

Fig.1 shows distribution-free plots for simulated 1:1 and 2:2 mechanisms. Plots of v vs S for these mechanisms were indistinguishable, indicating their apparent similarity in this conventional space. Fig.1a is a plot of a_1 vs b_1 for the 1:1 mechanism, the intersections giving $a_1 = 40$ and $b_1 = 3$. When the a_2 and b_2 intercepts were calculated (eqns 10,11), the values were between 0 and 10^{-17}, showing that the mechanism was indeed 1:1. Fig.1b shows a plot of a_1 vs b_1 for the simulated 2:2 mechanism, giving values of $a_1 = 40$ and $b_1 = 3$. These values were then used in constructing a plot of a_2 vs b_2 (fig.1c), the median intersection point giving the coordinates of $a_2 = 28$ and $b_2 = 3.0$. When the a_3 and b_3 intercepts were calculated (eqns 12,13) the values were between 0 and 10^{-15}, and no plot was possible, showing that the mechanism was indeed 2:2.

Fig.2 shows distribution-free plots for 1:2, 2:3, and 3:3 simulated mechanisms. Fig.2a shows a

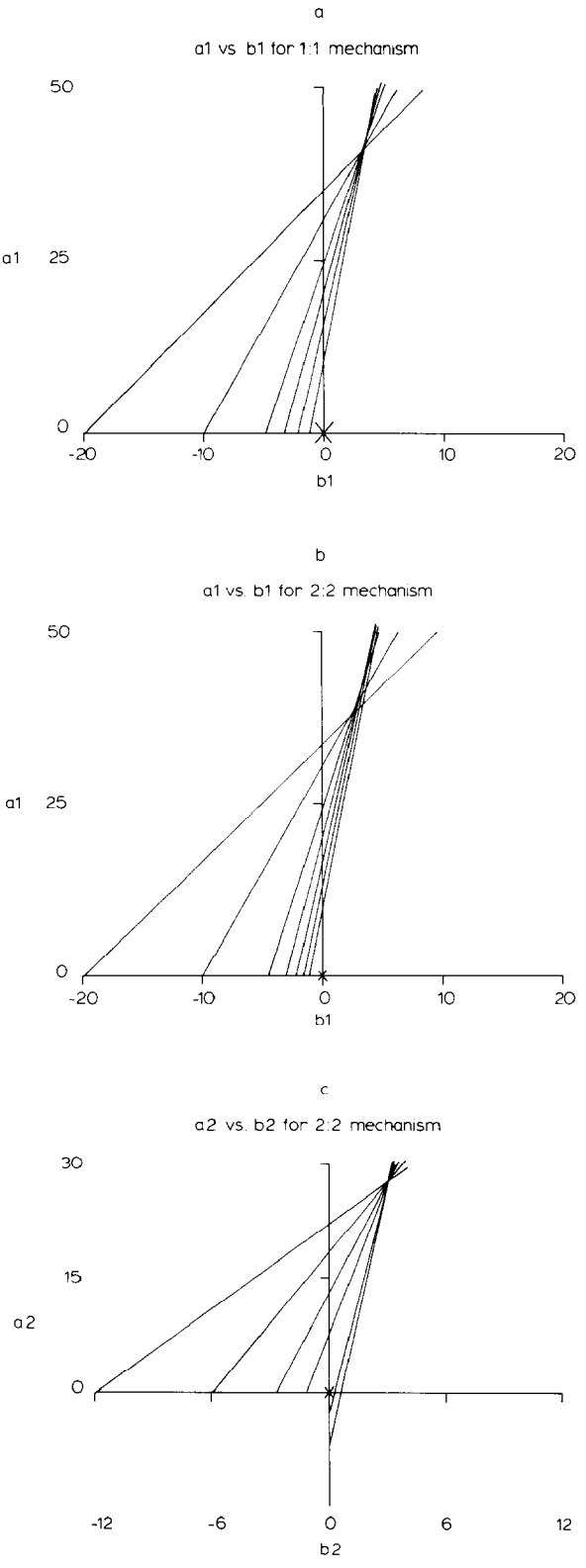

Fig.1. Distribution-free plots for simulated 1:1 and 1:2 mechanisms. For the 1:1 mechanism, $a_1 = 40$ and $b_1 = 3$; the 2:2 mechanism has $a_1 = 40$, $a_2 = 28$, $b_1 = 3$ and $b_2 = 3$. Plots of: (a) a_1 vs b_1 for the 1:1 mechanism; (b) a_1 vs b_1 for the 2:2 mechanism; (c) a_2 vs b_2 for the 2:2 mechanism.

plot of a_2 vs b_2 for a 1:2 mechanism, showing the intersections on the x axis, giving values of $a_2 = 0$ and $b_2 = 20$. Fig. 2b shows a plot of a_3 vs b_3 for a 2:3 mechanism, giving values of $a_3 = 0$ and $b_3 = 5$. Fig.2c shows a plot of a_3 vs b_3 for a 3:3 mechanism, with values $a_3 = 5$ and $b_3 = 5$.

Fig.3 shows this method used in real systems. Fig.3a shows a plot of a_1 vs b_1 for the oxidation of NADPH in the presence of DL-glyceraldehyde and potassium phosphate buffer. We have previously shown that this process proceeds via a free-radical-induced oxidation of the reduced nucleotide [17,19]. Parameter values were $a_1 = 1.2$ and $b_1 = 0.1$. Subsequent calculation of a_2 and b_2 intercepts gave values of between 0.001 and 10^{-19}, showing that NADPH oxidation under these conditions obeyed a 1:1 function. Fig.3b shows a plot of a_2 vs b_2 for bovine lens polyol dehydrogenase, values for a_1(2.4) and b_1 (0.6) having been found from a plot of a_1 vs b_1. Intersections occurred around the a axis, suggesting that the mechanism is 1:2, with $a_2 = 0$ and $b_2 = 0.0078$. These values were close to those estimated by non-linear regression (2.31, 0.72, 0 and 0.0070) [10].

Fig.3c shows a plot of a_1 vs b_1 for NADPH oxidation in the presence of DL-glyceraldehyde, potassium phosphate buffer and an NADPH-binding protein (bovine lens aldose reductase) which enhances the radical-induced oxidation of the NADPH [17]. There was a spread of values, suggesting a complex mechanism, but to a first approximation $a_1 = 6.5$ and $b_1 = 2.5$. These values were used in the construction of a plot of a_2 vs b_2 (fig.3d), with a median intersection giving $a_2 = 0.38$ and $b_2 = 0.08$. Calculation of intercepts for a plot of a_3 vs b_3 gave values close to 0, showing the mechanism to be 2:2 as had been suggested in [18]. Values for a_1 and b_1 were refined by including the a_2 and b_2 values in eqns 10 and 11, producing the a_1 vs b_1 plot shown in fig.3e, with refined values $a_1 = 8$ and $b_1 = 3.2$.

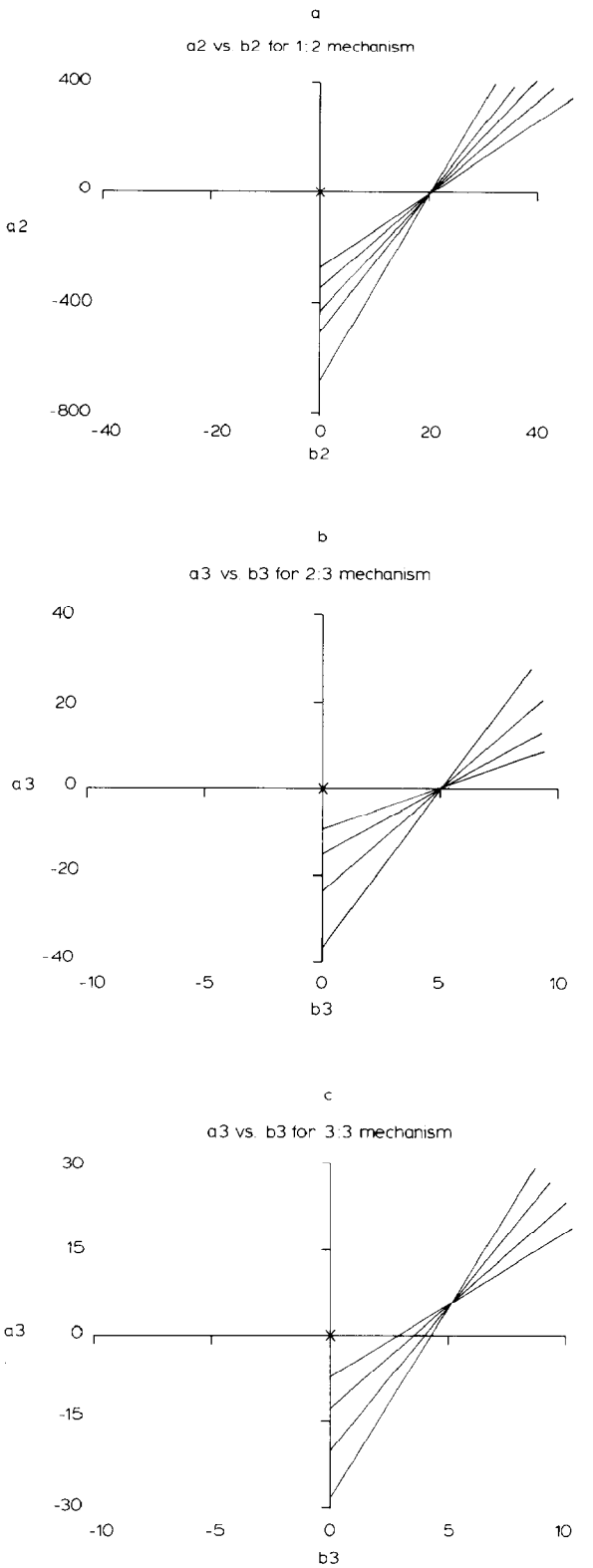

Fig.2. Distribution-free plots for simulated 1:2, 2:3 and 3:3 mechanisms. Plots of: (a) a_2 vs b_2 for a 1:2 mechanism where $a_1 = 400$, $b_1 = 2$ and $b_2 = 20$; (b) a_3 vs b_3 for a 2:3 mechanism where $a_1 = 40$, $a_2 = 29$, $b_1 = 3$, $b_2 = 4$ and $b_3 = 5$; (c) a_3 vs b_3 for a 3:3 mechanism with the addition of $a_3 = 5$.

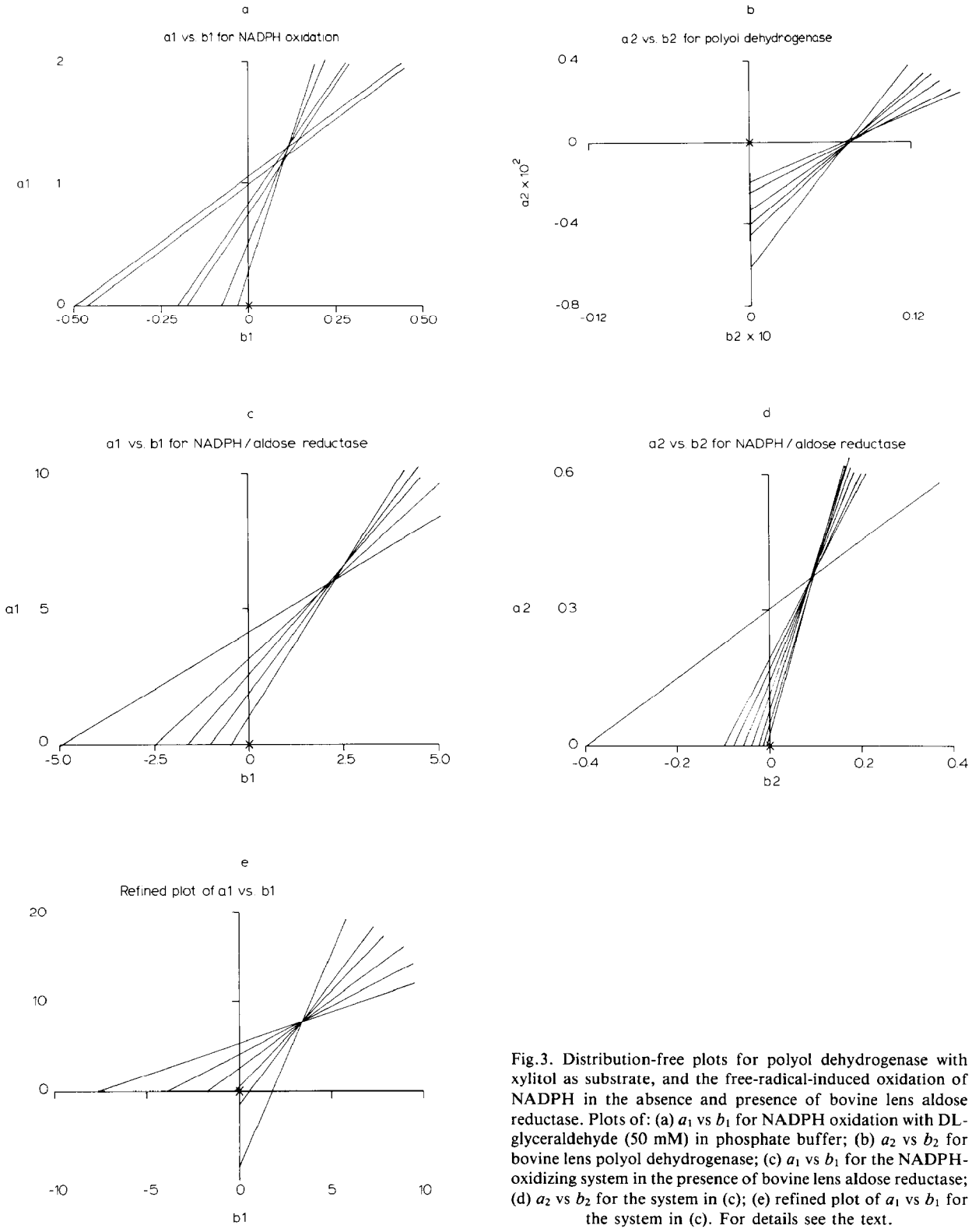

Fig.3. Distribution-free plots for polyol dehydrogenase with xylitol as substrate, and the free-radical-induced oxidation of NADPH in the absence and presence of bovine lens aldose reductase. Plots of: (a) a_1 vs b_1 for NADPH oxidation with DL-glyceraldehyde (50 mM) in phosphate buffer; (b) a_2 vs b_2 for bovine lens polyol dehydrogenase; (c) a_1 vs b_1 for the NADPH-oxidizing system in the presence of bovine lens aldose reductase; (d) a_2 vs b_2 for the system in (c); (e) refined plot of a_1 vs b_1 for the system in (c). For details see the text.

The methods developed here, which are also applicable to analysis by computer, distinguished between 1:1 and 2:2 functions which both produced hyperbolic plots in v vs [S] space, and showed whether inhibition at high concentrations of ligand was due to 2 or 3 receptor species reacting with ligand, i.e. whether the steady-state equation was of degree 1:2, 2:2, 2:3 or 3:3. These graphical procedures may be used as a preliminary test of the complexity of a process, to be followed by non-linear regression techniques to confirm initial estimates of mechanism and parameters. In the real examples described, the distribution-free parameter estimates agreed well with those found by non-linear regression computer techniques. Polyol dehydrogenase was shown to form a simple dead-end complex with xylitol at high substrate concentrations. The addition of an NADPH-binding protein to a system containing DL-glyceraldehyde, NADPH and phosphate buffer caused an increase in complexity from a 1:1 to a 2:2 function, indicating a functionally significant complex between NADPH and the protein which influenced the free-radical oxidation of the NADPH [19,20].

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