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A SIMPLE METHOD FOR THE DETERMINATION OF AFFINITY AND BINDING SITE CONCENTRATION IN RECEPTOR BINDING STUDIES

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Summary

In ligand binding studies, it is often difficult to apply kinetic analyses because of an uncertainty in experimental data obtained at high ligand concentrations. Under such circumstances, K_d value (an index of the affinity) and the binding site concentration may be estimated more accurately from the binding of a fixed concentration of labelled ligand observed in the presence of various concentrations of the non-labelled ligand, if the fraction of both labelled and non-labelled ligand bound is small. When there is no cooperative effect of the ligand binding, the K_d value may be calculated by subtracting the concentration of the labelled drug from the concentration of the non-labelled drug to cause a 50% reduction of the saturable binding of the labelled drug. From above values, the binding site concentration may be calculated. The proposed method is capable of examining the cooperativity of the ligand binding, the labelled drug concentration and the specific radioactivity of the labelled drug and does not require large amounts of the labelled drug.

Introduction

In recent years, the high affinity binding of drugs and hormones to tissue preparations was reported by a number of investigators. In those studies, a radiolabelled drug possessing high specific radioactivity is incubated in vitro with tissue preparations such as slices, homogenates, subcellular organells or purified proteins. After the separation of bound and unbound drugs by means of an ultrafiltration, ultracentrifugation, dialysis or partitioning between two solvent phases, the amount of the bound drug may be estimated. A non-specific, non-saturable binding which occurs simultaneously may be estimated from a concurrent binding assay performed either in the presence of excess non-labelled drug or in the absence of an essential cofactor for the binding. Non-

saturable binding is usually subtracted from the total binding to calculate the saturable portion of the binding.

Such a saturable binding is frequently called "receptor binding" with the assumption that the binding represents an interaction between the drug and its pharmacologically active or silent receptors. In fact, there is an impressive evidence which suggests that the high affinity saturable binding sites and pharmacologic receptors are related. For example, it has been generally observed with a number of agents that the saturable binding is displaced by pharmacologically active, but not by pharmacologically inactive, congeners and the affinities of binding sites for ligands parallel the pharmacologic potencies of these compounds.

Estimation of the affinity and binding site concentrations has been usually performed by means of a kinetic analysis such as Scatchard plots or Lineweaver-Burk type double-reciprocal plots [1]. These methods, however, are originally developed to handle enzyme studies in which large relative errors occur only at low substrate concentrations [2]. In contrast, large errors occur at high, as well as at low, ligand concentrations in receptor binding studies. At high ligand concentrations, the non-saturable binding tends to mask the saturable binding, and hence large relative errors occur in estimated values for the saturable binding. Thus, the present study was initiated to develop a method which is capable of accurately estimating kinetic parameters under the conditions of receptor binding studies.

Methods

As an example of in vitro receptor binding studies, [^{3}H]ouabain binding to rat brain homogenates and to partially purified rat brain ($Na^+ + K^+$)ATPase preparations in the presence of $MgCl_2$ and inorganic phosphate was employed. It has been shown that cardiac glycosides specifically bind to ($Na^+ + K^+$)ATPase under these conditions [3].

Male Sprague-Dawley rats weighing approximately 200 g were used. Brain homogenates were prepared by homogenizing the whole brain in 0.32 M sucrose solution containing 10 mM Tris · HCl buffer (pH 7.5) using a Dounce ball-type homogenizer. Partially purified ($Na^+ + K^+$)ATPase preparations were obtained from brain microsomal fractions by deoxycholic acid and sodium iodide treatment as previously described [4]. Brain homogenates (final concentration, 200 μg of protein per ml) or enzyme preparations (final concentration, 10 μg of protein per ml) were added to a pre-warmed incubation mixture producing a final concentration of 1 mM $MgCl_2$, 1 mM Tris-phosphate and 10 mM Tris · HCl buffer (pH 7.5) and various concentrations of ^{3}H-labelled and non-labelled ouabain. The mixture was incubated at 37° C for 90 min to attain the equilibrium of the binding reaction. After the incubation, [^{3}H]ouabain binding reaction was terminated by the addition of non-labelled ouabain (final concentration, 0.1 mM). The amount of bound [^{3}H]ouabain was estimated using a Millipore filter system to separate the unbound ouabain and liquid scintillation spectrometer to assay radioactivity [5]. Millipore filters were dissolved before counting in ethylene glycol monomethyl ether. Counting efficiency (approxi-

mately 30%) was monitored by the external standard channel ratio, which was calibrated with internal standards.

Protein concentrations were determined by the method of Lowry et al. [6]. [^{3}H]Ouabain was purchased from New England Nuclear Corporation, Boston, Mass. Other chemicals used were of reagent grade.

Theoretical Considerations and Experimental Results

Enzyme kinetics and receptor binding kinetics share many common features. In general, the binding of a ligand to its binding site may be described in the following reaction scheme:

$$D + R \rightleftharpoons DR \quad (1)$$

where D is the ligand (drug) and R is the binding site (receptor). If we can assume (a) that the reaction is reversible; (b) that the drug concentration is markedly greater than the binding site concentration; and (c) that no cooperative effects of the ligand binding occurs, then the following modified Michaelis-Menten equation may be derived:

$$B = \frac{B_{max} \cdot F}{K_d + F} \quad (2)$$

where F is the free drug concentration, B the bound drug concentration *, B_{max} the maximal binding, which is equal to the binding site concentration and K_d the apparent dissociation constant for reaction [1].

When $F = K_d$, then Eqn. 2 indicates that $B = 0.5\, B_{max}$. Thus, K_d is the concentration of a ligand which produces a half-maximal ligand binding (50% occupancy of the binding sites).

In ligand binding studies, data as shown in Fig. 1A are usually obtained when concentrations of radiolabelled drug are varied over a wide range starting from less than the K_d value and extending to approximately an order of magnitude greater than the K_d value. Eqn. 2, above, indicates that the highest concentration of the labelled drug should be at least 9 times the K_d value so that the observed binding approaches within 10% of the maximal binding. The use, however, of such a high concentration of labelled drug usually results in large errors in estimated saturable binding values (for example, ref. 7).

Fig. 1A shows the feasibility of studying the saturable binding of [^{3}H]ouabain to rat brain homogenates over a wide range of [^{3}H]ouabain concentrations because of the high affinity and low lipid solubility of this compound. The non-saturable binding accounts for less than 2% of the total binding even at a concentration of [^{3}H]ouabain as high as 500 nM, which is approximately 20 times the K_d value. Thus, the values for K_d and B_{max} can be estimated from double-reciprocal plots of data shown in Fig. 1B. The results indicate that K_d and B_{max} values are 25.3 nM and 63.7 pmol/mg protein, respectively.

Although conventional kinetic analyses can be performed with [^{3}H]ouabain binding studies, similar analyses are difficult when the compound to be ana-

* Bound drug concentration in the text denotes the concentration of specifically bound drug. This value may be obtained by subtracting the non-specific binding from the total binding.

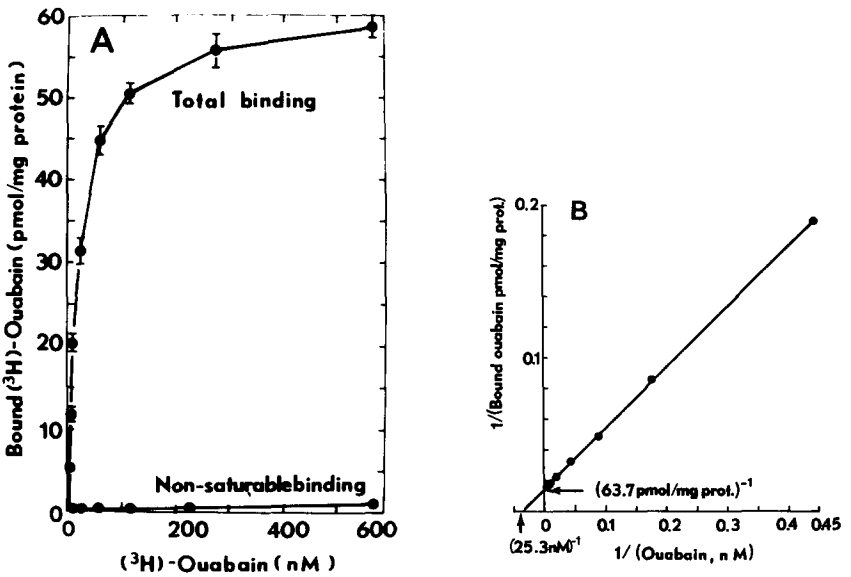

Fig. 1. The binding of [^{3}H]ouabain to rat brain homogenates in the presence of Mg^{2+} and inorganic phosphate. Rat brain homogenates were incubated with various concentrations of [^{3}H]ouabain (see Appendix 2 for the calculation of the ouabain concentration) at 37°C for 90 min in the presence of 1 mM $MgCl_2$ and 1 mM inorganic phosphate. Non-saturable binding was assayed by adding 0.1 mM non-labelled ouabain (final concentration) to the incubation mixture (A). The saturable binding was then calculated as the difference in the binding in the absence and presence of 0.1 mM non-labelled ouabain and then the double reciprocal plot was constructed (B). Each point represents the mean of three experiments.

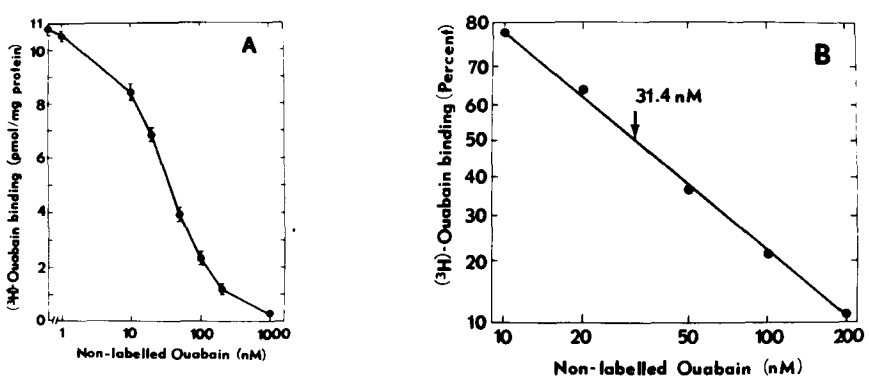

Fig. 2. The binding of 4 nM [^{3}H]ouabain to rat brain homogenates in the presence of Mg^{2+}, inorganic phosphate and various concentrations of non-labelled ouabain. Rat brain homogenates were incubated with 4 nM [^{3}H]ouabain at 37°C for 90 min in the presence of 1 mM $MgCl_2$, 1 mM inorganic phosphate and various concentrations of non-labelled ouabain. The binding of [^{3}H]ouabain in the presence of non-labelled ouabain is expressed either as pmol/mg protein (A) or as percent of that in the absence of the non-labelled ouabain in probit scale (B) and plotted against non-labelled ouabain concentration in logarithmic scale. Each point represents the mean of three experiments. In this and the following figures, only the saturable portion of the [^{3}H]ouabain binding is presented. The saturable [^{3}H]ouabain binding is calculated by subtracting the non-saturable binding observed in the presence of 0.1 mM non-labelled ouabain from the total binding.

lyzed has relatively high lipid solubility, because relatively high non-saturable binding is observed at high concentrations. Under such circumstances, data with smaller relative errors may be obtained when a fixed concentration of radiolabelled compound is used and the binding of the labelled drug is assayed in the presence of various concentrations of the non-labelled drug [7]. Consequently, the estimation of the values for K_d and B_{max} is more reliable when these values are derived from the type of studies such as shown in Fig. 2A.

Eqn. 2 may be modified to describe the binding of a ligand when both labelled and non-labelled drug are present in the incubation mixture.

$$B = \frac{B_{max} \cdot (a + C)}{K_d + a + C} \tag{3}$$

where a is the concentration of the labelled drug and C is the concentration of non-labelled drug. Although B in Eqn. 3 represents the binding of labelled plus non-labelled drug, only the binding of the labelled drug may be observed. The observed binding of the labelled drug in the presence of non-labelled drug is:

$$B_a = \frac{B_{max} \cdot (a + C)}{K_d + a + C} \cdot \frac{a}{a + C} \tag{4}$$

where B_a is the binding of the labelled drug. Eqn. 4 then may be rewritten as follows:

$$B_a = \frac{B_{max} \cdot a}{K_d + a + C} \tag{5}$$

Eqn. 5 describes the behavior of a labelled drug binding in the presence of various concentrations of non-labelled drug. Fig. 3 shows the plot of radiolabelled

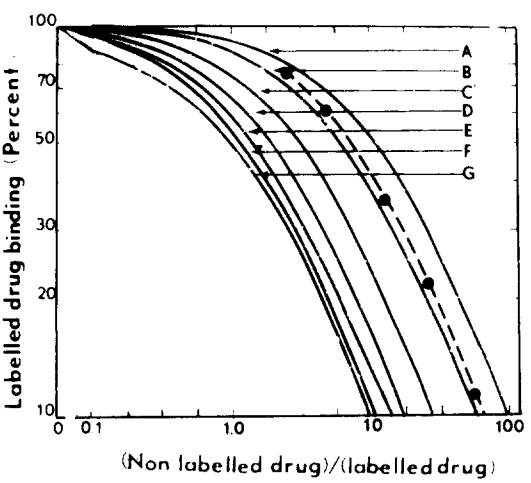

Fig. 3. The binding of a labelled drug in the presence of a fixed concentration of the labelled drug and various concentrations of the non-labelled drug. These curves are calculated from Eqn. 5. The binding of the labelled drug in the presence of a non-labelled drug is expressed as percent of that in the absence of the non-labelled drug and plotted against the ratio between non-labelled and labelled drug concentrations. Note that both axes are in logarithmic scale. Labelled drug concentrations are: A, 0.1 K_d; B, 0.2 K_d; C, 0.5 K_d; D, equal to K_d; E, 2 K_d; F, 5 K_d; G, 10 K_d. Filled circles represent data points for [^{3}H]ouabain binding calculated from experiments shown in Fig. 2.

drug binding as the function of the non-labelled drug concentrations calculated from Eqn. 5.

The binding of [^{3}H]ouabain to rat brain homogenates in the presence of various concentrations of non-labelled ouabain is also shown in Fig. 3 (filled circles: values are calculated from the data shown in Fig. 2). The dotted line in Fig. 3, which represents the saturable binding of ouabain, is similar to theoretical curves calculated from Eqn. 5. This finding indicates that the basic assumptions for drug-receptor interactions listed above are met for the ouabain binding (see Appendix 3). From Fig. 3, it is also possible to roughly estimate the K_d value for the ouabain binding.

Theoretical considerations, however, indicate that the affinity for the binding may be calculated more precisely and easily from the concentration of the non-labelled drug to produce a 50% inhibition of the labelled drug binding, i.e., a value which may be obtained from plots shown in Fig. 2B. If a non-labelled drug at the concentration of $C_{0.5}$ produces a 50% inhibition of a labelled drug binding, then:

$$\frac{1}{2} \cdot \frac{B_{max} \cdot a}{K_d + a} = \frac{B_{max} \cdot a}{K_d + a + C_{0.5}} \tag{6}$$

Solving Eq. 6, we obtain

$$K_d = C_{0.5} - a \tag{7}$$

Thus, K_d value may be calculated by subtracting the concentration of a labelled drug from the concentration of the non-labelled drug to cause a 50% inhibition of the labelled drug binding. From Eqn. 7, it may be observed that the calculated K_d value has a smaller relative error when the labelled drug concentration is smaller (i.e., when both a and $C_{0.5}$ are small). Thus, the use of a small labelled drug concentration which permits an accurate estimation of $C_{0.5}$ will yield the best estimate of the K_d value. From the data shown in Fig. 2B (the labelled drug concentration = 4.0 nM, $C_{0.5}$ = 31.4 nM), the K_d value for the interaction between ouabain and rat brain homogenates was estimated to be 27.4 nM.

The binding site concentration, i.e., B_{max} in Eqn. 5, may be calculated from the value of $C_{0.5}$, labelled drug concentration (a) and the bound drug concentration (B_a) observed in the absence of non-labelled drug (C = 0), as follows:

$$B_{max} = B_a \frac{K_d + a}{a} = B_a \frac{C_{0.5}}{a} \tag{8}$$

For the ouabain binding studies shown in Fig. 2A, the B_{max} value is estimated to be 64 pmol/mg protein. Above data were obtained with an assumption that the concentration of ouabain in the purchased [^{3}H]ouabain solution is 44.2 μM, instead of 82 μM as claimed by its supplier (see Appendix 2).

The proposed method is capable of examining the concentration of the radiolabelled drug if $C_{0.5}$ values were determined using three or more fixed concentrations of the labelled drug. Fig. 4 shows the results of such a study performed with partially purified ($Na^+ + K^+$)ATPase preparations. In Fig. 4, the concentration of labelled ouabain is calculated based on the concentration claimed by its supplier. Arrows in Fig. 4A show $C_{0.5}$ values estimated from a

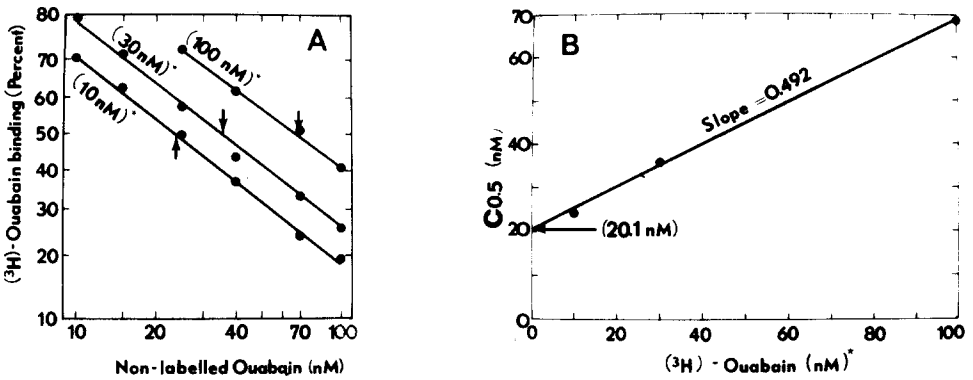

Fig. 4. The binding of [^{3}H]ouabain to partially purified ($Na^+ + K^+$)ATPase preparations in the presence of Mg^{2+} and inorganic phosphate. Partially purified rat brain ($Na^+ + K^+$)ATPase preparations were incubated at 37°C for 90 min with 10, 30, or 100 nM [^{3}H]ouabain * in the presence of 1 mM $MgCl_2$, 1 mM inorganic phosphate and various concentrations of non-labelled ouabain. Non-saturable binding was negligible under the present experimental conditions. A. The binding of the labelled ouabain in the presence of non-labelled ouabain is expressed as percent of that in the absence of non-labelled ouabain and plotted in probit scale against non-labelled ouabain concentration in logarithmic scale. Mean of two experiments. Regression lines are fitted by the least squares method. Number in parenthesis indicates the concentration of labelled ouabain. Arrows indicate concentrations of non-labelled ouabain to cause a 50% inhibition of the [^{3}H]ouabain binding ($C_{0.5}$). B. Values of $C_{0.5}$ are plotted against the concentration of [^{3}H]ouabain. * The concentration of [^{3}H]ouabain was calculated according to the value claimed by its supplier (see Appendix 2) in order to demonstrate that the present method can be used to obtain an accurate estimate of the K_d value independent of the labelled drug concentration.

linear regression line analysis. Eqn. 7 indicates that the plot of $C_{0.5}$ vs. labelled drug concentration should yield a straight line with a slope of 1.0, intercepting abscissa at the K_d value. When such a plot was constructed, a straight line was obtained (Fig. 4B). The slope of the regression line, however, was markedly lower than unity. A linear regression line analysis indicated that the slope is 0.492 and the abscissa intercept (K_d value) is 20.1 nM. Since the abscissa-intercept represents the concentration of non-labelled drug which occupies 50% of the binding site in the absence of the labelled drug, the estimate of K_d value with this method is not influenced by inaccuracies in the specific radioactivity (or concentration) of the labelled compound, which is rather difficult to estimate routinely. These data suggest that the concentration of the labelled drug is 49.2% of the value claimed by its supplier.

Discussion

In receptor binding studies performed in vitro, the binding site is characterized by two terms: B_{max}, or its equivalent, and K_d, or its equivalent. These values are usually estimated by kinetic analyses. In Lineweaver-Burk type double-reciprocal analysis, B_{max} is estimated from the extrapolation of a regression line to the infinite ligand concentration, and the K_d value is estimated as the concentration of the ligand to produce a half-maximal binding. Thus, K_d value is dependent on the estimated B_{max} value. Estimation of this latter value often involves substantial uncertainty [8], unless the binding at very high concentrations can be estimated accurately. In Scatchard type analysis, B_{max} is also

determined from the extrapolation of a regression line to the infinite ligand concentration. While the K_d value may be directly estimated from the slope of the regression line, independent of the B_{max} value, inaccuracies in observed values at both ends of ligand concentrations make it rather difficult to estimate the slope of the regression line accurately.

For the interaction of ouabain with $(Na^+ + K^+)$ATPase in the presence of Mg^{2+} and inorganic phosphate, above kinetic analyses yield accurate estimates of B_{max} and K_d values since the nonsaturable binding is extremely low due to the high affinity and low lipid solubility of this compound. Thus, the model system provides an opportunity to compare the proposed method with more conventional methods. The values of K_d and B_{max} estimated by the present method (27.4 nM and 64 pmol/mg protein, respectively) are in good agreement with those obtained with the double-reciprocal analysis (25.3 nM and 63.7 pmol/mg protein). Under these conditions, one advantage of the present method is that it is economical since a large concentration of labelled drug is not required for an accurate estimation of K_d and B_{max} values. Additionally, the estimated K_d value is not dependent on the concentration or specific radioactivity of the labelled drug in the present method. The present method rather provides an estimate of the labelled drug concentration. Thus, the proposed method should be employed when there are some doubts in the concentration or specific radioactivity of the labelled compound, or when the determination of these values for the labelled compound is more difficult than the determination of the concentration of non-labelled compound.

The present method may be inferior to other methods, such as Schild plots [9,10], if there is more than one binding site. Additionally, the present method is not applicable if the affinities of labelled and non-labelled ligands are different [11] or if one attempts to analyze the ligand displacement curves using non-labelled ligand which is chemically different from the labelled ligand. Methods which are capable of handling such a situation have been recently published [12,13]. In fact, Eqn. 7 of the present paper is a simplified form of equation 10 of ref. 12 or equation A31 of ref. 13. A complex kinetic handling described in these reports [12,13] is also capable of analyzing data when the fraction of bound drug is substantial. In contrast, the present method is based upon Michaelis-Menten type kinetics. Thus, similar to double reciprocal or Scatchard type analysis, the present method is valid only when the binding site concentration is sufficiently low to allow only a small fraction of both labelled and non-labelled ligand to be bound. Nevertheless, the present approach offers a simple method for the determination of affinity and binding site concentration in ligand binding studies in which the main interest is the determination of kinetic parameters for a specific ligand.

In conclusion, the present method is the preferable one to use under the condition in which non-saturable binding accounts for a substantial fraction of the total binding of a labelled drug at high concentrations. Either the double-reciprocal plot or Scatchard plot analysis fails to yield accurate estimates of K_d and B_{max} values under those conditions. The proposed method would yield relatively accurate estimates of K_d and B_{max} values under such conditions since a reasonably accurate estimation of the non-labelled drug concentration to cause a 50% inhibition of the labelled drug binding is possible when a relatively

low concentration of the labelled drug is used [7]. The proposed method for the determination of affinity and binding site concentration is summarized in Appendix 1.

Appendix 1

Algorithms for the determination of affinity and binding site concentration in receptor binding studies

1. Study the time course of the ligand binding using a concentration of labelled drug which is close to or lower than the expected K_d value. Following studies should be performed with the incubation time sufficient to attain equilibrium state.

2. Assay the binding of a fixed concentration of the labelled drug in the presence of various concentrations of the non-labelled drug. Lower concentration of the labelled drug is preferable. Concentrations of the non-labelled drug should be chosen so that the concentration to cause a 50% inhibition of the labelled drug binding may be accurately estimated. The nonspecific, non-saturable binding of the labelled drug should be estimated with an appropriate means. This value should be subtracted from observed values of the labelled drug binding for the following calculations.

3. Repeat step 2 with two other concentrations of the labelled drug.

4. Plot the data in the format of Fig. 2. The shape of curves indicates whether the conditions for this type of analysis are met. If the shape of curves resembles those of Fig. 5, positive cooperative effect of the ligand binding is suggested (see Appendix 3). Such data cannot be analyzed by the present method.

5. Plot the data in the format of Fig. 4A. Determine the concentrations of non-labelled drug to cause a 50% inhibition of the labelled drug binding for each concentration of the labelled drug.

6. Plot the data in the format of Fig. 4B. A straight line with a slope equal to unity should be obtained. The intercept of the regression line at abscissa represents the K_d value.

7. Calculate B_{max} from Eqn. 8.

8. This method is valid when conditions for Michaelis-Menten type kinetic analyses are satisfied.

Appendix 2

The specific radioactivity and the concentration of [^{3}H]ouabain

According to New England Nuclear Corporation, [^{3}H]ouabain (NET 211, lot number 747-186) was supplied as a solution containing 1 mCi and 0.048 mg of ouabain in 1 ml of an ethanol/benzene mixture (9 : 1, v/v). Thus, the concentration of ouabain (M_r 584.7) is calculated to be 82 μM and specific radioactivity, 12.2 Ci/mmol. Following studies, however, indicate that the ouabain concentration is only 54% of this value.

[^{3}H]Ouabain was diluted to 20 nM (based on the reported concentration and specific radioactivity) and the binding to partially purified ($Na^+ + K^+$)ATPase preparation was assayed in the presence of Mg^{2+} and inorganic phosphate. The amount of bound [^{3}H]ouabain after a 90-min incubation at 37°C was 233.3

TABLE I

ESTIMATION OF [^{3}H]OUABAIN CONCENTRATIONS

Partially purified rat brain ($Na^+ + K^+$)ATPase preparations were incubated with ouabain at 37°C for 90 min in the presence of 1 mM $MgCl_2$ and 1 mM inorganic phosphate. Values of non-saturable binding observed in the presence of 0.1 mM ouabain were subtracted.

[^{3}H]Ouabain (nM)	Non-labelled ouabain (nM)	[^{3}H]Ouabain binding (pmol/mg protein)
20 *	0	233.3
10 *	10.0	97.3
10 *	7.0	107.3
10 *	6.0	116.2
10 *	5.0	118.0
10 *	4.0	122.8

* [^{3}H]Ouabain was diluted according to the concentration claimed by its supplier.

pmol/mg protein. Evaporation of the ethanol/benzene mixture and subsequent reconstitution with distilled water failed to affect the [^{3}H]ouabain binding. In simultaneous experiments, [^{3}H]ouabain was diluted to 10 nM (again based on the reported concentration) and its binding was assayed in the presence of 10 nM non-labelled ouabain which was purchased from Sigma Chemical Company, St. Louis, Mo. (ouabain octahydrate, M_r = 728.6). If the concentrations of labelled and non-labelled ouabain were equal, this procedure would reduce the specific radioactivity of ouabain to 50% and therefore the saturable [^{3}H]ouabain binding to ($Na^+ + K^+$)ATPase should be 116.64 pmol/mg protein, i.e., 50% of the above value. However, the amount of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase under the latter condition was markedly lower than this value (Table I) indicating that either labelled drug concentration is lower or non-labelled drug concentration is higher than 10 nM. Table I shows additional data obtained with various concentrations of non-labelled ouabain. From the regression line analysis, it is estimated that "10 nM" labelled ouabain is equivalent to 5.39 nM non-labelled ouabain (i.e., this concentration of ouabain causes the [^{3}H]ouabain binding to be 116.64 pmol/mg protein).

Results shown in Fig. 4B in the text (slope of the regression line is less than unity) also support the contention that either the labelled drug concentration is lower or the non-labelled drug concentration is higher than the indicated value. Since several separate weighings and dilutions of the non-labelled ouabain yielded similar results, it was tentatively concluded that the concentration of ouabain in supplied [^{3}H]ouabain solution is 44.2 μM and the specific radioactivity is 22.6 Ci/mmol. These values were used for the calculation of [^{3}H]ouabain concentration in the test except for Fig. 4 and Table I.

Appendix 3

The binding of a labelled drug in the presence of various concentrations of the non-labelled drug when there is a cooperativity for the ligand binding

When there is a cooperative effect for the ligand binding, Eqn. 2 should be

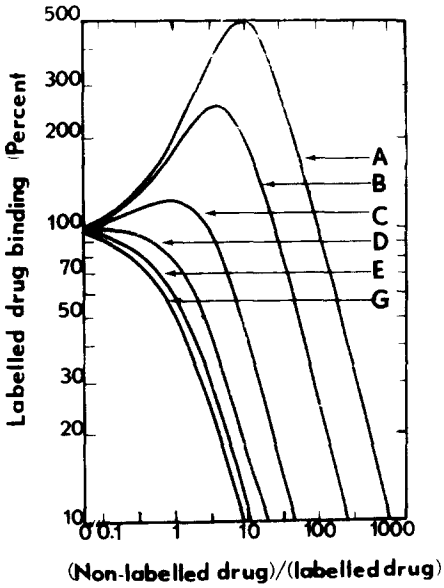

Fig. 5. The binding of a labelled drug in the presence of a fixed concentration of the labelled drug and various concentrations of the non-labelled drug when there is a positive cooperativity for the binding. For details see Fig. 3. Curves are calculated from Eqn. 5a with $n = 2$, instead of Eqn. 5.

modified as follows:

$$B = \frac{B_{max} \cdot F^n}{K_d{}^n + F^n} \tag{2a}$$

Where n is the index of cooperativity. Under this condition, Eqn. 4 should be modified as follows:

$$B_a = \frac{B_{max} \cdot (a + C)^n}{K_d{}^n + (a + C)^n} \cdot \frac{a}{a + C} \tag{4a}$$

Examples of curves which represents Eqn. 4a are shown in Fig. 5. This figure indicates that by the use of a labelled drug concentration which is 10—20% of the K_d value, it may be determined whether there is a cooperative effect for the ligand binding. Under these circumstances, kinetic parameters should be determined using methods such as polynomial approximation, non-linear curve fitting technique [14] or Hill plot analysis [15].

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