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A DOUBLE INHIBITION KINETIC ANALYSIS OF [^{3}H]-MUSCIMOL BINDING TO THE γ-AMINOBUTYRIC ACID RECEPTOR ON CALF BRAIN SYNAPTIC MEMBRANES

FURTHER STUDIES ON THE MECHANISM OF HOMOCYSTEINE-INDUCED SEIZURES

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Abstract—The *simultaneous* action of pyridoxal 5′-phosphate (PLP) and L-homocysteine on specific [^{3}H]muscimol binding to the γ-aminobutyric acid receptor on freeze-thawed, Triton-treated calf-brain synaptic membranes was examined kinetically by double inhibition analysis. PLP was found to be a *pure* inhibitor and L-homocysteine, a *partial* inhibitor, with respect to [^{3}H]muscimol.

Diagnostic analysis of the experimental data showed that the interaction constant (α) of the two inhibitors for the free receptor is between 0 and 1, confirming that the two inhibitors act synergistically.

Further double inhibition analysis showed that no quaternary receptor–[^{3}H]muscimol–homocysteine–PLP complex is formed although the ternary receptor–homocysteine–PLP complex is present.

The localization and relationship of binding groups for both inhibitors is discussed as in their association with the ligand binding site.

γ-Aminobutyric acid (GABA)* is a major inhibitory neurotransmitter of the central nervous system and has been associated with numerous normal and abnormal aspects of brain function [1]. Disturbances of GABA-mediated inhibition can lead to the appearance of seizures [2]. Griffiths *et al.* [3] first reported that inhibition of post-synaptic GABA receptor binding by the combined action of L-homocysteine and pyridoxal 5′-phosphate (PLP) might be involved in the genesis of seizures which occur following administration of an excess of L-homocysteine to experimental animals [4, 5]. Such involvement could be incorporated into a biochemical explanation for the appearance of seizures in human homocystinuria, a genetic disorder of methionine metabolism [6]. Various kinetic models were proposed [3] to explain the inhibitory phenomenon although it was not possible to discriminate between the rival schemes or to determine information regarding the binding relationship between ligand and inhibitors.

A number of workers have utilized the method of double inhibition analysis to investigate enzyme mechanisms [7–13], the same theory being applicable to receptor systems at equilibrium. Theoretical analyses of double inhibitions [14, 15] have shown that inhibitors or modifiers may exert varying kinetic effects on the action of the functional protein (receptor or enzyme) depending on the nature of the inhibition. Such analysis has shown how the simultaneous use of two inhibitors can be used to elucidate, for example, changes in the steric structure caused by one or other inhibitor, and the mechanism of action of the inhibitors.

The present study thus reports on the double inhibition by L-homocysteine and PLP of [^{3}H]muscimol (a GABA-mimetic) binding to the GABA receptor on calf brain synaptic membranes, and the subsequent diagnostic analysis used in an attempt to evaluate the kinetic mechanism of inhibition.

MATERIALS AND METHODS

Materials. L-Homocysteinethiolactone and pyridoxal 5′-phosphate were purchased from the Sigma Chemical Company (Poole, Dorset). [Aminomethyl-^{3}H]muscimol (15.5 Ci/mmole) was purchased from Amersham International Limited (Amersham, U.K.). Triton X-100 was obtained from BDH (Poole, Dorset). All other chemicals used were of the purest grade available. L-Homocysteine was prepared freshly from L-homocysteinethiolactone as described by Mudd *et al.* [16].

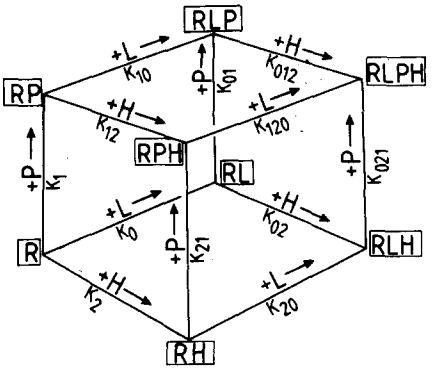
Preparation of Triton X-100-treated crude synaptic membranes. Synaptic membranes from 2-year-old cattle were prepared as described previously [3]. Essentially, the membranes obtained by differential centrifugation of a brain homogenate were initially frozen at −20° for at least 24 hr, thawed, and then treated with 0.05% (w/v) Triton X-100. The synaptic membranes were then washed thoroughly with 0.05 M Tris/citrate buffer (free of Na^+ and Triton X-100), pH 7.1, by resuspension and centrifugation. Following the final wash, the membrane pellet was resuspended in 0.05 M Tris/citrate (Na^+-free) buffer, pH 7.1, to give the required protein concentration.

* Abbreviations used: GABA, γ-aminobutyric acid; PLP, pyridoxal 5′-phosphate.

Measurement of [^{3}H]*muscimol binding in the absence and presence of inhibitors.* For the [^{3}H]-muscimol binding assay, aliquots of synaptic membrane suspension (1 mg protein in 100 μl) were added to plastic Eppendorf microfuge tubes (1.4 ml capacity) containing 0.05 Tris/citrate (Na^+-free) buffer, pH 7.1, [^{3}H]muscimol (15.5 Ci/mmole) alone, or, in the presence of inhibitors (the concentration of radioligand and inhibitors used are specified in each figure legend). Tubes used for non-specific binding determinations also contained non-radioactive GABA (0.4 mM final concentration). Triplicate samples, in a total volume of 1 ml, were incubated for 30 min at 4° followed by centrifugation at 8800 *g* in an Eppendorf 5413 microcentrifuge. The supernatants were quickly and carefully discarded by gentle suction and the pellets rinsed rapidly and superficially (without disruption) with 1 ml of 0.05 M Tris/citrate (Na^+-free) buffer, pH 7.1. The pellets were solubilized with 0.1 ml 8 M urea and, after an overnight incubation at room temperature, 1 ml of Packard toluene/Triton X-100 (2:1, v/v) scintillation fluid was added to each tube. The contents of each tube were mixed thoroughly prior to determination of radioactivity in a Packard 300 C liquid scintillation spectrometer at a counting efficiency of 40%. Specific binding was determined by subtracting the amount of [^{3}H]muscimol bound in the presence of 0.4 mM non-radioactive GABA (non-specific binding) from that bound in the absence of excess non-radioactive GABA (total binding). Non-specific binding was consistently 3–5% that of specific binding.

Data analysis. Kinetic binding data were analysed where applicable by non-linear regression using a VAX computer as described previously [3].

Analysis and general mechanism of double inhibitions. In the following theoretical treatment, the work of Keleti and Fajszi [14] and Fajszi [15] has been adapted to describe receptor interactions at equilibrium. For the general case, if two inhibitors interact with a receptor, the following complexes can be formed (assuming that all complexes contain the receptor, that is, complexes PH, LP, LH or LPH are not formed): RL, RP, RH, RLP, RLH, RPH, RLPH; where R is the receptor, L the ligand ([^{3}H]-muscimol), P and H are the inhibitors (P = PLP and H = L-homocysteine). In the general case for receptors, no product will be formed from RL species, since unlike enzymes, only a physiological response will be noted.



Scheme 1. Equilibria among receptor species for the general mechanism of double inhibitions. The vertices (boxes) of the scheme correspond to the free receptor and to the various complexes formed between ligand (L) and the inhibitors, L-homocysteine (H) and PLP (P). The line joining any two vertices represents a reversible binding step, the equilibrium dissociation constants (K) being indicated on this line. The subscripts of K are as defined previously [14, 15]; 0 = ligand, 1 = P and 2 = H. From any complex, the ligand or inhibitor which dissociates corresponds to the last digit in the combined subscript of the dissociation constant term.

The general mechanism is illustrated in Scheme 1. For such a system, at equilibrium, the following relationship exists between the equilibrium dissociation constants:

$$K_0K_{01}K_{012} = K_0K_{02}K_{021} = K_1K_{12}K_{120} = K_1K_{10}K_{012} = K_2K_{20}K_{021} = K_2K_{21}K_{120} \quad (1)$$

from which,

$$K_1K_{12} = K_2K_{21} \quad (2)$$

Table 1. Interpretation of the meaning of the interaction constants, α and β

Magnitude of interaction constant α or β	Binding of one inhibitor:
1	is independent of the binding of the other
∞	excludes the binding of the other
>1 and $<\infty$	hinders the binding of the other
>0 and <1	facilitates the binding of the other

The magnitude of the interaction constant α and β can convey information regarding the relationship between inhibitors. The interaction of the inhibitor on free receptor is defined by α while β refers to the interaction on the RL complex. Thus, α is only meaningful when R (inhibitor$_1$) and R (inhibitor$_2$) complexes exist and β when RL (inhibitor$_1$) and RL (inhibitor$_2$) complexes exist.

that is,

$$\frac{K_{12}}{K_2} = \frac{K_{21}}{K_1} = \alpha \quad (3)$$

where α may be defined as an interaction constant of the two inhibitors on the free receptor. Also,

$$K_{01}K_{012} = K_{02}K_{021} \quad (4)$$

that is,

$$\frac{K_{012}}{K_{02}} = \frac{K_{021}}{K_{01}} = \beta \quad (5)$$

where β may be defined as an interaction constant of the inhibitors on the RL complex. Various other constants may be defined as necessary [14]. It should be appreciated that α and β define the effect of the binding of one inhibitor to the receptor and the receptor–ligand complex, respectively, on the binding of the other inhibitor to these complexes. The significance of the interaction constant can be seen when different values of the term are used (Table 1).

One aim of double inhibition studies is to determine the value of an interaction constant within the limits described in Table 1. Such information can be used to evaluate a great deal about the exact mechanisms involved. Prior to a determination of interaction constants is the need to ascertain whether both inhibitors are complete (pure) or partial with respect to the ligand ([^{3}H]muscimol). In pure inhibition, the binding of ligand to receptor can be blocked completely; while in partial inhibition, the species containing inhibitor is still able to bind ligand but less than in the absence of inhibitor. Keleti and Fajszi [14] have described seventeen different permutations by which ligand and inhibitors may interact depending upon the type of inhibition. These permutations are, however, condensed to three general categories, for which rapid equilibrium equations have been derived [14, 15].

Thus, for the present system we have: CASE I where both P and H are pure inhibitors; CASE II where P is pure and H is partial inhibitor or vice versa; CASE III where both P and H are partial inhibitors.

Although separate use of inhibitors can be used to determine the nature of the inhibition, it is advisable to endorse this by undertaking experiments in which both inhibitors are applied *simultaneously*. Differentiation of these cases can be made by suitable analysis (Table 2).

Table 2. Differentiation of CASES I, II and III

	Inhibition by		Plot of $1/b_{1,2}$ against	
CASE	i_1	i_2	i_1 at fixed i_2	i_2 at fixed i_1
I	pure	pure	straight line	straight line
II	pure	partial	straight line	hyperbola
III	partial	pure	hyperbola	hyperbola

Binding experiments should be undertaken at constant radioligand concentrations in each case. The terms $b_{1,2}$ = radioligand bound in the presence of both inhibitors; i_1 = PLP; i_2 = L-homocysteine.

Once the correct category (CASE I, II or III) has been established, it is necessary to determine whether there is full competition between the inhibitors. If this is so, then the binding of one inhibitor to R or RL excludes the binding of the other, that is, neither RPH nor RLPH exist. If either the RPH or RLPH complexes exist, it is then possible to determine the interaction constants which can provide information about binding groups on the receptor or about the binding of ligand and inhibitors [14].

In summary, the protocol followed in the following experiments is:

(i) by simultaneous application of both inhibitors and subsequent analysis, determine whether CASE I, II or III operates;

(ii) evaluate whether full competition exists between the inhibitors (α and/or $\beta = \infty$);

(iii) if full competition does not exist between inhibitors, then determine the order of magnitude of the interaction constants.

RESULTS

Affinity and capacity of [^{3}H]*muscimol binding*

Synaptic membranes, prepared from calf brain cortex, were frozen at −20° for 24 hr, thawed, and then treated with 0.05% (w/v) Triton X-100. Specific [^{3}H]muscimol binding to Triton-washed, freeze-thawed synaptic membranes, measured as a function of the free [^{3}H]muscimol concentration (0.5–20 nM) exhibited saturable kinetics (Fig. 1). Scatchard analysis of the binding data derived from these experiments yielded one binding site having a dissociation constant (K_d) = 3.6 ± 0.3 nM, with a maximum number of specific receptor sites (B_{max}) = 3.94 ± 0.14 pmoles/mg protein.

Effect of simultaneous application of L-*homocysteine and PLP*

Equilibrium binding studies were carried out at a fixed concentration of 4 nM [^{3}H]muscimol and in the presence of both inhibitors, varying L-homocysteine concentrations between 0 and 4.0 mM and PLP concentrations between 0 and 8.0 mM. Figure 2 shows the experimental data plotted according to Yonetani and Theorell [10]. By reference to Table 2, it can be seen that the present system conforms to the CASE II category of Keleti and Fajszi [14]. The straight lines of Fig. 2A indicate that PLP is a pure inhibitor of specific [^{3}H]muscimol binding to the GABA receptor. However, the hyperbolic nature of the curves (Fig 2B) indicate that L-homocysteine is a partial inhibitor of [^{3}H]muscimol binding to the receptor. It should be noted that the nature of inhibitions at this stage of the analysis reflect only whether inhibition is pure or partial. On the basis of experiments described in Fig. 2 it is not possible to predict whether the inhibitions are competitive or non-competitive. However, it has been shown [3] that when both inhibitors are analyzed *separately*, PLP and L-homocysteine, demonstrated pure and partial competitive modes of inhibition, respectively. Furthermore, these workers showed by statistical

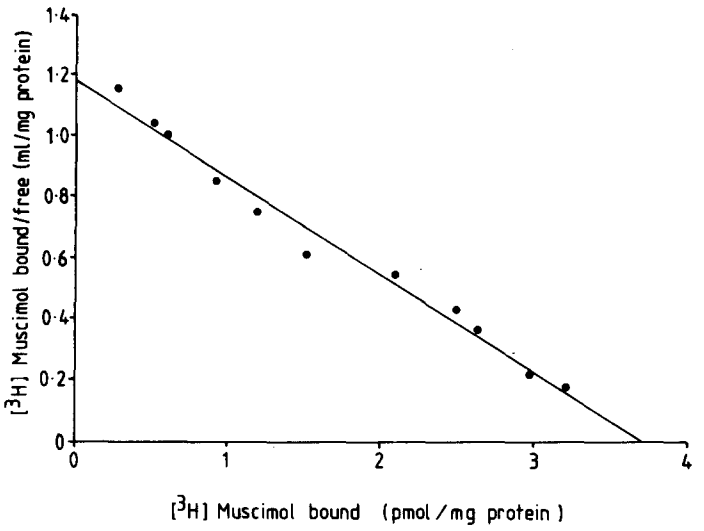


Fig. 1. Specific [^{3}H]muscimol binding to calf brain synaptic membranes. Freeze-thawed, Triton-treated synaptic membranes (1 mg protein/100 μl) were incubated with varying concentrations of [^{3}H]muscimol up to 20 nM as described in Materials and Methods. Non-specific binding was determined in the presence of 0.4 mM GABA. Specifically bound radioactivity was determined and the data subjected to non-linear regression analysis for computation of kinetic binding parameters. The same data was utilized for Scatchard analysis as shown. Each experimental point represents the mean of triplicate determinations (variation < 4%) and the experiment is typical of three.

analysis that inhibition by L-homocysteine could be explained satisfactorily by a partial non-competitive system. The implications of this will be discussed later.

Evaluation of full competition between inhibitors

Several methods have been proposed to evaluate full competition between two inhibitors [8–10, 17]. Of the available methods, that of Loewe [8] was used. The method of Loewe [8] involves construction of appropriate isobolograms. Isobols are equi-effective combinations of active compounds plotted such that the co-ordinates represent the concentration of the compounds. In the present system (Fig. 3) the co-ordinates are PLP and L-homocysteine and the isobols correspond to combinations of the inhibitors yielding (at a fixed [^{3}H]muscimol concentration of 4 nM) a constant $b_{1,2}$ (or $b_{1,2}/b_0$) value, where b_0 = specific [^{3}H]muscimol bound in the absence of either inhibitor and $b_{1,2}$ = specific [^{3}H]muscimol bound in the presence of both inhibitors. For a CASE II system the presence of straight lines with a common intercept at a negative L-homocysteine co-ordinate would be indicative of full competition implying that neither the RPH or RLPH complexes existed. It can be seen that the presence of an essentially curvilinear plot suggests that full competition between both inhibitors does not exist.

Determination of possible ternary and quaternary complexes

Experiments were conducted to examine whether either or both the ternary RPH and quaternary RLPH complexes are formed. The specific binding of [^{3}H]muscimol was measured in the presence of both inhibitors, as a function of the variable [^{3}H]-muscimol concentration. The assay system comprises the partial inhibitor, L-homocysteine, and the pure inhibitor, PLP, varied so as to generate a curve for diagnostic analysis. For a CASE II system, a plot of $1/b_{1,2}$ against $1/$[^{3}H]muscimol concentration at variable PLP concentrations should generate straight lines, which are parallel when RPH does not exist. Alternatively, if the quaternary RLPH complex does not exist, straight lines with a common intercept is expected [15]. Figure 4 illustrates the double reciprocal plot of $b_{max}/b_{1,2}$ against $1/$[^{3}H]muscimol concentration from which it can be deduced that the quaternary RLPH complex does not exist. The interaction constant, β, which represents the affinity of both inhibitors for the RL complex therefore has no meaning in this system. Keleti and Fajszi [14] describe seven ways of inhibition of CASE II systems, when one of the inhibitors inhibits partially. Of these, only two are meaningful when the quaternary RLPH complex does not exist. It is now possible to reduce the options to two feasible systems, viz.

(i) PLP is a pure competitive inhibitor, and L-homocysteine a partially competitive inhibitor, or,

(ii) PLP is a pure competitive inhibitor, and L-homocysteine a partial non-competitive inhibitor.

In both instances, $K_{01} = K_{012} = \infty$, but additionally, in the latter case, $K_2 = K_{02}$.

Determination of interaction constants

The absence of full competition between L-homocysteine and PLP (Fig. 3) indicates that RPH, RLPH or both complexes could exist. The conclusion from Fig. 4 further indicates that no quaternary RLPH complex can be formed. Since the ternary RPH complex is formed, determination of the interaction

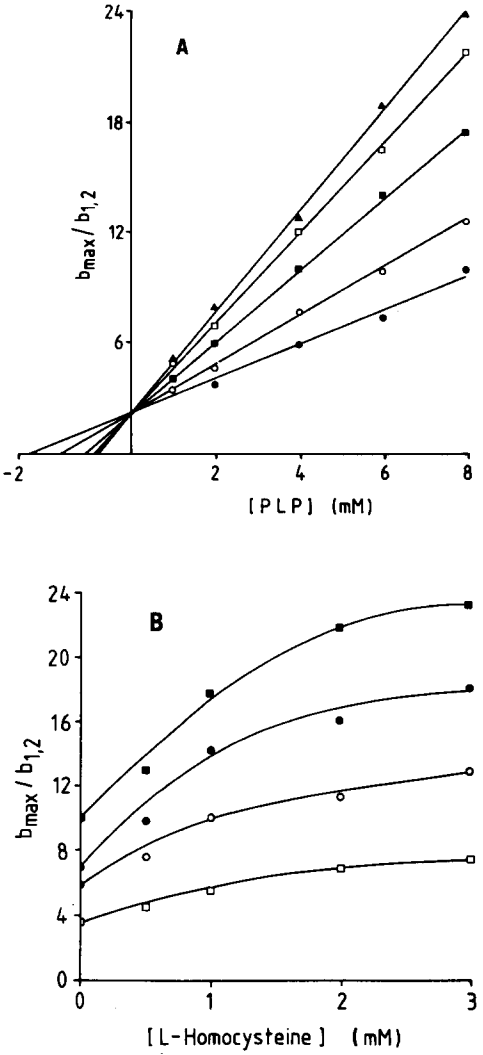


Fig. 2. Yonetani–Theorell plot of double inhibition of [^{3}H]-muscimol binding by L-homocysteine and PLP. Calf brain synaptic membranes (1 mg protein/100 μl) were incubated for 30 min at 4° in the presence of 4 nM [^{3}H]muscimol (A) with varying concentrations of PLP (0–8.0 mM) at fixed concentrations of (●) 0, (○) 0.5 mM, (■) 1.0 mM, (□) 2.0 mM, and (▲) 4.0 mM L-homocysteine, and (B) with varying concentrations of L-homocysteine (0–3.0 mM) at fixed concentrations of (□) 2.0 mM, (○) 4.0 mM, (●) 6.0 mM and (■) 8.0 mM PLP.

Specific [^{3}H]muscimol binding was determined as described in Materials and Methods. The curves were constructed with the aid of computer estimates, by non-linear regression analysis, as described previously [3]. Each experimental point represents the mean of triplicate determinations (variation < 4%) and the experiment is typical of two: b_{max} = maximum concentration of specific receptor sites for [^{3}H]muscimol; $b_{1,2}$ = [^{3}H]muscimol bound in the presence of both L-homocysteine and PLP.

constant (α) should provide some information regarding the binding characteristics of both inhibitors on the free receptor. To achieve this, the intercepts on the ordinate (negative PLP axis) of Fig. 2A were further plotted as a function of the concentration of L-homocysteine, this intercept value

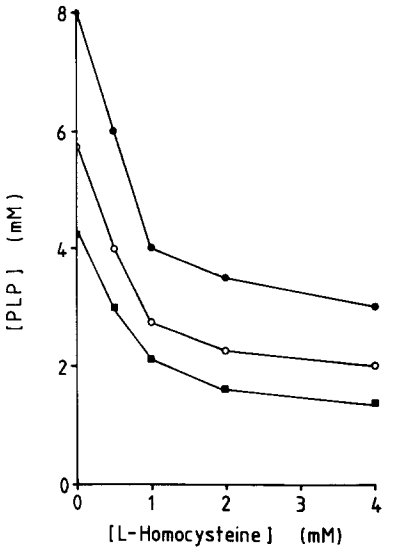


Fig. 3. Isobologram analysis for determination of possible full competition between L-homocysteine and PLP. Data obtained from Fig. 2 was used in constructing the isobologram for analysis by the method of Loewe [8]. The experimental points were plotted such that the co-ordinates represent inhibitor (L-homocysteine and PLP) concentration at which constant $b_{1,2}$ values were obtained. The significance of the figure is described in the text. $b_{1,2}$ = [^{3}H]-muscimol bound in the presence of both inhibitors.

being a function of ligand and L-homocysteine concentration [14]. The resultant decreasing hyperbola (Fig. 5) indicates that the interaction constant (α) of the two inhibitors, for free receptor, is between 0 and 1.

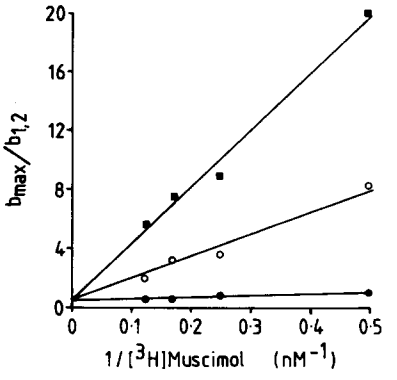


Fig. 4. Modified Keleti–Fajszi double-reciprocal plot used as a diagnostic test to determine the existence of ternary RPH and quaternary RLPH complexes. Calf brain synaptic membranes (1 mg protein/100 μl) were incubated for 30 min at 4° in the presence of varying concentrations of [^{3}H]muscimol (2.0–8.0 nM), L-homocysteine fixed at 15.0 mM, and varying: (●) 0 mM, (○) 1.8 mM and (■) 3.6 mM concentrations of PLP. Each experimental point represents the mean of triplicate determination (variation < 4%) and the experiment is typical of two. Lines were fitted to experimental points by non-linear regression analysis of the data. b_{max} and $b_{1,2}$ are as defined earlier.

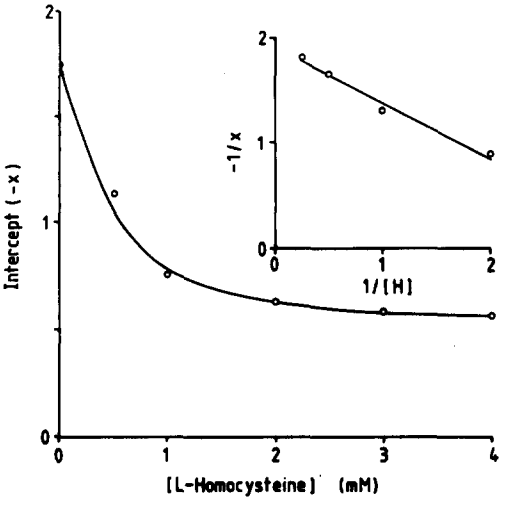


Fig. 5. Determination of the interaction constant (α) of both inhibitors on the free receptor. The method of Keleti and Fajszi [14] was adopted to determine the interaction constant (α) from data described in Fig. 2. Intercepts on the negative PLP-axis ($-x$) in Fig. 2 were plotted against the respective concentrations of L-homocysteine, 0, 0.5, 1.0, 2.0, 3.0 and 4.0 mM. Insert: the straight line replot of $1/x$ against 1/L-homocysteine indicating hyperbolic nature of main curve: H = concentration of L-homocysteine.

DISCUSSION

A general approach to the analysis of multiple inhibition has been described by Keleti and Fajszi [14]. More precisely, their theoretical approach in the present context describes how the relationships of two inhibitors acting on the same receptor can be characterized. In this study, analysis by double inhibition has been used essentially as a qualitative tool. The purpose was to ascertain more information regarding the kinetic mechanism of synergistic inhibition of [^{3}H]muscimol binding to the GABA receptor on brain synaptic membranes by L-homocysteine and PLP *in vitro*. The observation that L-homocysteine enhances PLP-induced seizures *in vivo* [5] gives direct relevance to this study.

It has been shown [3] that PLP, used *independently* of L-homocysteine, is a two-site, pure competitive inhibitor of [^{3}H]muscimol binding. L-Homocysteine when used independently of PLP, was, however, shown to be a partial competitive inhibitor of [^{3}H]-muscimol binding although a partial non-competitive mode of inhibition could also be fitted to the experimental data. Kinetic models were proposed by these workers to explain the observed synergistic inhibition, although models incorporating complexes in which both PLP sites are occupied were not strong contenders on statistical grounds. Use of statistical optimisation routine, in which both inhibitors were present *simultaneously*, indicated that a model, whereby only one PLP site was involved in complex formation, appeared most adequate.

The method of double inhibition analysis, followed in the present study [14, 15] assumes that for every inhibitor, the protein has only a single binding site. The extent to which the results fit the framework of double inhibition analysis can thus be taken as a measure of its suitability to a kinetic model in which only one PLP site participates directly with L-homocysteine. Indeed the kinetic binding data obtained fit remarkably well to specific diagnostic systems defined by others [14, 15]. On the basis of appropriate kinetic plots, it can be concluded that a suitable model to explain the present results conforms to the general mechanism (Scheme 1) in which no quaternary RLPH or ternary RLP complex exists. This is identical to one of the models (model I) proposed by Griffiths *et al.* [3].

The use of double inhibition analysis also provides information regarding the binding characteristics of the inhibitors. Semi-quantitative determination of the interaction constant (α) showed that $1 > \alpha > 0$. On the basis of the present analysis, PLP was shown to be a pure competitive inhibitor of [^{3}H]muscimol binding. L-Homocysteine can be characterized as a partial inhibitor; the nature of the inhibition, either competitive or non-competitive being unresolved. If the partial inhibition by L-homocysteine is competitive, it suggests that the binding sites of two inhibitors partially overlap. A partial non-competitive mode of inhibition would suggest that PLP is bound by several groups, one part of which is shared with [^{3}H]muscimol, another part with L-homocysteine.

In the context of brain function, small changes of metabolite levels or neurotransmitter-mediated functions are enough to disturb the delicate equilibrium which maintains the electrical stability of the central nervous system. Such changes of GABA binding may well result in a significant disturbance of GABA-mediated neuroinhibitory function. The present kinetic approach can be used to postulate that binding of the natural ligand, GABA, to its post-synaptic receptor could be disturbed under conditions where L-homocysteine or PLP are elevated. Indeed, seizures are a characteristic of L-homocysteine [4] and PLP [17] excess, while evidence exists to support some interaction between PLP and L-homocysteine in seizure formation [5]. Furthermore, it has been shown [18] that other endogenous sulphur-containing compounds can replace L-homocysteine in displaying synergistic inhibition in combination with PLP. Such interactions would however, be more relevant under clinical circumstances of pyridoxine excess.

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