J. Pharm. Pharmacol. 1984, 36: 776–778
Communicated March 28, 1984

Scatchard plots with a positive slope: dependence upon ligand concentration

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The inverse dependence of binding constants (nK) upon albumin concentration for 2-(4′-hydroxybenzeneazo) benzoic acid (HABA) has been investigated with bovine and human albumin by equilibrium dialysis. Data obtained by the use of a range of concentrations of either bovine or human albumin, together with a single ligand concentration, gave positive Scatchard plots indicative of binding constants inversely dependent upon protein concentration. The slope of each plot was influenced by the particular ligand concentration used and an increase in the ligand concentration in the case of both bovine and human albumin resulted in a decrease in the slope of the positive Scatchard plot. Higher concentrations of ligand produced a transformation from a positive to a negative plot with both bovine and human albumin.

It is generally assumed that the apparent association constant (K) and the number of binding sites (n) are independent of protein concentration. Inverse dependence of binding constants upon albumin concentration has been demonstrated, however, for a variety of ligands (Bowmer & Lindup 1978b), including the anionic dyes methyl orange (Klotz & Urquhart 1949; Breyer & Bauer 1953) and 2-(4′-hydroxybenzeneazo) benzoic acid (HABA) (Zia & Price 1975; Bowmer & Lindup 1980). There are several other proteins besides albumin for which the affinity of the ligand varies inversely with the protein concentration (Klapper & Klotz 1968) but in general the phenomenon has received little attention. Recently, however, conflicting evidence has appeared with regard to the occurrence of this effect with methyl orange and HABA (Ford & Winzor 1981; Sakurai et al 1981) which are two ligands commonly used for the investigation of protein binding.

Recent reports (Judis 1980; Mueller & Potter 1981; Shami et al 1984) have shown that ligand concentration may affect the degree of dependence of binding constants upon protein concentration. Positive Scatchard plots, indicative of the inverse dependence of binding constants upon protein concentration, had lower slopes when higher ligand concentrations were used in conjunction with a range of protein concentrations. It has subsequently been shown (Clegg & Lindup 1982) that for methyl orange, a positive Scatchard plot obtained with a range of protein concentrations and a single ligand concentration will undergo transformation to a negative plot of the ligand concentration is raised sufficiently.

HABA is structurally similar to methyl orange and so it seemed likely that HABA would provide a second example of a ligand where a transformation of the Scatchard plot could occur. This has been investigated by equilibrium dialysis with human and bovine albumin.

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Materials and methods

Human and bovine albumin (fraction V, lot nos. 30F-02271 and 118C-0026 respectively, Sigma Chemical Co., Poole, Dorset) were used without further purification. HABA and methyl orange (4-[*p*-(dimethylamino)-phenylazo] benzenesulfonic acid) were purchased from BDH Ltd (Poole, Dorset) and Koch-Light Laboratories Ltd (Haverhill, Suffolk) respectively.

The binding of HABA to each albumin preparation was measured at 37 °C in phosphate buffer at pH 7·4 by equilibrium dialysis (Bowmer & Lindup 1980). The binding of HABA to three different initial bovine albumin concentrations (1, 2 and 4% w/v i.e. about 150, 300 and 600 μM) was studied using a range of initial ligand concentrations (50 to 5000 μM), whilst the binding of HABA to a range (0·05 to 4% w/v) of bovine and human albumin concentrations was determined for four different initial ligand concentrations (75, 150, 250, 500 μM).

The results were plotted by the method of Scatchard (1949) as previously described (Bowmer & Lindup 1980). Du represents the unbound ligand concentration and r the molar ratio of bound drug to albumin. Representative error bars for standard deviations are included in Fig. 1.

Results

Binding of a range of HABA concentrations to bovine albumin. The Scatchard plots for the binding of HABA to 1, 2 and 4% (w/v) bovine albumin are shown in Fig. 1. The results of these three experiments are superimposable for most values of r, although there is some discrepancy between the plots at r values less than 0·5, particularly with the results for 4% bovine albumin where the plot shows a peak or maximum (Fig. 1). Our findings are similar to those of Sakurai et al (1981) in that a change in the concentration of bovine albumin in the range 0·5 to 4% did not affect the Scatchard plot at r values in excess of one. A more sensitive assay for HABA, for example a radiolabelled preparation of the dye, is required to confirm that a positive plot could be obtained for the interaction with 4% bovine albumin by the use of a range of low (50 to 250 μM) initial HABA concentrations.

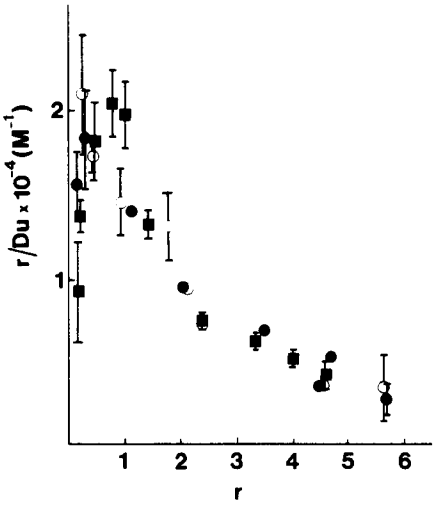

FIG. 1. Scatchard plots for the binding of a range (50 to 5000 μM) of HABA concentrations to 1% w/v (○), 2% (●) and 4% (■) bovine albumin. Each point is the mean of 4 to 6 measurements and the error bars represent ± one s.d. unless too small to show.

Binding of HABA to a range of bovine albumin concentrations. Fig. 2 shows Scatchard plots of the results obtained in experiments where the binding of each of four different concentrations of HABA to a range of bovine albumin concentrations was studied. The binding of a 75 μM concentration of HABA gave a positive slope. An increase in the ligand concentration to 150 μM produced a decrease in the slope of the plot and an increase in the ligand concentration to 250 μM, produced a conventional negative slope (Fig. 2). A further increase in HABA concentration to 500 μM produced results indistinguishable from those obtained at the 250 μM concentration, which were also in agreement for most values of r with those obtained by variation of the ligand concentration.

These results demonstrate that the slope of the positive Scatchard plot for the binding of HABA to bovine albumin is not only dependent upon albumin concentration but also that the ligand itself plays a role in the determination of albumin activity.

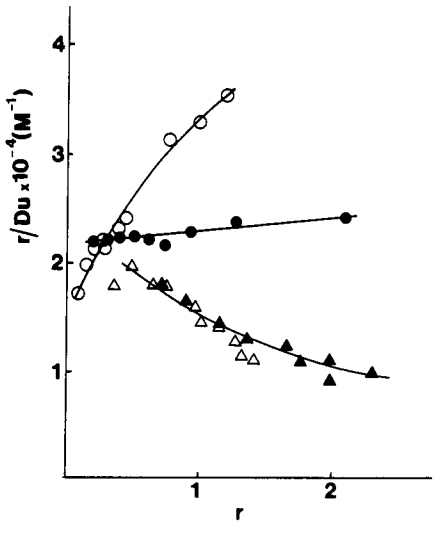

FIG. 2. Scatchard plots for the binding of HABA (○ 75 μM; ● 150 μM; △ 250 μM; ▲ 500 μM) to a range (7·5 to 600 μM) of bovine albumin concentrations. Each point is the mean of 4 to 6 measurements.

Binding of HABA to a range of human albumin concentrations. The binding of HABA to a range of human albumin concentrations (Fig. 3) gave a positive Scatchard plot, the slope of which was influenced by the ligand concentration in a similar way to the binding to bovine albumin. Binding of 150 μM HABA gave a positive slope in agreement with the findings of Bowmer & Lindup (1980). Increases in the ligand concentration to 250 and 500 μM produced successive decreases in the slope of the positive plot and a further increase in ligand concentration to 750 μM resulted in a transformation from a positive to a negative slope (Fig. 3).

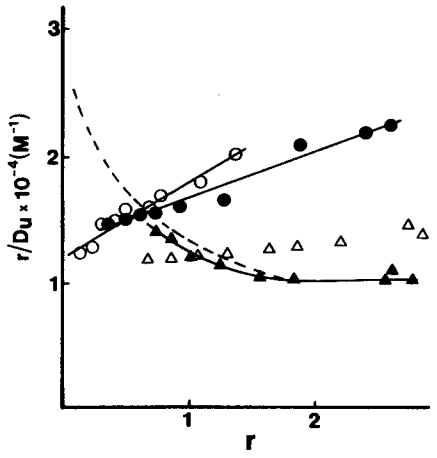

FIG. 3. Scatchard plots for the binding of HABA (○ 150 μM; ● 250 μM; △ 500 μM; ▲ 750 μM) to a range (7·5 to 600 μM) of human albumin concentrations. The broken line represents the data obtained by variation of HABA concentration (50 to 5000 μM) with a single concentration 150 μM) of albumin.

Discussion

Changes in the concentration of bovine albumin in the range 1 to 4% (w/v) did not affect the Scatchard plot for the binding of HABA to bovine albumin in that the plots obtained were superimposable for most values of r (Fig. 1), in agreement with the findings of Sakurai et al (1981). However, a low r values ($r \leq 0{\cdot}5$), there were

differences between the results obtained for the three protein concentrations and at the higher bovine albumin concentrations the values of r/Du were diminished. This suggests that, for bovine albumin as with human albumin, there is some inverse dependence of the association constant and/or the number of binding sites upon protein concentration. This effect was not reported by Sakurai et al (1981). There appears to be a peak in the Scatchard plot for the binding of HABA to 2% (w/v) and to 4% (w/v) albumin, although this was not observed with 1% (w/v) albumin (Fig. 1).

Scatchard plots with a maximum have been predicted theoretically (Nichol & Winzor 1976; Dahlquist 1978; Cann 1978) but to date there has been little experimental evidence to support this although Carlson & Breslow (1981) obtained such a plot for the binding of bromophenol blue to bovine neurophysin I. Several mechanisms for the phenomenon of binding constants which are inversely dependent upon protein concentration have been discussed (Bowmer & Lindup 1978a, b, 1980; Boobis & Chignell 1979; Zini et al 1981). There is insufficient and conflicting evidence (Boobis & Chignell 1979; Zini et al 1981) about the contribution of protein–protein interactions. Polymers of albumin do not appear to be involved in the case of the similar type of interaction which occurs between L-tryptophan and albumin (Clegg & Lindup 1984).

Co-operativity has been implicated frequently in unusual ligand–protein interactions (Cook & Koshland 1970; Eldefrawi & Eldefrawi 1973; Shen & Gibaldi 1974; Pfaff et al 1975; Henriksen & Jackson 1975; Kolb & Weber 1975) and could explain positive Scatchard plots. Mueller & Potter (1981) favoured positive co-operativity as the explanation for their findings with cortisol but protein–protein interactions could not be excluded. Scatchard plots with a maximum (Fig. 1) suggest a mixture of positive and negative co-operativity but on this basis, positive Scatchard plots should result when binding data are obtained by either of the two possible experimental approaches (Bowmer & Lindup 1980) but so far as we know no convincing experimental proof has been obtained. The maximum can also indicate ligand-mediated and ligand-facilitated association of protein molecules (Cann 1978). Several models may therefore fit the data but binding measurements alone are unlikely to eliminate incorrect models and further experiments, with, for example molecular probes of albumin conformation, are needed to gain more insight into the mechanism involved.

The transformation of a Scatchard plot from one of positive slope to a negative slope by an increase in the ligand concentration adds another dimension to the problem of ligand-protein interactions where nK is inversely dependent upon protein concentration. It now appears that with albumin and, so far, at least four substances, namely cortisol (Mueller & Potter 1981), methyl orange (Clegg & Lindup 1982), mianserin (Shami et al 1984) and HABA, that the slope of a positive plot can be decreased by an increase in the ligand concentration. In the case of methyl orange and HABA it has been possible to transform a positive plot to a negative one by an appropriate increase in the ligand concentration and so far as we are aware these are the first examples where this has been demonstrated experimentally with albumin.

The implications of this phenomenon for drug-receptor interactions have not been studied but a similar inverse dependence of affinity upon protein concentrations has been observed for [^{3}H]domperidone binding to rat striatal homogenates (Larenzo & Nahorski 1982) and this did not appear to be explicable solely in terms of the limitations of their experimental method. Characterization of any such ligand–macromolecule interaction by the use of only one concentration of macromolecule may well therefore prove inadequate.

L. S. C. was supported by a University of Liverpool research studentship and we are grateful to Professor A. M. Breckenridge for his interest and encouragement.

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J. Pharm. Pharmacol. 1984, 36: 779–781
Communicated April 12, 1984

Evidence for a pharmacokinetic interaction between ibuprofen and meptazinol in the mouse

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Of a variety of non-steroidal anti-inflammatory drugs administered concurrently with meptazinol (p.o.), only ibuprofen potentiated the antinociceptive response (mouse, hot-plate test) to the opioid. In addition, the brain tritium concentration of mice given [^{3}H]meptazinol (p.o. or i.v.) was significantly raised by the oral administration of ibuprofen. It is argued that the interaction between these drugs is pharmacokinetic in nature, due probably to an action of ibuprofen on the biotransformation of meptazinol.

Introduction

Some formulations containing centrally active opioid analgesics and non-steroidal anti-inflammatory agents have been prescribed for the control of mild-to-moderate pain. The theoretical advantages of this approach are that: (a) with idiopathic pain there is a greater likelihood of effective analgesia if two drugs are used which modify different parts of the mechanisms involved in pain induction and perception than if a single treatment is used, and (b) if the analgesic effects are additive or supra-additive, lower doses of the two components (hence less severe side effects) should be required to achieve a particular degree of analgesia than would be necessary if either drug were used alone. There is little published evidence to support these contentions but Woodbury & Fingl (1975) have reported that aspirin and opioid analgesics produce additive analgesic effects in man.

During routine pre-clinical investigations, results were obtained from a writhing procedure which suggested a supra-additive antinociceptive interaction in the mouse between the new opioid analgesic agent, meptazinol, and ibuprofen – but not other non-steroidal anti-inflammatory drugs. Both individual drugs were active in this test but subsequent studies, the results of which form the basis of the report, utilized a hot-plate procedure. As anti-inflammatory drugs do not affect responses to thermal noxious stimuli, it was considered that any potential effect of ibuprofen on meptazinol might be easier to demonstrate by this means.

Methods

Antinociception experiments. In all experiments, the reaction latencies of mice placed on a hot-plate (Woolfe & MacDonald 1944) maintained at 55 °C were measured immediately before dosing and at 30, 60 and 90 min thereafter. In pilot experiments various doses of meptazinol (p.o.) were used to estimate the dose required to induce a doubling of the control reaction latency and the time of peak antinociceptive activity. In the first interaction experiment, the following treatment groups (n = 10) were examined (mg kg^{-1}): meptazinol 40; meptazinol 80; meptazinol 40 in combination with ibuprofen 240; ibuprofen 240 and vehicle p.o. The same treatments were used in subsequent experiments except that ibuprofen was replaced by each of the anti-inflammatory agents at the doses shown in Table 1, in turn. In each experiment, mice from the five treatment groups were tested in a balanced order to militate against any temporal influence on the results. Also, the observer was unaware of the treatments each mouse had received.

The reaction latency of each mouse before treatment was subtracted from that obtained at each time after treatment. The mean changes in latency so obtained for each single drug treatment group were compared with the appropriate change of the vehicle control group using Student's *t*-test. The same test was used to compare the effects of meptazinol alone (40 mg kg^{-1}) with those obtained when meptazinol was given in combination with the anti-inflammatory agents.

Brain concentration of meptazinol. Groups of 50 mice were treated orally with 40 mg kg^{-1} [^{3}H]meptazinol (50 μCi/mouse) alone or in combination with 240 mg kg^{-1} ibuprofen. Thirty minutes later the animals were killed by cervical dislocation, their brains (minus cerebellum, pons and medulla) homogenized in 10 ml ice-cold Tris buffer and centrifuged at 20 000 rev min^{-1} for 5 min. Aliquots of the supernatant from each brain