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# DIFFERENT EFFICACIES OF *d*- AND *l*-γ-AMINO-β-HYDROXYBUTYRIC ACIDS IN GABA RECEPTOR AND TRANSPORT TEST SYSTEMS[1]

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## Abstract

The structure of the GABA receptor was investigated by determining the relative effects of two stereoisomers of the GABA agonist, γ-amino-β-hydroxybutyric acid (GABOB), in several quantitative receptor-related assay systems. (3*S*)-(+)-4-Amino-3-hydroxybutanoic acid (*d*-GABOB) was found to be about twice as potent as (3*R*)-(−)-4-amino-3-hydroxybutanoic acid (*l*-GABOB) in displacing [$^3$H]muscimol from specific binding sites in mouse brain membrane fractions. [$^3$H]Muscimol is thought to bind to the GABA recognition site of postsynaptic GABA receptor·anionophore complexes. A similar order of potency for the GABOB enantiomers was observed for the cerebrovascular GABA receptor in [$^3$H]muscimol binding assays using bovine cerebral blood vessels. In contrast to the binding results, *l*-GABOB was significantly more potent than *d*-GABOB in mimicking the postsynaptic action of GABA, which was measured as increases in membrane input conductance in the isolated crayfish stretch receptor neuron. Both GABOB enantiomers have some affinity for GABA transport processes, and *d*-GABOB was found to be more potent than *l*-GABOB in inhibiting GABA uptake into rat brain synaptosomes and $Na^+$-dependent GABA binding to mouse brain membranes. *l*-GABOB was more potent than *d*-GABOB when conductance measurements were made in the presence of $10^{-3}$ M nipecotic acid or L-α,β-diaminopropionic acid, two specific GABA transport blockers. The greater effectiveness of *l*-GABOB found in the crayfish assay is congruent with work by others that showed that *l*-GABOB, but not *d*-GABOB, is an effective inhibitor of induced seizure activity in cat brain and rabbit motor cortex. Thus, *d*-GABOB is more potent than *l*-GABOB in membrane binding and uptake systems, while the reverse is found in physiologically responding systems. These findings suggest that the structural asymmetry of the GABA recognition site *in vitro* is different from that related to activation of GABA receptors *in vivo*. A model is proposed that is consistent with the above observations.

One of the major current objectives of our work on the GABA system is to determine the quantitative relationships between *in vitro* ligand binding measurements and the physiological responsivity of excitable membranes to the ligands (Roberts et al., 1978a). We have studied a variety of chemical substances employing quantitative conductance measurements in the GABA-responding crayfish stretch receptor neuron (Hori et al., 1978); Swagel et al., 1973a, b; Krause et al., 1978) and have examined the effects of GABA and some of these substances on the relaxation of tension in isolated cerebral blood vessels (Edvinsson and Krause, 1979). The same compounds also were examined using adaptations of previously published procedures to measure GABA binding in brain membrane preparations to presumed GABA recognition sites of receptor·anionophore complexes (Enna and Snyder, 1975) and of the GABA transport system (Roberts et al., 1978b) and to GABA receptor sites in cerebral blood vessels (Krause et al., 1980). In general, we found a good quantitative correspondence between the physiological and biochemical measurements (Roberts et al., 1978a; Krause et al., 1980; D. N. Krause, K. Ikeda, and E. Roberts, manuscript in preparation).

In the course of the above studies, we investigated the relative effects of two stereoisomers of the GABA agonist γ-amino-β-hydroxybutyric acid (GABOB), (3*S*)-(+)-4-amino-3-hydroxybutanoic acid (*d*-GABOB) and (3*R*)-(−)-4-amino-3-hydroxybutanoic acid (*l*-GABOB). Much to our surprise, the results of the present investigation showed that *d*-GABOB was more potent than *l*-GABOB in *in vitro* binding and uptake systems, while the reverse was found to be true in our conductance studies with the crayfish stretch receptor neuron and in the physiological

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studies reported by others using invertebrate and vertebrate systems (Hayashi, 1959; Katayama, 1976; Takeuchi et al., 1977; Aishita et al., 1978; Katayama and Mori, 1977). This suggests the possibility that the structural asymmetry of GABA recognition sites *in vitro* may be different from that of such sites *in vivo*. A possible rationale for this will be presented in the discussion. A preliminary report of this work has been presented (Krause et al., 1979).

## Materials and Methods

*Materials.* [$^3$H]Muscimol (12 Ci/mmol) and [$^3$H]-GABA (34.5 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA) and [$^{14}$C]GABA (224 mCi/mmol) from Amersham/Searle (Arlington Heights, IL). GABA and Lubrol PX were obtained from Sigma Chemical Co. (St. Louis, MO), muscimol from Research Organics Inc. (Cleveland, OH), L-$\alpha,\beta$-diaminopropionic acid from Calbiochem (San Diego, CA), Triton X-100 from Packard Co. (Downers Grove, IL), and Tween 20 from J. T. Baker (Phillipsburg, NJ). Nipecotic acid was a gift of Dr. P. Krogsgaard-Larsen, Copenhagen. (3*R*)-(−)-4-Amino-3-hydroxybutanoic acid (*l*-GABOB, m.p. 224 to 225°C (decomp.), $[\alpha]_D^{23}$ −20.71° (c = 0.92, $H_2O$)) and (3*S*)-(+)-4-amino-3-hydroxybutanoic acid (*d*-GABOB, m.p. 224 to 225°C (decomp.), $[\alpha]_D^{23}$ +20.13° (c = 1.165, $H_2O$)) were kindly supplied by Dr. M. Kurono, Research Laboratory of Ono Pharmaceutical Co., Osaka. The same stock solutions of the latter two compounds were used for both the binding and the conductance assays.

*Membrane conductance measurements.* The methods used for the electrophysiological experiments were similar to those described previously (Hori et al., 1978; Swagel et al., 1973a, b). The slowly adapting stretch receptor neuron with innervating nerves and attached receptor muscle was dissected from the second or third abdominal tergum of the crayfish, *Procambarus clarkii*, mounted in a chamber, and constantly perfused with Van Harreveld's solution (Van Harreveld, 1936) maintained at 18 ± 1°C. The neuron was impaled with two glass pipette microelectrodes filled with 4 mM $K^+$ acetate. A hyperpolarizing current pulse of 200 msec duration was applied through one electrode, while the resultant potential across the membrane was measured by the other electrode. Membrane input conductance was calculated as the ratio of applied current to potential. In each instance, control conductance measurements in Van Harreveld's solution were made before and after perfusion of Van Harreveld's solution containing a given concentration of test substance, and the results were averaged. This value was subtracted from the maximum conductance observed during application of the test solution to obtain the value for the conductance change produced by the test substance.

*Preparation of membrane fractions.* $P_2$ and $P_3$ membrane fractions were prepared from whole brains of Swiss mice. The tissue was homogenized in 10 vol of ice cold 0.25 M sucrose and centrifuged at 1050 × *g* for 10 min. The resulting supernatant was centrifuged at 17,000 × *g* for 15 min to obtain the $P_2$ pellet. For some studies, the final supernatant was centrifuged at 70,000 × *g* for 1 hr to pellet the $P_3$ fraction.

The $P_2$ pellet was subjected further to osmotic shock and then twice to a freeze-thaw-wash procedure as described previously (Wang et al., 1979). The $P_2$ fraction was stored frozen in double-distilled water (10 to 11 mg of protein/ml) until the time of assay when it was thawed, diluted in 7 vol of distilled water, and centrifuged at 70,000 × *g* for 20 min. The final $P_2$ pellet was resuspended in distilled water and assayed immediately.

The $P_3$ pellet was also washed, but not frozen, by suspension in 50 mM Tris-HCl (pH 7.3). After 10 min on ice, the suspension was centrifuged at 70,000 × *g* for 10 min. This procedure was repeated two more times using distilled water for the resuspensions. The final $P_3$ pellet was resuspended in 0.32 M sucrose, 0.01 M dithiothreitol and assayed for binding.

A crude membrane fraction from bovine cerebral blood vessels was obtained (Krause et al., 1978). The blood vessels were dissected with forceps from pia-arachnoid membranes and homogenized in 10 vol of 0.25 M sucrose using a Brinkmann Polytron. The homogenate was centrifuged at 900 × *g* for 10 min, and the resulting supernatant was centrifuged at 39,000 × *g* for 10 min to pellet the membrane fraction. The pellet was washed three times by resuspension in ice cold distilled water and centrifugation at 39,000 × *g* for 20 min. The final pellet was resuspended in distilled water (2 to 3 mg of protein/ml) and stored frozen until the time of assay.

Crude synaptosomal fractions were prepared from cerebral cortical tissue of Sprague-Dawley rats. The tissue was homogenized in 10 vol of 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 12,000 × *g* for 20 min, and the resulting pellet was washed with the sucrose solution and recentrifuged. The final crude synaptosomal pellet was resuspended in the sucrose solution at 10% (w/v) based on original cortex weight for use in the uptake experiments.

*Binding assays.* [$^3$H]Muscimol binding assays (Wang et al., 1979) were usually performed in duplicate. Typically 0.4 to 0.6 mg of protein of either frozen-thawed mouse brain $P_2$ or bovine cerebral vessel membranes were incubated in 1 ml of 0.05 M Tris-HCl (pH 7.3) containing 8.4 nM (brain $P_2$) or 20 nM (cerebral vessels) [$^3$H]muscimol for 30 min at 4°C with no addition or containing $10^{-4}$ M unlabeled muscimol or various concentrations of *d*- or *l*-GABOB. Following incubation, the mixtures were centrifuged at 28,000 × *g* for 15 min. The pellets were rinsed twice superficially with 3 ml of distilled water, and then were solubilized in 0.5 ml of Soluene at 60°C prior to liquid scintillation counting.

Assays for sodium-dependent [$^3$H]GABA binding were carried out in duplicate by incubating aliquots of the $P_3$ suspension (0.2 to 0.4 mg of protein) in 50 mM Tris-HCl (pH 7.3) containing 70 mM NaCl and 19 nM [$^3$H]GABA with and without additions of $10^{-2}$ M unlabeled GABA or various concentrations of *d*- or *l*-GABOB. After incubation for 1 min at 4°C, the experiments were terminated as described previously (Roberts et al., 1978b) by rapid filtration through 0.65-$\mu$m pore size Millipore filters which then were washed twice with Tris buffer and counted in a liquid scintillation counter.

The radioactivity bound in the presence of excess unlabeled muscimol or GABA was subtracted from all

results. Such nonspecific binding represented about 2% of the total $Na^+$-dependent [$^3$H]GABA binding and about 10% (brain $P_2$) and 70% (cerebral vessels) of the total [$^3$H]muscimol binding. Binding in the presence of *d*- or *l*-GABOB was expressed as the percentage of the control specific binding determined in the absence of these compounds.

*Synaptosomal uptake assays.* Assays were performed in triplicate employing published procedures (Nelson-Krause and Howard, 1978) with slight modification. A 0.2-ml sample of the synaptosomal fraction (0.6 to 0.8 mg of protein) was added to 1.8 ml of Krebs medium, pH 7.4, which in some cases contained *d*- or *l*-GABOB. The suspensions were preincubated for 15 min at 37°C on a metabolic shaker, and the [$^{14}$C]GABA was added to a final concentration of 0.045 μM. Following an additional incubation for 5 min, uptake was terminated by the addition of 5 ml of Krebs medium and rapid filtration on 0.8-μm pore size Millipore filters. The filters were washed with an additional 15 ml of medium and counted by liquid scintillation spectrometry. Background counts (about 1% of the total uptake) were determined by mixing tissue, buffer, and [$^{14}$C]GABA on ice and filtering immediately, and these counts were routinely subtracted from the uptake values.

## Results

*Membrane conductance changes.* Noncumulative log concentration versus conductance change plots were obtained for GABA, *l*-GABOB, and *d*-GABOB in the crayfish slowly adapting stretch receptor neuron preparation (Fig. 1). Both GABOB enantiomers acted like GABA to produce an increase in membrane input conductance in a dose-dependent manner with no apparent receptor desensitization. Both enantiomers appeared capable of producing the same maximal response as GABA. *l*-GABOB was found to be significantly more potent than *d*-GABOB in increasing input membrane conductance. The concentration of *d*-GABOB required to produce a half-maximal response ($5.5 \times 10^{-4}$ M) was more than twice that required for *l*-GABOB ($2.3 \times 10^{-4}$ M). As expected, a racemic mixture of 50% *l*-GABOB and 50% *d*-GABOB produced a conductance response midway between those observed for the pure isomers alone (data not shown). A Hill plot analysis of the data gave a slope ($n_H$) of 2.3 ± 0.2 for *l*-GABOB and 2.1 ± 0.1 for *d*-GABOB, which indicates some degree of cooperativity in the process by which the GABOB enantiomers produce a membrane conductance increase.[2]

*Receptor binding assays.* In contrast to the results in the GABA receptor conductance assay, *d*-GABOB was found to be about twice as potent as *l*-GABOB in displacing [$^3$H]muscimol from specific binding sites in mouse brain membrane fractions (Fig. 2*A*). The greater potency of *d*-GABOB was found consistently in seven independent experiments, and the difference between the

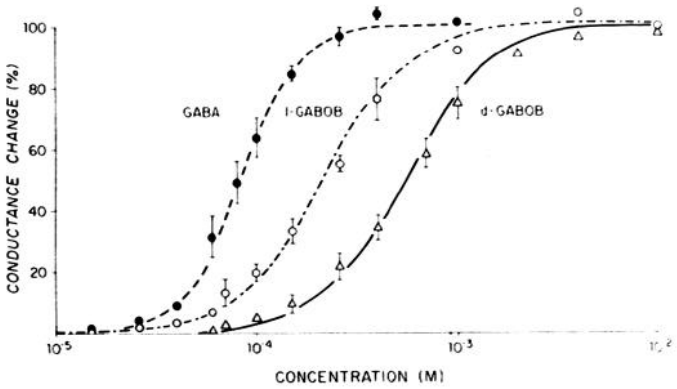


*Figure 1.* Concentration-conductance change relationships in the postsynaptic membrane of the crayfish stretch receptor neuron by GABA (●), *l*-GABOB (○), and *d*-GABOB (△). Conductance change is expressed as the percentage of the maximum change produced by GABA in the same neuron. *Points* represent the mean ± SEM for five separate experiments. Curves were drawn according to the equation

$$y = \frac{y_{max}}{1 + K_a/A^n}$$

where $n = 2$ for *d*- and *l*-GABOB and $n = 3$ for GABA.

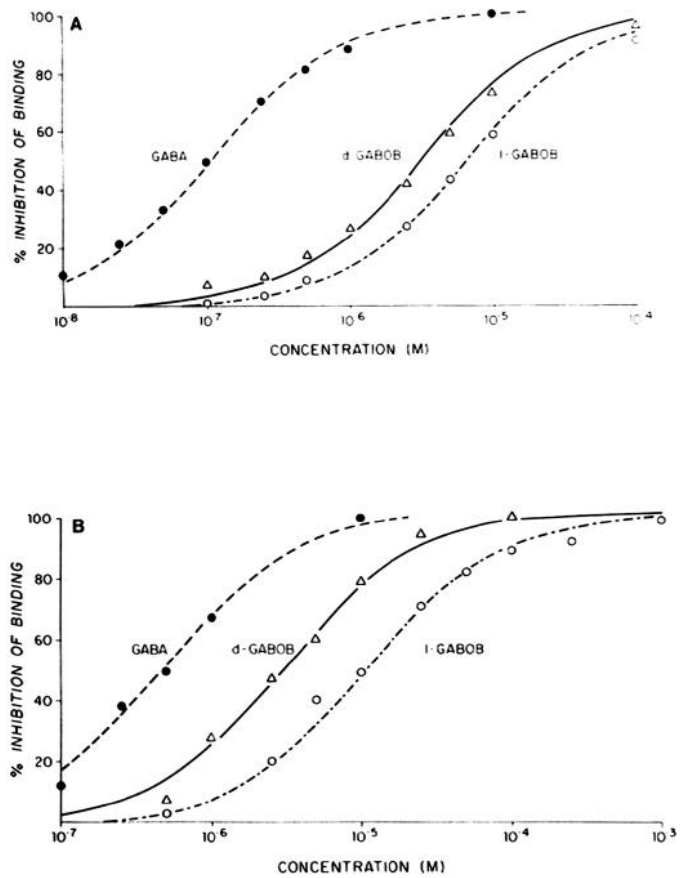


*Figure 2.* Inhibition of specific [$^3$H]muscimol binding by various concentrations of GABA and *d*- and *l*-GABOB was determined using the standard assay procedure. *A*, Mouse brain $P_2$ membrane fractions. *B*, Bovine cerebral blood vessel membranes. Curves were theoretical (see legend to Fig. 1) with $n = 1$ in all cases.

[2] While $n_H \simeq 3$ for GABA in this study, we have found that when GABA uptake processes in the preparation are inhibited, $n_H$ is about 2 for GABA (Krause et al., 1978; D. N. Krause, K. Ikeda, and E. Roberts, manuscript in preparation; see also Hori et al., 1978; Adams et al., 1980).

$IC_{50}$ values for the two enantiomers was highly significant (Table I). In four experiments, a 50:50 mixture of *d*- and *l*-GABOB was also tested, and in all cases, this curve fell between those for the two enantiomers alone. No apparent cooperativity was indicated by a Hill plot analysis of the binding curves for either *d*-GABOB ($n_H = 0.86$) or *l*-GABOB ($n_H = 1.03$).

The relative potencies of the GABOB enantiomers also were assessed for the cerebrovascular GABA receptor recently characterized in our laboratory with [$^3$H]muscimol binding assays using bovine cerebral blood vessels (Krause et al., 1978). As shown in Figure 2*B*, the results were similar to those obtained in the brain receptor binding assays. *d*-GABOB was consistently more potent than *l*-GABOB in five separate experiments (Table I).

Several variations of the standard binding assay condition also were tested (Table I). The relatively high concentration of NaCl (207.5 mM) present in the crayfish physiological experiments has been reported to reduce the potency of the GABA agonist, 3-aminopropanesulfonic acid, in GABA receptor-related binding assays (Enna and Snyder, 1977). However, we found no effect of 200 mM NaCl on the potencies of the GABOB enantiomers in the [$^3$H]muscimol binding assay (Table I). Detergent pretreatment of brain membranes also has been reported to alter the potencies of some GABA analogs for the [$^3$H]muscimol binding site (Wang et al., 1979). While detergent treatment decreased the $IC_{50}$ values for both *d*- and *l*-GABOB, the relative potencies of the two enantiomers were unchanged (Table I). A 2-fold greater potency of *d*-GABOB also was seen when binding was assayed at room temperature or when [$^3$H]GABA was used as ligand under $Na^+$-free conditions (Enna and Snyder, 1975). During the course of our work, a brief report appeared which confirms the greater potency of *d*-GABOB compared with *l*-GABOB in inhibiting $Na^+$-independent [$^3$H]GABA binding to rat brain membranes (Galli et al., 1979).

The results in Figure 3 show that both isomers of GABOB are competitive inhibitors of the specific binding of [$^3$H]muscimol to the $P_2$ fraction. Thus, the isomers of GABOB appear to occupy the same receptor sites as GABA.

*GABA transport recognition site.* Both GABOB enantiomers were found to have some affinity for GABA transport processes. *d*-GABOB was more potent than *l*-GABOB as an inhibitor of [$^3$H]GABA accumulation by rat cerebral cortex synaptosomes (Fig. 4*B*). The same relative potencies were found when the enantiomers were tested on $Na^+$-dependent, [$^3$H]GABA binding to $P_3$ membrane fractions from mouse brain (Fig. 4*A*). This latter assay is thought to reflect binding to the GABA recognition site associated with GABA transport (Enna and Snyder, 1975; Roberts et al., 1978a, b).

GABA uptake processes also are present in the crayfish preparation (Kuffler and Edwards, 1958; Sisken and Roberts, 1964; Roberts et al., 1978a; Krause et al., 1978; Adams et al., 1980). Therefore, conductance measurements were made in the presence of nipecotic acid or L-$\alpha,\beta$-diaminopropionic acid (L-DAPA), two specific GABA uptake blockers which, by themselves, do not affect membrane conductance at $10^{-3}$ M (Krause et al., 1978). GABA uptake processes in the preparation were found to have some influence on the measured $EC_{50}$ values for the GABOB isomers. For example, in the experiment illustrated in Figure 5, the effect of *l*-GABOB was significantly potentiated in the presence of L-DAPA. Nipe-

TABLE I

*Inhibition of GABA receptor binding by d- and l-GABOB*

Mouse brain $P_2$ or bovine cerebral blood vessel membranes (lysed, well washed, and frozen and thawed) were incubated for 30 min with 8.4 nM (brain $P_2$) or 20 nM (cerebral vessels) [$^3$H]muscimol or 19 nM [$^3$H]GABA in 50 mM Tris-HCl (pH 7.3) at 0°C except where modifications are indicated. Various concentrations of *d* or *l*-GABOB were included in the assays which were run in duplicate and terminated by centrifugation. Where applicable, the mean ± SEM are given for the number of separate experiments indicated in parentheses.

| GABA Receptor Binding Assay | $IC_{50}$ | |
|---|---|---|
| | *d*-GABOB | *l*-GABOB |
| | | $\mu M$ |
| I. [$^3$H]Muscimol binding | | |
| Mouse brain $P_2$ | 2.8 ± 0.6 | 6.0 ± 1.3[a] (7) |
| 1. +200 mM NaCl | 2.8 | 6.4 |
| 2. Room temperature | 3.7 | 7.0 |
| 3. 37°C pretreatment[b] | 1.4 | 3.0 |
| 4. 0.1% Triton X-100[b] | 0.8 | 1.9 |
| 5. 0.1% Lubrol PX[b] | 0.9 | 1.8 |
| 6. 25% Tween 20[b] | 1.0 | 2.0 |
| Bovine cerebral blood vessels | 4.4 ± 1.3 | 9.6 ± 2.2[c] (5) |
| II. $Na^+$-independent [$^3$H]GABA binding | | |
| Mouse brain $P_2$ | 1.3 ± 0.1 | 2.4 ± 0.3[c] (2) |

[a] Significantly different from *d*-GABOB with $p < 0.001$.

[b] Membranes were pretreated at 37°C for 30 min with or without detergent and then pelleted (20,000 × *g* for 10 min). The pellet was resuspended in $H_2O$ and assayed as usual at 0°C.

[c] Significantly different from *d*-GABOB with $p < 0.05$.

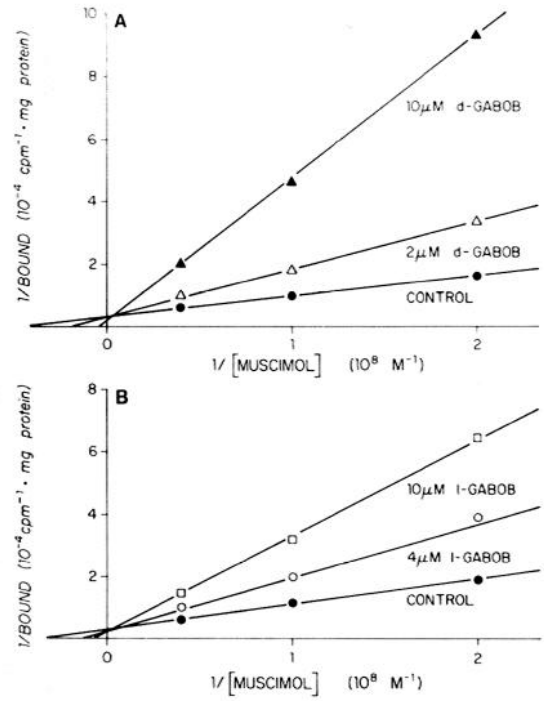


*Figure 3.* Double reciprocal plots of concentration-binding relationships in mouse brain $P_2$ membranes for muscimol alone (●) and in the presence of (*A*) 2 μM (△) or 10 μM (▲) *d*-GABOB and (*B*) 4 μM (○) or 10 μM (□) *l*-GABOB.

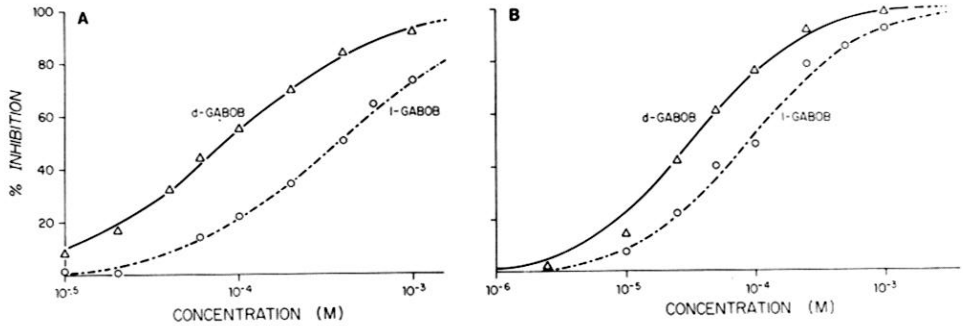


*Figure 4.* Inhibition by *d*- and *l*-GABOB of (*A*) $Na^+$-dependent [$^3$H]GABA binding to mouse brain $P_3$ membrane fractions at 0°C and of (*B*) [$^3$H]GABA uptake by rat brain synaptosomes at 37°C. Details of both assays are given in the text. Curves were theoretical (see legend to Fig. 1) with $n = 1$.

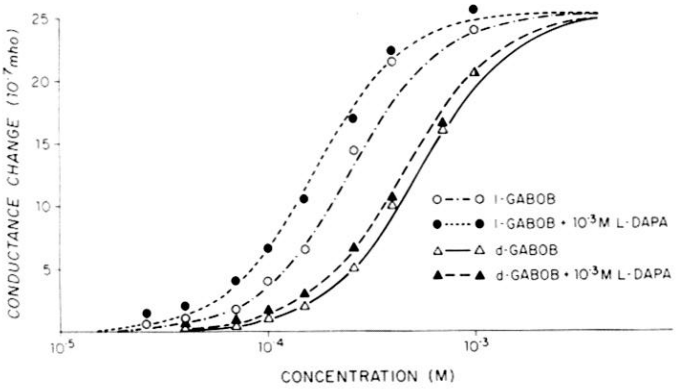


*Figure 5.* Concentration-conductance curves obtained from the same crayfish stretch receptor neuron showing the conductance changes produced by *d*- and *l*-GABOB alone and in the presence of L-DAPA, a specific GABA uptake blocker which by itself has no effect on input conductance at $10^{-3}$ M.

cotic acid also was found to have a small potentiating effect on both *l*- and *d*-GABOB (data not shown). The rank order of potency for the enantiomers (*l*-GABOB > *d*-GABOB) was unchanged by either L-DAPA or nipecotic acid.

## Discussion

Identical groups in symmetrical compounds may be rendered chemically nonequivalent by combination with asymmetric surfaces, such as may exist on enzymes or membrane receptors. Thus, GABA would exhibit prochirality if it were to combine with asymmetric membrane receptors or transport recognition sites. Optical isomers of several GABA analogs have shown stereoselectivity in interacting with GABA receptor and transport sites (Krogsgaard-Larsen et al., 1978; Schousboe et al., 1979). In the present study, we have used the enantiomers of β-hydroxy GABA (GABOB) to probe a possible asymmetry of GABA receptors in the physiologically responsive crayfish stretch receptor neuron (SRN) and of the GABA recognition sites of receptor-related complexes in membrane preparations from mouse brain and bovine pial vessels. In addition, the GABA binding sites of GABA transport systems of rat cortical synaptosomes and highly comminuted $P_3$ membrane fractions from mouse brain were studied.

In each instance, it appeared that both *d*- and *l*-GABOB combine with the same sites as GABA. In the case of the SRN, both isomers of GABOB produced the same maximal changes in conductance as GABA or muscimol (Krause et al., 1978). The *l* isomer consistently was approximately twice as potent as the *d* isomer in producing increases in input membrane conductance, an observation consistent with the finding that *l*-GABOB was the more effective isomer in inhibiting the electrical activity of a tonically active giant neuron in the subesophogeal ganglion of the African giant snail (Katayama, 1976; Takeuchi et al., 1977). A similar order of potency for the GABOB isomers also has been reported for several vertebrate species. Following intracisternal or intraventricular administration, the *l* isomer was found to have a much stronger inhibitory action in preventing generalized seizures induced electrically (Hayashi, 1959; Aishita et al., 1978) or chemically (Katayama and Mori, 1977) in dog, rabbit, and cat brain, respectively. *l*-GABOB also was more effective than *d*-GABOB in cat cerebral cortex when applied topically to epileptic foci induced by potassium benzyl penicillin (Katayama, 1976). *l*-GABOB but not *d*-GABOB, injected intraventricularly, mimicked the ability of GABA to stimulate growth hormone release in the rat (Abe et al., 1977).

It was surprising, therefore, when we observed that *d*-GABOB was considerably more effective than *l*-GABOB in competing with GABA or muscimol for the $Na^+$-independent receptor-related binding sites of mouse brain membranes. These *in vitro* binding sites are thought to reflect GABA receptor sites *in vivo* (Enna and Snyder, 1975; DeFeudis, 1980; Wang et al., 1979), and until now, we have found a very good correlation between our crayfish physiological data and mouse brain binding data for a series of GABA analogs (Krause et al., 1978; D. N. Krause, K. Ikeda, and E. Roberts, manuscript in preparation). The crayfish stretch receptor neuron, in particular, has been considered to be a good quantitative model for studying mammalian cortical GABA receptors (Nistri and Constanti, 1979).

Another puzzling feature of our results was that Hill plots of the effects on the SRN of *d*- and *l*-GABOB gave slopes of approximately 2, consistent with the cooperativity seen for GABA responses in a variety of preparations (see Nistri and Constanti, 1979). However, our binding measurements with these compounds and pre-

vious work on [$^3$H]muscimol (Wang et al., 1979) and [$^3$H]GABA (Enna and Snyder, 1975) binding showed invariably that with *in vitro* membrane preparations, Hill slopes of 1 were obtained. In addition, the $IC_{50}$ values found in physiological measurements of the SRN were approximately two orders of magnitude greater than those found in the *in vitro* binding measurements. For example, the $IC_{50}$ values for *d*- and *l*-GABOB, respectively, were 550 and 230 μM in the SRN test system but were 2.8 and 6.0 μM in the membrane binding assay employed. Low concentrations of the racemate, *d,l*-GABOB, also inhibited [$^3$H]muscimol binding to crayfish muscle membrane preparations ($IC_{50}$ = 0.6 μM) (Meiners et al., 1979). However, no distinction between the potencies of the pure *d* and *l* isomers could be made in the latter assay, perhaps due to the large (50%) error involved in the measurements (R. Olsen, personal communication).

Such discrepancies in affinity and degree of cooperativity between physiological and binding measurements are common for various receptor types, and many possible explanations have been discussed (see Changeux et al., 1976; Colquhoun, 1978; Nistri and Constanti, 1979). One likely possibility is that the manipulations involved in making and assaying the membrane preparations alter the properties of the receptor. Under binding assay conditions, acetylcholine receptors have been found to shift to a desensitized state (Changeux et al., 1976). Cooperative interactions may be disrupted at the relevant GABA recognition sites in the membrane fragments so that individual GABA molecules are bound independently of each other. A plausible model that can accommodate the above results is presented below.

It has been suggested that GABA acts on its receptors in the extended form (Roberts et al., 1978a; see Defeudis, 1977, for review). We have proposed that *face 1* of the GABA molecule, as shown in Figure 6, may resemble the surface of GABA that would attach to its receptor, while *face 2* of the GABA model may resemble the one that attaches to the binding site(s) involved in the transport of GABA (Roberts et al., 1978a). In keeping with considerable data on the apparent cooperativity involved in GABA responses (Nistri and Constanti, 1979), let us further assume that two GABA molecules fit the GABA recognition site in the extended close-packed, head-to-toe fashion shown in Figure 7 to cooperatively activate a unit conductance change. Manipulation of CPK space-filling molecular models shows that such tightly packed dimeric arrangements are feasible. The charge neutralization that would occur as a result of the interaction of juxtaposed oppositely charged amino and carboxyl groups would be expected to greatly decrease the degrees of hydration of the polar ends of the GABA molecules, and both coulombic and van der Waal's forces would tend to hold two molecules together in the proposed configurations. It would be expected that, because of increased nonpolar properties of the dimers relative to the monomers, the water solubility of such dimers would be decreased and lipid solubility increased, favoring their existence in lipidic membrane environments. Self-association of pyridine (Huyskens et al., 1977) and nicotinamide and isonicotinic hydrazide (Kopecký et al., 1978) has been demonstrated in aqueous solutions. We are

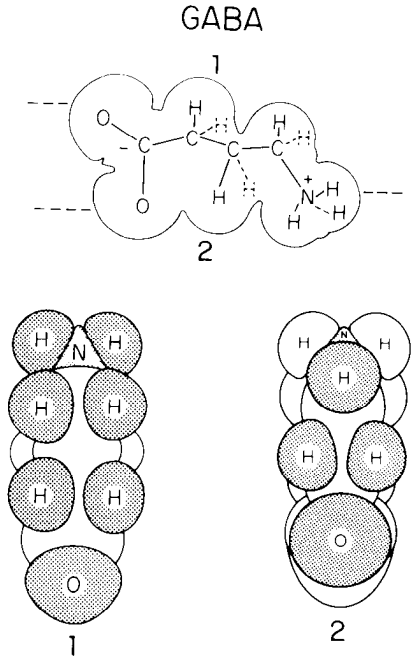


*Figure 6.* The faces of GABA as seen on projections of CPK space-filling models.

planning direct physical experiments to determine whether GABA molecules can form dimers or higher associates at concentrations expected to be present at GABAergic synaptic junctions.

We now postulate that there are two equivalent, nonidentical membrane sites into which pairs of GABA molecules could fit (Fig. 7, *A* and *B*). These are presumed to be mirror images of each other. While these binding sites would be chirally blind insofar as GABA molecules are concerned, they would have markedly different affinities for asymmetrically β-substituted derivatives of GABA, such as *l*- or *d*-GABOB. As illustrated in Figure 7, *A* and *B*, the active sites could accommodate external OH groups of the GABOB molecules, but the GABOB molecules would not pack tightly enough to fit the proposed receptor binding sites if the OH groups were in the internal positions. Thus, *l*-GABOB would not fit the site of Figure 7*A* and *d*-GABOB would not fit that of Figure 7*B*. The extra bulk and the hydrophilic character of the OH group should greatly decrease the affinity of the GABOB isomers relative to GABA. In this connection, it is of interest that no effects were observed by us with millimolar concentrations of (±)-β-(*p*-chlorophenyl)-GABA in either the SRN system or in the GABA receptor binding assays employed (Krause et al., 1978; Wang et al., 1979). If it is assumed that the affinities of the *A* site for *d*-GABOB and the *B* site for *l*-GABOB are similar, the 2-fold difference in $IC_{50}$ values found in the SRN system would suggest that there may be approximately twice as many accessible *B* sites as *A* sites.

The destruction of cooperativity, possibly associated with desensitization of receptors, is modeled in Figure 7, *C* to *F*. The manipulations required to perform *in vitro* binding measurements with the preparations employed to date result in the functional (but not necessarily physical) dissociation of the subunits of the binding sites so that individual molecules of ligand can bind to them

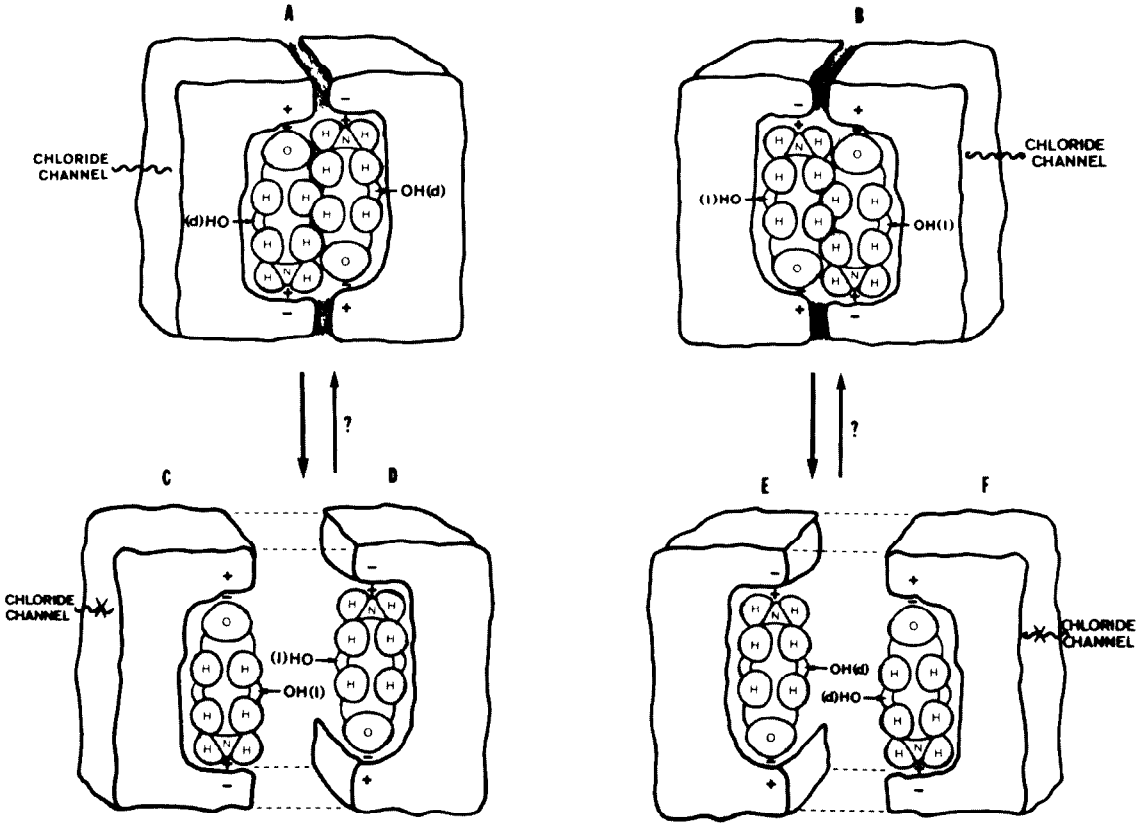


*Figure 7.* A model of the GABA recognition sites of the GABA receptor system. It is proposed that there are two chirally different sites, *A* and *B*, that represent low affinity states that are coupled with membrane anion gating mechanisms. Both *A* and *B* accommodate tightly packed head-to-toe dimers of GABA with approximately equal affinity, but *d*-GABOB attaches preferentially to *A* and *l*-GABOB to *B* for reasons stated in the text. It is presumed that procedures for preparing brain membranes lead to a conversion to high affinity sites that are uncoupled from anion channels (*sites C* to *F*). The possible structural reason for the increases of affinities of the latter sites for GABA and *d*- and *l*-GABOB and for the reversal of affinities for the GABOB enantiomers from those found for *sites A* and *B* are discussed in the text. It is possible that the transformations suggested may take place in a reversible fashion when desensitization in responsive membranes occurs during continued action of agonist (see Katz and Thesleff, 1957, for relevant model for desensitization of the nicotinic cholinergic receptor). The figure is drawn so that the reader views it from behind the presumed GABA recognition site.

independently of each other. The probability of attachment of single zwitterionic GABA molecules in a given orientation to a doubly charged binding site would, in all likelihood, be greater than would that of two GABA molecules to a binding site with four charges. In the case of the GABOB isomers, it is shown that preferential binding would take place with the OH groups facing away from the structure of the binding site and toward the aqueous environment; that is, *d*-GABOB would bind to subunits of the *B* site and *l*-GABOB to the *A* sites, resulting in approximately twice as much binding being observed for the isomer that originally was only one-half as active as the other one in producing conductance changes in physiologically responsive membranes. We feel that the model proposed is the most consistent with our observations, but other explanations also might account for the data.

Synaptosome and $P_3$ membrane preparations were used to study *in vitro* the GABA recognition sites related to GABA transport. In both assay systems, *d*-GABOB was the more potent enantiomer, and dose-response curves were consistent with a Hill slope of 1. In the SRN system, GABA uptake processes have been shown to influence the dose-response curves of those GABA agonists which also have affinity for the transporter (Roberts et al., 1978a; Krause et al., 1978; Adams et al., 1980). In the current study, the GABA uptake blocker, L-DAPA, appeared to have more effect on the dose-response curve of *l*-GABOB than of *d*-GABOB (Fig. 5). However, clear interpretations of this data are not possible, since higher concentrations of *d*-GABOB were needed to produce comparable conductance changes. In studies with intact mice, it was found that label from intraperitoneally administered *l*-[$^{14}$C]GABOB appeared more rapidly and extensively in blood and tissues than the label from *d*-[$^{14}$C]GABOB. The *l* isomer also penetrated into brain to some extent, while hardly any label from *d*-GABOB was detected (Katayama, 1976). The latter results suggest that uptake systems for *l*-GABOB may be more prevalent or more active than for *d*-GABOB in various membranes in the intact organism. The limitations of our current knowledge of the specific GABA transport system(s) in brain have been attributable to our inability to perform accurate relevant quantitative measurements in physio-

logically intact preparations. Even though the *d*-GABOB-preferring isolated synaptosome preparation showed GABA binding and transport, it cannot be inferred that this preparation more truly reflects a situation that exists *in vivo* than does the less intact $P_3$ membrane fraction. Membrane vesicles (Kanner, 1978a) and even liposomes into which have been incorporated detergent-solubilized GABA transporter (Kanner, 1978b) were able to catalyze GABA transport with similar characteristics.

We have previously advanced the following hypothesis (Roberts et al., 1978a; Roberts, 1979, 1980): When GABA molecules attach to the receptor sites in the extended form, the other sides of the GABA molecules (*face 2*, Fig. 6) already are in the configuration that is recognized by the transport binding sites. If receptor and transport sites are in close juxtaposition in the region of the receptor·anionophore complex, the immediate removal of GABA from receptor sites would be facilitated by the transport system. If the intact $Na^+$-dependent GABA uptake sites (not shown) act in a coordinated fashion with the receptor complex, as suggested above, they would attach to the surface of the GABA dimer that faces the extracellular environment, as depicted in Figure 8 for the *d*-GABOB-preferring site. This predicts that in the *in vivo* situation, there would be two asymmetric GABA recognition sites associated with the GABA transport system in a manner analogous to that proposed for the receptor sites, and their properties might be altered *in vitro* in a fashion similar to that postulated for the GABA recognition sites. If the hypothesis set forth above is correct, measurements in suitably intact preparations with the GABA transport system should show Hill slopes of 2 and preference for the *l* isomer.

Our own studies and those of others have been handicapped by the absence of single preparations on which all desired parameters can be measured. Thus, ideally, studies of changes in conductance produced by GABA, receptor-related $Na^+$-independent binding to isolated membranes, $Na^+$-dependent GABA uptake, and transport-related $Na^+$-dependent membrane binding all should be carried out on one cell type. This goal may now be approachable with the use of genetically stable, cloned neural cultured cell lines that have become available. We are beginning such experiments in order to probe further the questions raised in the present study, such as whether or not the change in preference of the GABA receptor from *l*- to *d*-GABOB is a reversible phenomenon *in vivo* and whether it is related to the desensitization phenomenon observed at high GABA concentrations in physiologically responsive systems.

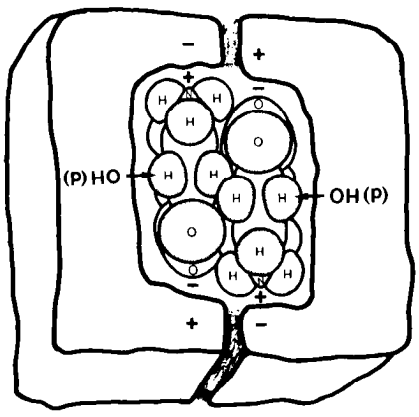


*Figure 8.* A view of the back side of *site A* in Figure 7. It is presumed that this is the molecular configuration that would be recognized by the GABA transport system when removing GABA from the low affinity, channel-coupled receptor site (see "Discussion").

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