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CAGE CONVULSANTS AND ANTICONVULSANTS

ANNUAL REPORT

EUGENE M. BARNES, JR., PH.D.

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The interactions of organophosphorus compounds with γ-aminobutyric acid (GABA)-ergic neurons have been studied by the specific binding of the cage convulsant, t-butylbicyclophosphorothionate (TBPS). Scatchard analysis of [^{35}S]TBPS binding to cortical membranes from adult rat and chicken brain yielded

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two binding constants (Kd = 1 nM and 200 nM). The anticonvulsants etomidate and etazolate were potent displacers of TBPS ~~(K_i = 1-3 μM).~~ Secobarbital was moderately effective, while phenobarbital and diazepam were poor displacers. Organophosphorus insecticides, such as O-ethyl-O-p-nitrophenyl phenylthiophosphonate (EPN), paraoxon, and Dyfonate, were moderately effective displacers of TBPS binding (K_i = 100-200 μM).

The interaction of organophosphorus cage convulsants with GABA-gated chloride channels was studied by $^{36}Cl^-$ flux measurements in neurons cultured from the chick embryo cerebrum. TBPS proved to be a potent inhibitor of GABA-dependent Cl^- uptake (IC_{50} = 0.30 μM). Scatchard analysis of [^{35}S]TBPS to binding to neurons gave K_d values of 3.1 nM and 270 nM. The TBPS binding constant for this lower affinity site agrees well with the IC_{50} value for TBPS inhibition of Cl^- flux, suggesting that this site is physiologically relevant to GABA antagonism.

These results indicate that TBPS acts as a GABA antagonist via direct blockade of neuronal Cl^- channels. Thus the convulsive effects of cage compounds and perhaps other organophosphorus compounds may be exerted by interference with GABAergic rather than cholinergic neurotransmission.

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SUMMARY

The interactions of organophosphorus compounds with γ-aminobutyric acid (GABA)-ergic neurons have been studied by the specific binding of the cage convulsant, t-butylbicyclophosphorothionate (TBPS). Scatchard analysis of [^{35}S]TBPS binding to cortical membranes from adult rat and chicken brain yielded curvilinear plots. Nonlinear regression methods indicated binding to two sites that differ in affinity. For rat cerebrum, $K_{d(1)}$ = 1.2 nM, $K_{d(2)}$ = 230 nM; and for chicken cerebrum, $K_{d(1)}$ = 1.4 nM, $K_{d(2)}$ = 170 nM. This multiplicity of [^{35}S] TBPS binding was further confirmed when unlabeled TBPS or picrotoxinin displaced radioligand. The IC_{50} values obtained from the displacement studies were similar to the K_d values obtained by Scatchard analysis. Of the anticonvulsants tested, etomidate, cartazolate, and etazolate were the most potent displacers of TBPS with K_i values in the 1-3 μM range for both rat and chicken membranes. Secobarbital and CL 218,872 were moderately effective, while phenobarbital, clonazepam, and diazepam were poor displacers. Organophosphorus insecticides, such as O-ethyl-O-p-nitrophenyl phenylthiophosphonate (EPN), paraoxon, and Dyfonate were moderately effective displacers of TBPS binding (K_i = 100-200 μM). All compounds were nearly equipotent with rat or chicken brain membranes.

The interaction of organophosphorus cage convulsants was also examined by studies of GABA-gated chloride channels using $^{36}Cl^-$ flux measurements in neurons cultured from the chick embryo cerebrum. TBPS proved to be a potent inhibitor of GABA-dependent Cl^- uptake (IC_{50} = 0.30 μM). Analysis of the kinetics of this process revealed that TBPS is a noncompetitive inhibitor (K_i = 0.15 μM) with respect to GABA. Scatchard analysis of direct binding of [^{35}S]TBPS to membranes isolated from neuronal cultures also gave curvilinear plots. These could be resolved into two components with K_d values of 3.1 nM and 270 nM. The TBPS binding constant for this lower affinity site agrees well with the IC_{50} and K_i values for TBPS inhibition of Cl^- flux, suggesting that this site is physiologically relevant to GABA antagonism. GABA was a noncompetitive displacer of TBPS binding from its low affinity site. The IC_{50} value for this displacement by GABA (0.8 μM) was comparable to the $K_{0.5}$ value for GABA enhancement of Cl^- flux. The addition of 150 mM Cl^- produced a 9-fold increase in TBPS binding to its low affinity site on neuronal membranes. The EC_{50} value for Cl^- enhancement of TBPS binding (160 mM) agreed well with the K_m for Cl^- influx via GABA-gated channels (140 mM).

These results indicate that TBPS acts as a GABA antagonist via direct blockade of neuronal Cl^- channels. Thus the convulsive effects of cage compounds and perhaps other organophosphorus compounds may be exerted by interference with GABAergic rather than cholinergic neurotransmission.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. Statement of the Problem

Although a prominent effect of organophosphorus intoxication is the induction of convulsions, the target of this action in the body is not understood. Yet anticonvulsant antidotes such as benzodiazepines and barbiturates act primarily by potentiation of γ-aminobutyric acid (GABA)-ergic transmission. It is also apparent that cage convulsants, a family of bicycloorganophosphorus compounds, are potent GABA antagonists but lack anticholinesterase activity. This has led to the hypothesis that certain organophosphorus compounds interact with GABA-gated chloride (Cl^-) channels in the vertebrate cerebral cortex. The problem under study is to test this hypothesis.

II. Background

GABA receptors. GABA has been identified as the major inhibitory neurotransmitter in the vertebrate brain (1). At the postsynaptic membrane, the inhibitory effect of GABA is manifested by the opening of Cl^- channels (2,3). This GABA response is enhanced by anticonvulsant drugs, such as benzodiazepines (4) and barbiturates (5), and blocked by convulsant agents such as picrotoxin and bicuculline (6). These modulators of Cl^- conductance interact with at least three pharmacologically distinct membrane components: GABA recognition sites, benzodiazepine receptor sites, and picrotoxin sites which are arranged together in a supramolecular complex (7,8).

Chloride channels. The effects of GABA and GABA antagonists on neuronal Cl^- channels have been extensively studied by electrophysiological techniques (2,9). However, it has been difficult to extract kinetic constants by this approach. Biochemical studies of Cl^- channels in the central nervous system (CNS) have been limited by deficiencies in experimental systems. Although GABA-dependent Cl^- efflux from rat brain slices has been demonstrated (10), the concentrations of agonists and antagonists in situ are indeterminate. Studies of Cl^- fluxes in neurosomal preparations from rodent brain (11,12) have yielded important information, but the presence of endogenous GABA and subcellular heterogeneity pose problems.

We have employed monolayers of neurons from the chick embryo cerebrum for the study of ionic channels (3,13). These preparations are relatively enriched in GABAergic neurons (14). Only a single population of low affinity $GABA_A$ receptors was present in membranes from cultured neurons in comparison with the receptor heterogenity of preparations from adult tissues (15). Development of biochemical markers for presynaptic and postsynaptic elements of GABA synapses in culture is similar to that observed in vivo (15,16). In these preparations, a $^{36}Cl^-$ uptake technique allowed kinetic constants for GABA agonists and antagonists to be determined (3). More recently, the properties of GABA-gated Cl^- channels in these neuronal cultures have been analyzed by patch-clamp methods (17). The potency of GABA for stimulation of $^{36}Cl^-$ uptake is in agreement with these biophysical measurements.

Cage convulsants. Alkyl derivatives of phosphobicyclooctane were originally developed for use as flame retardants, vinyl resin stabilizers,

and antioxidants (18). These bicyclophosphorus compounds were found to be extremely toxic convulsants. The poisonous smoke from burning flame-retarded polyurethane foams contains the ethyl derivative. The LD_{50} for t-butylbicyclophosphate is 40 μg/kg (i.p. in mice) or about 100 times more toxic than diisopropylfluorophosphate (19). In contrast to the usual mechanism of organophosphorus toxicity, the bicyclophosphorus compounds are not acetylcholinesterase inhibitors. Rather, Bowery et al. (20) found that these agents are potent antagonists of GABA-gated conductances in rat brain.

Squires et al. (21) reported that [^{35}S]t-butylbicyclophosphorothionate (TBPS) binds with high affinity (K_d = 17 nM) to membranes from rat brain. Since over 70% of the total TBPS bound could be displaced by picrotoxin, this ligand is far superior to others previously employed for the picrotoxin receptor.

III. Rationale

Our previous studies with picrotoxin and ethyl bicyclophosphorothionate (3) led to the hypothesis that these convulsants block GABA-gated Cl^- channels in cerebral neurons. The availability of [^{35}S]TBPS made possible direct binding measurements to brain membranes which could be correlated with inhibition of Cl^- fluxes. This seemed to be an important approach in defining the physiological relevance of the TBPS binding site and in testing the hypothesis that this site is the Cl^- channel. In addition, the [^{35}S]TBPS binding and Cl^- flux assays provided the opportunity of studying the interactions of other organophosphorus compounds, e.g., insecticides and nerve agents, with these specific GABA sites in the CNS. The interactions of putative anticonvulsants, e.g., benzodiazepines and barbiturates, with these sites could be approached by similar methods. We felt that such studies would elucidate the mechanism of convulsant action for organophosphorus compounds and identify anticonvulsant drugs which might attenuate this action.

IV. Methods

Membrane preparations. Cerebral hemispheres were dissected from adult female Leghorn chickens or adult male Sprague-Dawley rats. Cultured neuronal tissues were obtained by washing monolayers on petri dishes with ice-cold 0.32 M sucrose and then harvesting by scraping with a plastic policeman. The cells were collected in 0.32 M sucrose. All tissues were stored at -80°C. After thawing the tissue, crude synaptic membranes were prepared as described earlier (22). In brief, packed cells were resuspended in 50 mM Tris Cl (pH 7.4), homogenized with a Polytron (Brinkmann Instruments, Westbury, N.Y.), and centrifuged at 19,000 g for 10 min. All centrifugation steps were carried out in a SS34 rotor of an RC-2B centrifuge (Dupont Sorvall, Newtown, CT). The pellet was washed three times by suspension in Tris buffer and centrifugation. This pellet was homogenized in 4 volumes of Tris buffer and frozen at -80°C overnight. After thawing, the suspension was centrifuged as before and the pellet was washed once with buffer and twice with deionized water. The final pellet was homogenized in 1 volume of 1 mM Tris Cl (pH 7.4) and stored at -80°C. The protein content was determined by the method of Lowry et al. (23).

Binding assay. [^{35}S]TBPS binding was performed by filtration using GF/B filters (Whatman, Inc., Hillsboro, OR) according to Tehrani et al.

(22). Membranes (approximately 100 µg protein) were incubated for 90 min at 25°C in 20 mM HEPES-Tris (pH 7.4), 250 mM NaBr (or NaCl when indicated), and 2 nM [^{35}S]TBPS (specific activity 89.1 Ci/mmol) in a final volume of 0.1 ml. For displacement studies, the test compound was included in the 90 min incubation. Assays for nonspecific binding also contained 100 µM picrotoxinin. All data were corrected by subtracting these values for nonspecific binding.

Cell cultures. Cultures of pure neurons were prepared essentially as described by Thampy et al. (14). In brief, cerebral hemispheres of chick embryos, 8 1/2 days in ovo, were dissociated by sieving through nylon mesh (44 µm pore size) and then plated on polylysine-coated vinyl plastic coverslips (22 x 22 mm, Fisher Scientific Co., Houston, TX) or petri dishes (100 mm Falcon 1029 plates, Becton, Dickinson and Co., Oxnard, CA). Cells were incubated in Dulbecco's minimum essential medium (DMEM, Gibco Laboratories, Grand Island, NY), supplemented with 2% fetal calf serum (Gibco Laboratories, Grand Island, NY), 8% donor calf serum (Gibco Laboratories, Grand Island, NY), 50 units of penicillin per ml, and 50 µg streptomycin per ml, at 37°C in a humidified 10% CO_2 atmosphere for 8-10 days.

$^{36}Cl^-$ uptake. The assay for $^{36}Cl^-$ uptake by neuronal monolayers, 8-9 days in culture, was as described by Thampy and Barnes (3). Coverslips were incubated in fresh DMEM saturated with 10% CO_2 for 5-10 min at 37°C, briefly rinsed in HEPES-buffered saline (3), drained rapidly, and then transferred to 2 ml of assay medium (96 mM NaCl, 40 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, and 20 mM HEPES adjusted to pH 7.4 with Tris base) at 22°C for 10 sec. This solution also contained ^{36}Cl (5 µCi/ml) and other additions as indicated. Uptake was terminated by a 6-8 sec immersion of coverslips in 600 ml of ice-cold stop solution (150 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, and 5 mM HEPES adjusted to pH 7.4 with Tris base), which was stirred continuously. Each coverslip was drained on tissue paper and transferred to a scintillation vial containing 1 ml of 0.5% sodium dodecylsulfate. After overnight extraction at room temperature, a 0.2 ml aliquot was withdrawn for protein estimation (23). The remainder was mixed with 10 ml counting fluid (ACS, Amersham Corp., Arlington Heights, IL) and counted in a LS-100 liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Materials. Standard convulsants (including picrotoxinin), anticonvulsants, antichonergic antidotes, GABA, sodium dodecylsulfate, and antibiotics were from Sigma Chemical Co. (St. Louis, MO). Organophosphorus insecticides were obtained from Chem Service Inc. (West Chester, PA). Labeled TBPS was a product of New England Nuclear (Boston, MA). Unlabeled TBPS was obtained either from New England Nuclear or from Dr. John Verkade (Iowa State University, Ames, Iowa). Both samples appeard to be identical. ^{36}Cl was from ICN, Irvine, CA. Compound R5135 was provided by Dr. Peter Hunt (Roussel Uclaf, Romainville, France). Standard chemicals were from standard sources and of the highest available purity.

V. Results

A. Characterization of Binding Parameters for [^{35}S]TBPS to Membranes from Rat and Chicken Cerebral Hemispheres

Scatchard analyses of [^{35}S]TBPS binding to cerebral membranes resulted in curvilinear plots as shown in Fig. 1. In both rat and chicken cerebrum, a receptor model based on two binding sites (high and low affinity) was statistically preferable ($p < 0.01$) to a model based on one or three binding sites analyzed according to Munson and Rodbard (24). Values of apparent dissociation constants (K_d) and binding site densities (B_{max}) obtained from an iterative curve-fitting computer program (24) are given in Fig. 1. The apparent dissociation constants for high and low affinity sites and the maximum binding site densities are comparable in rat and chicken cerebrum. Addition of R5135 or (+)bicuculline did not increase TBPS binding to these preparations. When the experiments shown in Fig. 1 were repeated in the presence of 10 μM R5135 (not shown), neither the shape of the Scatchard plot nor the derived binding parameters were altered. The solubility of TBPS poses no limitation because it is approximately 0.3 mM under these conditions.

B. Displacement of [^{35}S]TBPS from Brain Membranes

Standard convulsants. The reduction of specific [^{35}S]TBPS binding by unlabeled TBPS and picrotoxinin was dose-dependent. Both compounds produced biphasic displacement curves. Similar effects were observed in both rat and chicken cerebral membranes (Fig. 2). The Hill coefficients (n_H) for both compounds were below unity (Fig. 2, legend). The displacement curves were resolved into high and low affinity components and the corresponding IC_{50} and K_i values are given in Table 1. Although picrotoxinin was a potent displacer, (+)bicuculline failed to produce any effect. This is consistent with their different sites of convulsant action (6).

Anticonvulsants. Secobarbital was also a potent displacer of TBPS from rat and chicken cerebral membranes (Fig. 3). Phenobarbital, which fails to potentiate GABAergic responses, was only about one tenth as active (cf. Fig. 3; Table 1). The benzodiazepines, clonazepam and diazepam, had no effect on TBPS binding at pharmacologically relevant concentrations. On the other hand, the pyrazolopyridines, cartazolate and etazolate, were among the most effective anticonvulsants. Other anticonvulsants, CL 218,872 and etomidate, were also active (Fig. 4). Their IC_{50} and K_i values (Table 1) were comparable to those for secobarbital and etazolate, respectively.

Organophosphorus insecticides. Paraoxon, a moderately toxic compound (LD_{50} = 1.8 mg/kg), was also a moderately potent displacer of TBPS from both rat and chicken brain membranes (Fig. 5; Table 2). Malathion and parathion were less effective. EPN was the most potent organophosphorus insecticide yet tested as a TBPS displacer (Fig. 6). It was somewhat more effective in rat brain than in chicken brain (Fig. 6; Table 2). Tetraethylpyrophosphate (1 mM) failed to produce a significant reduction of TBPS binding. But Dyfonate, Leptophos, and Phenamiphos were about as effective as paraoxon (Fig. 7; Table 2).

Anticholinergic antidotes. Both atropine and 2-pralidoxime chloride (2-PAM) failed to displace TBPS binding at concentrations up to 1 mM

(Table 2).

C. Chloride Ion Flux Studies

Kinetics of GABA-gated chloride channels. The effect of GABA on $^{36}Cl^-$ uptake by monolayers of chick cerebral neurons is shown in Fig. 8. The optimum rates of $^{36}Cl^-$ influx were observed at GABA concentrations approaching 40 μM. Previous work (3) established that determination of initial rates of Cl^- entry from the 10 sec uptake time used in these experiments underestimates the true rate by less than 10%. The data of Fig. 8 have been corrected by subtraction of values for Cl^- uptake which occurred in the absence of GABA. The rates for this basal Cl^- influx are typically 30-50% of the rate observed in the presence of GABA (not shown). A non-GABAergic pathway is responsible for this basal entry because it is insensitive to picrotoxin or bicuculline (3). The hyperbolic dose-dependence for GABA-mediated Cl^- uptake is consistent with first-order kinetics for GABA receptor occupancy and Cl^- channel activation. The double-reciprocal plot of these data (Fig. 8) yields a $K_{0.5}$ value of 1.3 μM for GABA.

As shown in Fig. 9A, the GABA-mediated Cl^- uptake increases as a function of the external Cl^- concentration. Analysis of these data by double reciprocal plot (Fig. 9B) gave a K_m = 140 ± 12 mM and a V_{max} = 10.9 ± 1.1 nmol mg^{-1} sec^{-1}. The Cl^- concentration used in subsequent experiments is at this K_m value.

TBPS inhibition kinetics of chloride influx. TBPS inhibited both the basal Cl^- uptake by neurons, observed in the absence of GABA, and the GABA-dependent entry (Fig. 10). However, the potency of TBPS differed in blocking these two pathways. IC_{50} values of 0.30 ± 0.02 μM and 11.4 ± 0.4 μM were obtained for the GABA-dependent and basal Cl^- influx, respectively. Using concentrations of TBPS near the IC_{50} value, it was found that TBPS inhibited Cl^- uptake noncompetitively with respect to GABA (Fig. 11). The kinetic parameters derived from this series of experiments are given in Table 3. Although TBPS profoundly reduced the V_{max} values for GABA-dependent Cl^- entry, it had little effect on the $K_{0.5}$ for GABA. These experiments gave a mean K_i value of 150 ± 30 nM for noncompetitive TBPS inhibition of GABA-gated Cl^- uptake by cerebral neurons.

[^{35}S]TBPS binding to neuronal membranes. The binding of [^{35}S]TBPS to membranes from cultured neurons was studied over a wide range of radioligand concentrations (0.2-500 nM). Under these conditions, 50-80% of the total TBPS binding was displaced by picrotoxinin (not shown) and is defined as specific binding. The Scatchard plot of this specific binding was curvilinear (Fig. 12). Analysis of binding data by an iterative curve-fitting computer program (24) indicates the presence of two binding sites which differ in both affinity and density ($K_{d(1)}$ = 3.1 ± 0.6 nM, $B_{max(1)}$ = 0.031 ± 0.018 pmol/mg; $K_{d(2)}$ = 270 ± 20 nM, $B_{max(2)}$ = 3.9 ± 0.7 pmol/mg).

For subsequent studies of [^{35}S]TBPS binding, we have examined only the low affinity binding process, since its dissociation constant is in agreement with K_i values for TBPS inhibition of GABA-gated Cl^- influx (Table 3). It was also apparent that little inhibition of Cl^- uptake was observed at TBPS concentrations below 10 nM (Fig. 10), suggesting that only the lower affinity site of TBPS binding site is physiologically relevant to these

experiments. The 25 nM concentration of [^{35}S]TBPS employed in the latter studies permits over 90% of the specific binding to be assigned to the low affinity site. As shown in Fig. 13, the addition of GABA reduced TBPS binding to the low affinity site. An IC_{50} value of 0.8 μM was estimated from this plot. This effect of GABA was also studied by Scatchard analysis (Fig. 14). The range of TBPS concentrations used in this experiment (10-500 nM) permitted a linear analysis of the low affinity site. The addition of 1 μM GABA caused a parallel shift of the plot to the left, indicating a reduction in the B_{max} with little change in the affinity. This is borne out by the binding parameters given in Table 4. A K_i value of 1.7 μM for noncompetitive inhibition by GABA was determined from the data of Fig. 14.

The binding of TBPS to neuronal membranes was strongly dependent on the addition of halide anions, as was shown previously for membranes from adult rat brain (21). The effect of Cl^- on TBPS binding is illustrated in Fig. 15. An EC_{50} value of 160 mM for NaCl was determined from the Hill plot (Fig. 15B).

VI. Discussion and Conclusions.

Previous equilibrium binding studies with [^{35}S]TBPS (8,21) have revealed only a single binding site in mammalian brain with dissociation constants ranging from 16 to 120 nM, depending on the assay conditions. However, heterogeneity of TBPS binding sites has been suggested by several observations: i) polyphasic dissociation of radioligand from its receptor (21); ii) polyphasic action of NaCl and GABA in protecting binding sites against heat inactivation (21); and iii) biphasic displacement of TBPS by suriclone and zopiclone (25).

In this report we demonstrate the multiplicity of TBPS binding using both equilibrium and displacement studies. The direct observation of binding heterogeneity was made possible by using a wide range of ligand concentrations. Nonlinear regression analysis of these data suggests two TBPS binding sites that differ in affinity. Previous studies of TBPS binding were carried out over a limited range of ligand concentrations. This probably accounts for the failure to detect the low affinity site. Indeed, when our data (Fig. 1) are fitted for TBPS concentrations from 0.5 to 25 nM, similar to those employed by others (8,21), a linear Scatchard plot was obtained (not shown). The K_d value of 21 nM derived from this plot is in good agreement with reported values. We also observed biphasic displacement of specifically bound [^{35}S]TBPS by unlabeled TBPS and by picrotoxinin (Fig. 2), and both displacers gave Hill coefficients below unity. This provides further support for heterogeneity of TBPS binding sites. The comparable K_d, B_{max}, and IC_{50} values for rat and chicken cerebrum indicate similarity of TBPS sites in avian and mammalian brain. If the B_{max} values for low affinity binding (Fig. 1) are assumed to represent the level of sites per milligram of membrane protein, the TBPS sites are 8-fold and 4-fold higher, respectively, than the levels of benzodiazepine and GABA receptors in adult brain (15).

The $^{36}Cl^-$ flux studies reported here represent the first examination of the functional effects of TBPS on central neurons. TBPS was shown to be the most potent inhibitor of GABA-gated Cl^- channels yet described. Antagonism of GABA channels by other bicyclophosphorus compounds has been previously reported (3,20). In one study (3), the IC_{50} value for ethyl bicyclophosphorothionate was 100 times that for TBPS; a reasonable correlation was found with their 21-fold difference in toxicity in mice (19). Although TBPS was also an inhibitor of basal Cl^- influx in cultured neurons, 40-fold higher concentrations were required to produced the same degree of inhibition observed for GABA-dependent flux (Fig. 10). Ethyl bicyclophosphorothionate was nearly equipotent in blocking these two pathways for Cl^- entry (3).

As shown above for membranes from adult brain, Scatchard plots of specific TBPS binding to membranes from cultured neurons (Fig. 12) were also curvilinear but resolvable into two binding components. The K_d value for the low affinity site (0.27 μM) correlates well with the IC_{50} (0.30 μM) and K_i values (0.15 μM) for TBPS inhibition of GABA-gated Cl^- flux. Thus we suggest that the low affinity binding site for TBPS is physiologically relevant to GABA antagonism. The relevance of the high affinity site is not clear at present.

Additional correlations were found between the low affinity TBPS binding site and Cl^- channels. The binding of TBPS to neuronal membranes was increased more than 9-fold by the addition of Cl^- (Fig. 15). The EC_{50} for Cl^- enhancement of TBPS binding (160 mM) was quite similar to the K_m value (140 mM) for GABA-gated Cl^- influx. GABA also produced a noncompetitive displacement of TBPS binding from neuronal membranes (Figs. 13,14). The IC_{50} (0.80 μM) and K_i values (1.7 μM) for this effect agreed reasonably with the $K_{0.5}$ value (1.3 μM) for GABA stimulation of Cl^- entry into intact neurons (Fig. 8). Although no rationale has yet been given for the GABA displacement of TBPS binding previously observed (26), GABA receptor desensitization seems an attractive possibility for further study.

Taken together, these results indicate that TBPS exerts its effects as a GABA antagonist by direct blockade of Cl^- channels. The observation that TBPS inhibition of Cl^- influx is noncompetitive with respect to GABA (Fig. 14) is open to further interpretation. Noncompetitive inhibition predicts interaction of inhibitor with both free enzyme and the enzyme-substrate complex. Thus we would like to suggest that TBPS binds to both closed and open channels. Although other mechanisms are conceivable, such as allosteric effects of TBPS at some other site on the GABA receptor complex, channel interaction is the most facile interpretation of our data. Ultimate resolution of this issue must await reconstitution of Cl^- channels from purified GABA receptor preparations.

This work represents a validation of the [^{35}S]TBPS binding technique for screening agonists and antagonists of GABA channels. Since many compounds in this class of substances are convulsants or anticonvulsants, TBPS binding is a useful pharmacological tool. This is demonstrated in the studies of Tables 1 and 2. Picrotoxinin, a potent convulsant (LD_{50} = 3 mg/kg i.p. in mice), was also a very effective displacer of TBPS. This interaction was found to be competitive (not shown), supporting the hypothesis that TBPS and picrotoxinin interact at the same site. Bicuculline, on the other hand, failed to displace TBPS. This is consistent with the hypothesis that the GABA recognition site is distinct from the Cl^- channel (7). Secobarbital, an anticonvulsant barbiturate, was able at physiologically relevant concentrations (5) to displace TBPS from cortical membranes. Phenobarbital, which fails to potentiate GABA inhibition, was a much less effective displacer of TBPS. The lack of a significant benzodiazepine effect on TBPS binding is consistent with separate sites for picrotoxin and benzodiazepines (7). Benzodiazepines seem to potentiate GABA conductances via an allosteric rather than a direct interaction with Cl^- channels. Our studies identified two pyrazolopyridines, cartazolate and etazolate, as quite potent displacers of TBPS binding. Although these compounds have pharmacological effects similar to those of the benzodiazepines, it is apparent that their sites of interaction with brain membranes may be different. Etomidate, a compound with a pharmacological profile similar to that of sedative barbiturates, also had high potency for TBPS displacement. The latter three compounds merit further study as modulators of Cl^- fluxes and antidotes for organophosphorus toxication.

Certain organophosphorus insecticides also proved to be moderately potent displacers of TBPS binding to cortical membranes (Table 2). Of these, EPN and paraoxon were the most potent, with K_i values in the 100 μM range. Dyfonate was also effective in similar doses. Two nerve agent antidotes, atropine and 2-PAM, were not displacers, as would be expected

from their cholinergic mechanisms of action. The findings so far obtained support the hypothesis that certain organophosphorus convulsants act through a GABAergic rather than a cholinergic mechanism. This mode of action is clearest for the cage convulsants such as TBPS. However, the effects of paraoxon and other insecticides on TBPS binding suggest that GABA antagonism may contribute to their convulsant effects. This is of interest because Lebeda and Rutecki (27) have shown that soman produces an epileptiform discharge activity in rat hippocampus that is not related to blockade of acetylcholinesterase. Thus the interaction of nerve agents with GABAergic neurons merits attention. These experiments are in progress.

Table 1

Parameters for TBPS Displacement from Cortical Membranes by Convulsants and Anticonvulsants

Compound	Rat		Chicken	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
Barbiturates				
Secobarbital	64 ± 8	24 ± 0.3	54 ± 12	25 ± 0.5
Phenobarbital	490 ± 40	190 ± 15	380 ± 50	180 ± 20
Benzodiazepines				
Clonazepam	>100		>100	
Diazepam	>100		>100	
Pyrazolopyridines				
Cartazolate	5.4 ± 0.9	2.1 ± 0.3	6.1 ± 0.2	2.8 ± 0.1
Etazolate	5.2 ± 0.8	2.0 ± 0.3	5.4 ± 0.7	2.5 ± 0.3
Other Anticonvulsants				
CL 218,872	53 ± 12	20 ± 5	56 ± 2	26 ± 1
Etomidate	3.7 ± 0.4	1.4 ± 0.2	2.6 ± 0.2	1.2 ± 0.1
Standard Convulsants				
Picrotoxinin	0.0079 ±0.0057	0.0030 ±0.0022	0.0057 ±0.0034	0.0026 ±0.0016
	0.49 ±0.07	0.18 ±0.03	0.42 ± 0.11	0.19 ±0.05
(+)Bicuculline	>100		>100	

The IC_{50} values were determined from the data of Figs. 2-4 and represent the mean ± S.D. of two experiments, each carried out in triplicate. K_i values were determined according to $K_i = IC_{50} / [1 + L/K_d]$ where L = 2 nM. K_d values were determined from Scatchard analysis (Fig. 1).

Table 2

Parameters for TBPS Displacement from Cortical Membranes by Organophosphorus Compounds and Antidotes

Compound	Rat		Chicken	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
Organophosphorus Insecticides				
Paraoxon	410 ± 40	160 ± 15	300 ± 20	140 ± 9
Parathion	1300 ± 30	500 ± 11	1900 ± 400	880 ± 190
Malathion	750 ± 50	290 ± 20	990 ± 30	460 ± 10
EPN	180 ± 80	69 ± 30	400 ± 60	190 ± 30
Tetraethylpyrophosphate	>3000		>3000	
Dyfonate	230 ± 40	88 ± 15	340 ± 40	160 ± 20
Leptophos	390 ± 70	150 ± 30	430 ± 60	200 ± 30
Phenamiphos	360 ± 30	140 ± 10	520 ± 50	240 ± 20
Anticholinergic Antidote				
Atropine	>1000		>1000	
Oxime Antidote				
2-Pralidoxime chloride	>1000		>1000	

IC_{50} values were determined from the data of Figs. 5-7 and represent the mean ± S.D. of two experiments, each carried out in triplicate. K_i values were determined as described in the legend of Table 1.

Table 3

Kinetic Parameters for GABA-gated $^{36}Cl^-$ Uptake by Cerebral Neurons

TBPS	$K_{0.5}$ (GABA)	V_{max}	K_i (TBPS)
μM	μM	nmol/mg·sec	μM
-	1.32 ± 0.05*	5.6 ± 0.4	
0.2	1.45†	3.3 ± 0.6	0.158 ± 0.027
1.0	1.25	0.85	0.135

The values shown were obtained from plots as shown in Fig. 11.
*Mean ± S.D. of three independent experiments. †Mean of two independent experiments.

Table 4

Effects of GABA on TBPS Binding Parameters

GABA	$K_{d(2)}$ nM	B_{max} pmol/mg
-	200 ± 10	3.9 ± 0.7
1 μM	184 ± 10	2.0 ± 0.3

The values shown were obtained from linear regression analysis of the data in Fig. 14.

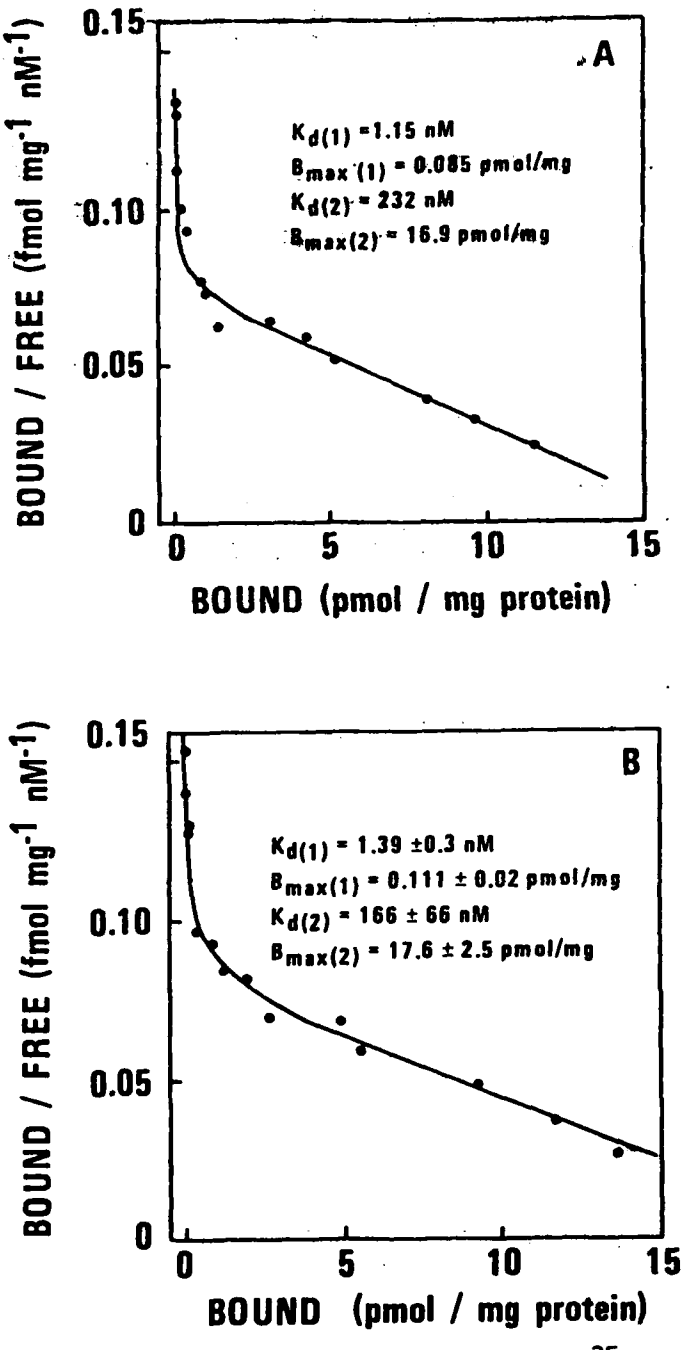

Fig. 1. Scatchard plots of specific binding of [^{35}S]TBPS to cerebral membranes from adult rat (A) or chicken (B). The solid lines represent the theoretical curves of the best computer fit based on two binding sites having the parameters shown. Binding studies were carried out as described under "Methods" except that the TBPS concentration varied from 0.25 to 500 nM. Each experimental point was determined in triplicate.

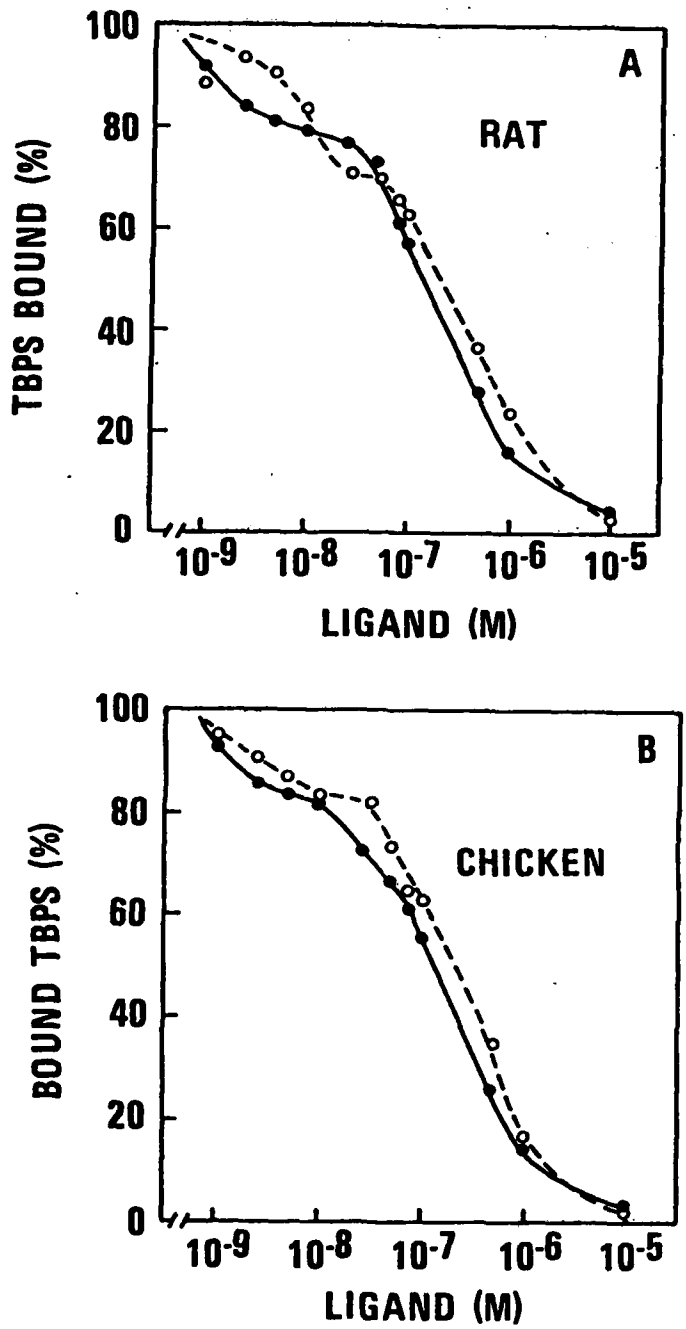

Fig. 2. Displacement of [^{35}S]TBPS binding by unlabeled TBPS (●) and picrotoxinin (○) in cerebral membranes of adult rat (A) and chicken (B). Experiments were carried out as described under "Methods" except that the labeled TBPS concentration was 2 nM. For TBPS, each point is the mean of two experiments in rat and three in chicken, each carried out in triplicate. Hill plots (not shown) of TBPS displacement data gave n_H = 0.65 ± 0.05 (rat) and 0.70 ± 0.08 (chicken), and for picrotoxinin displacement, n_H = 0.70 (rat) and 0.64 (chicken). These latter values were not statistically verified.

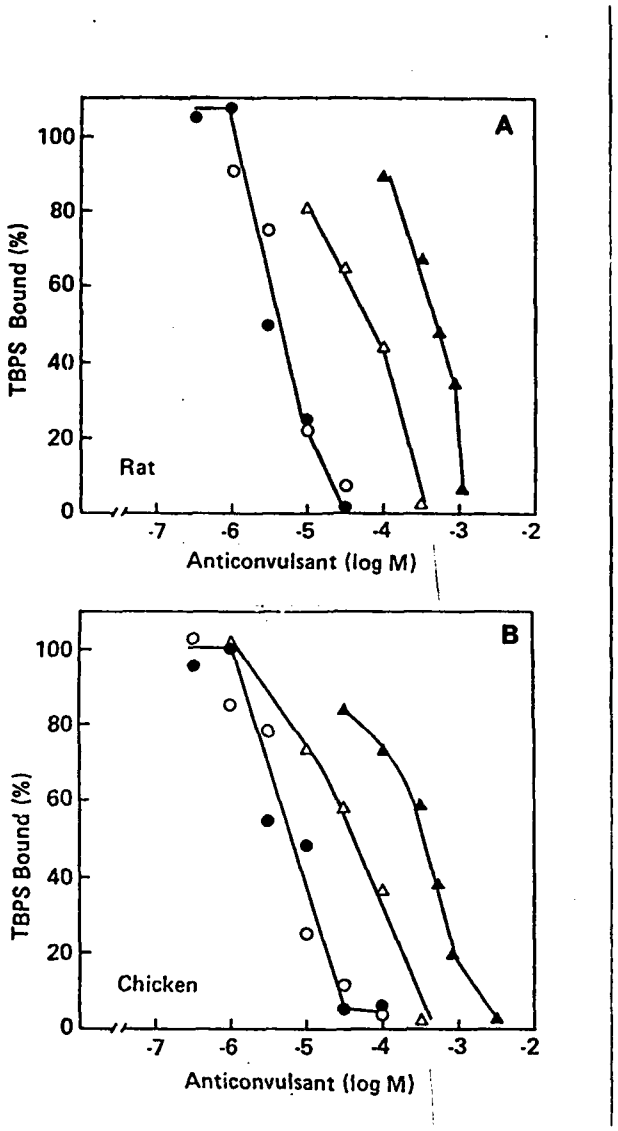

Fig. 3. Displacement of TBPS from rat (A) and chicken (B) cortical membranes by etazolate (●), cartazolate (○), secobarbital (△), and phenobarbital (▲). Assays were carried out as in the legend of Fig. 2. Data points are the mean of two experiments, each carried out in triplicate.

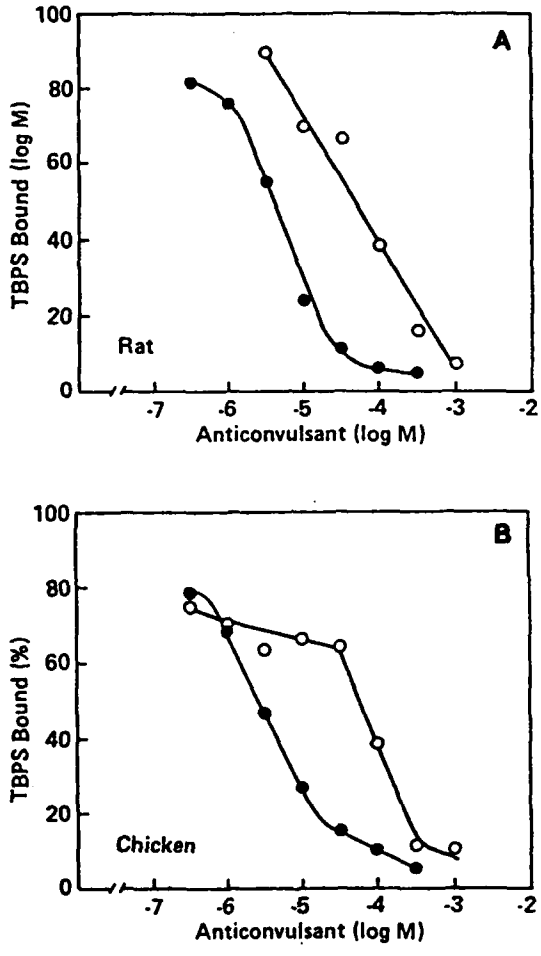

Fig. 4. Displacement of TBPS from rat (A) and chicken (B) cortical membranes by etomidate (●) and CL 218,872 (○). Assays were carried out as in the legend of Fig. 2. Data points are the mean of two experiments, each carried out in triplicate.

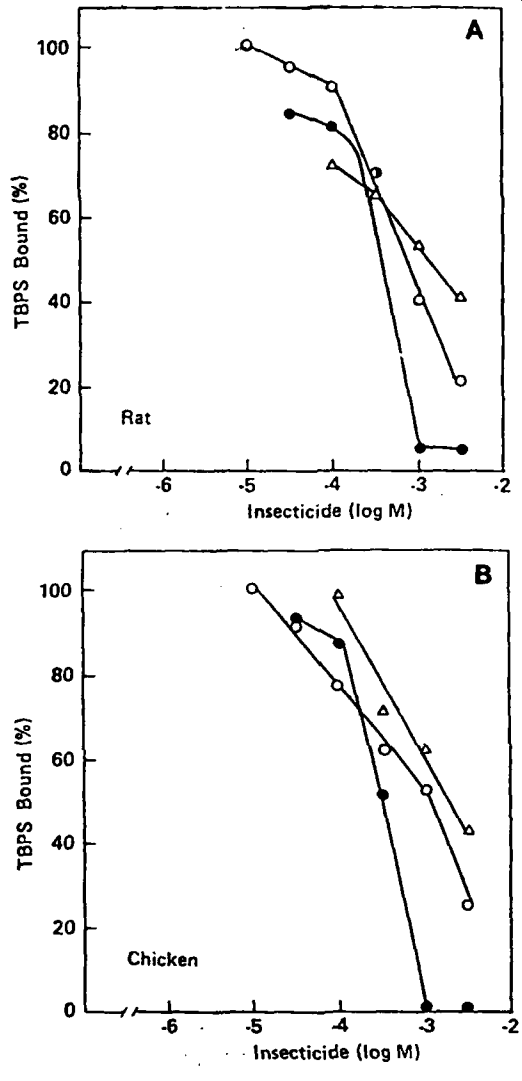

Fig. 5. Displacement of TBPS from rat (A) and chicken (B) cortical membranes by paraoxon (●), malathion (○), and parathion (Δ). Assays were carried out as in the legend of Fig. 2. Data points are the mean of two experiments, each carried out in triplicate.

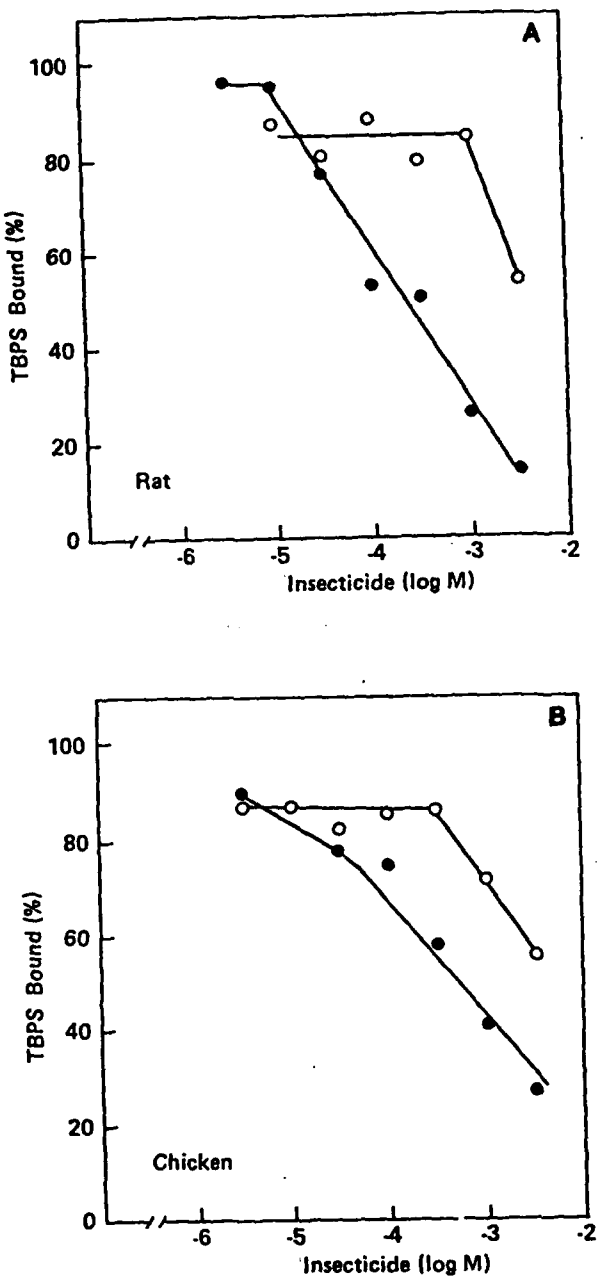

Fig. 6. Displacement of TBPS from rat (A) and chicken (B) cortical membranes by EPN (●) and tetraethylpyrophosphate (○). Assays were carried out as in the legend of Fig. 2. Data points are the mean of two experiments, each carried out in triplicate.

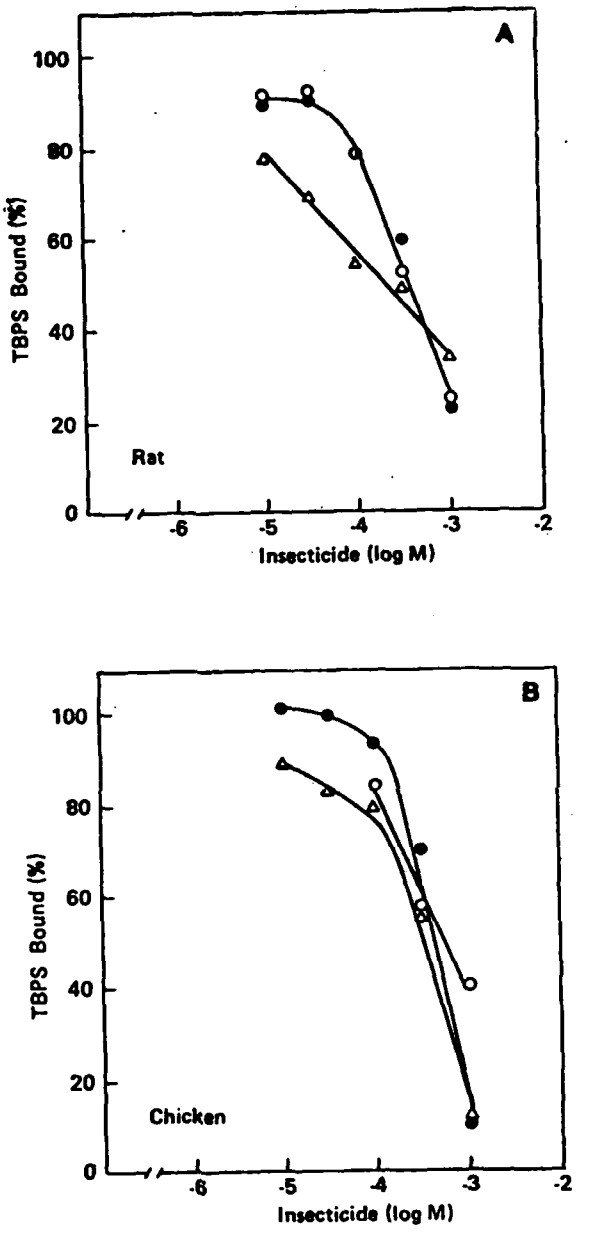

Fig. 7. Displacement of TBPS from rat (A) and chicken (B) cortical membranes by Leptophos (●), Phenamiphos (○), and Dyfonate (△). Assays were carried out as in the legend of Fig. 2. Data points are the mean of two experiments, each carried out in triplicate.

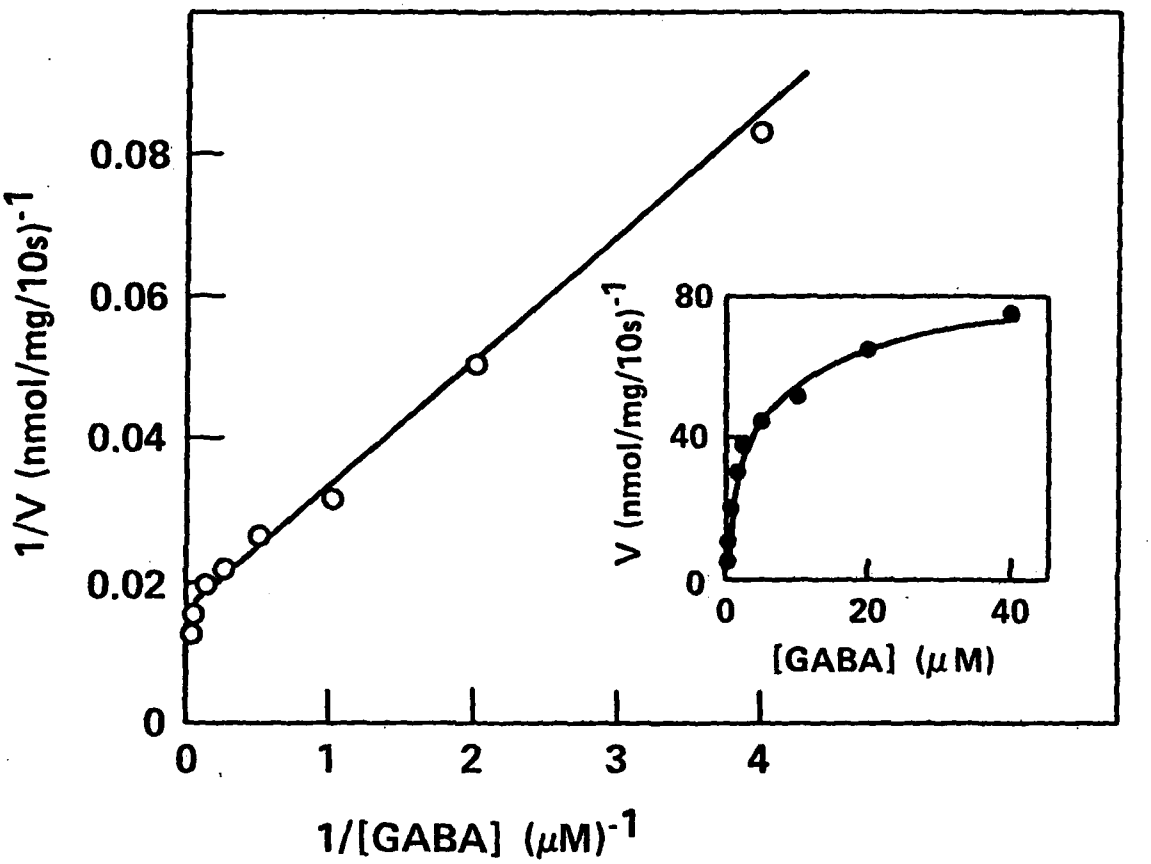

Fig. 8. Effect of GABA on $^{36}Cl^-$ uptake by cultured cerebral neurons. Assays were carried out at 22°C in HEPES-buffered saline containing 40 mM K^+ as described in under "Methods" except that the GABA concentration varied as indicated in the inset. Uptake was terminated after 10 sec. All values were corrected for the basal uptake which occurred in the absence of GABA. The line on the double reciprocal plot was obtained by linear regression ($r = 0.99$).

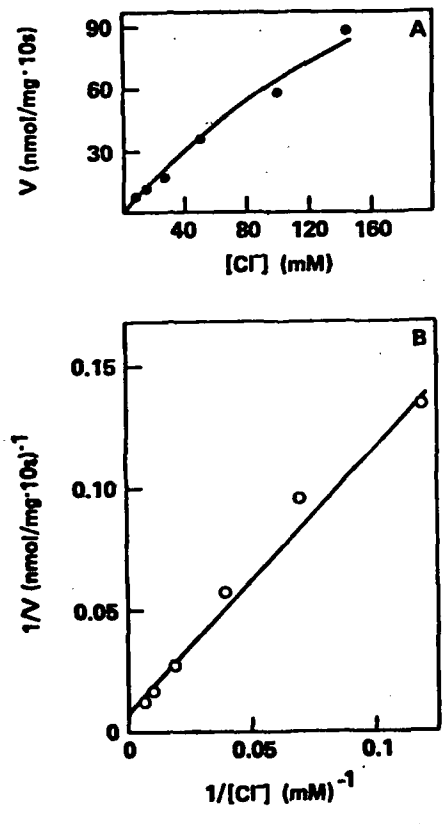

Fig. 9. Effect of external Cl^- on GABA-dependent $^{36}Cl^-$ uptake by cerebral neurons. Assays were carried out as described in the legend of Fig. 8 except that 50 μM GABA was present and the Cl^- concentration varied as shown in Panel A. The ionic strength was held constant by addition of sodium gluconate. The line in Panel B was obtained by linear regression ($r = 0.98$).

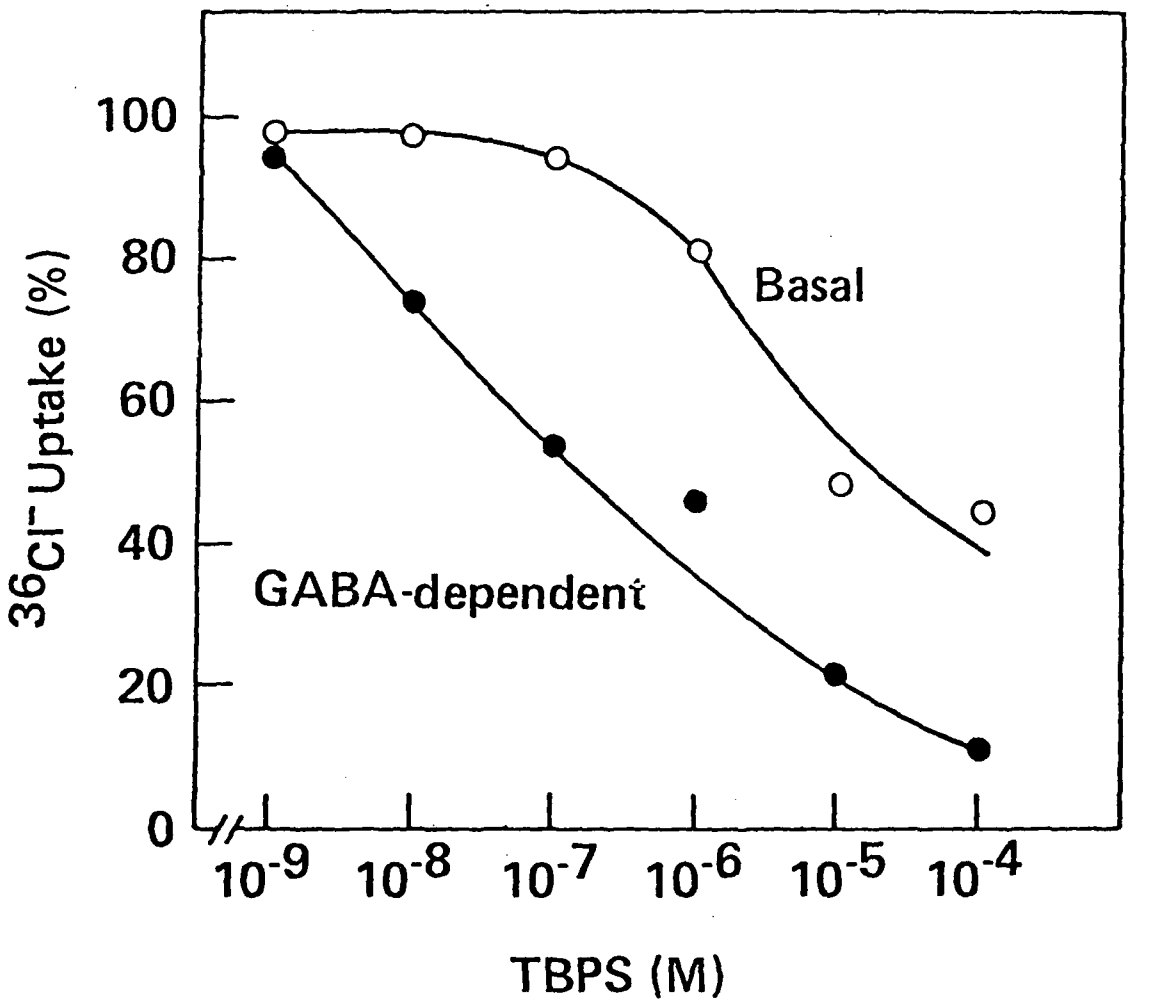

Fig. 10. Effect of unlabeled TBPS on Cl^- uptake by cerebral neurons. Experiments were carried out as described in the legend of Fig. 8 except that TBPS was added as indicated. Values for the rate of Cl^- entry are shown as a percentage of the control rate which occurred in the absence of TBPS. (O), Uptake rates for basal Cl^- uptake observed in the absence of GABA; (●), Rates for GABA-dependent Cl^- uptake observed with 50 μM GABA.

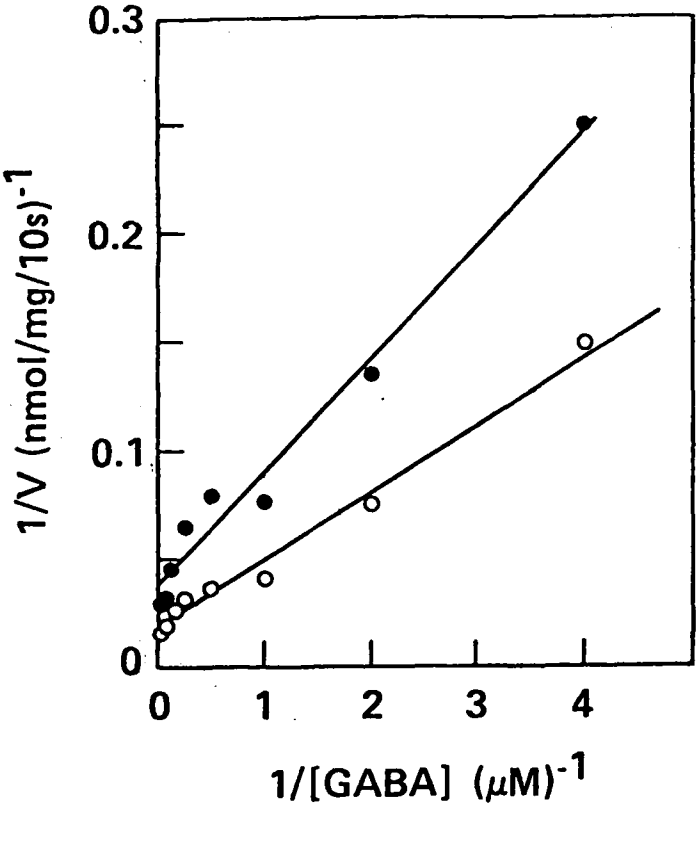

Fig. 11. Kinetics of TBPS inhibition of GABA-dependent Cl^- uptake. Assays were carried out as described in the legend of Fig. 8 except that TBPS was present where indicated. (O), Control. The r value from linear regression was 0.98.; (●), 0.2 µM TBPS (r = 0.97).

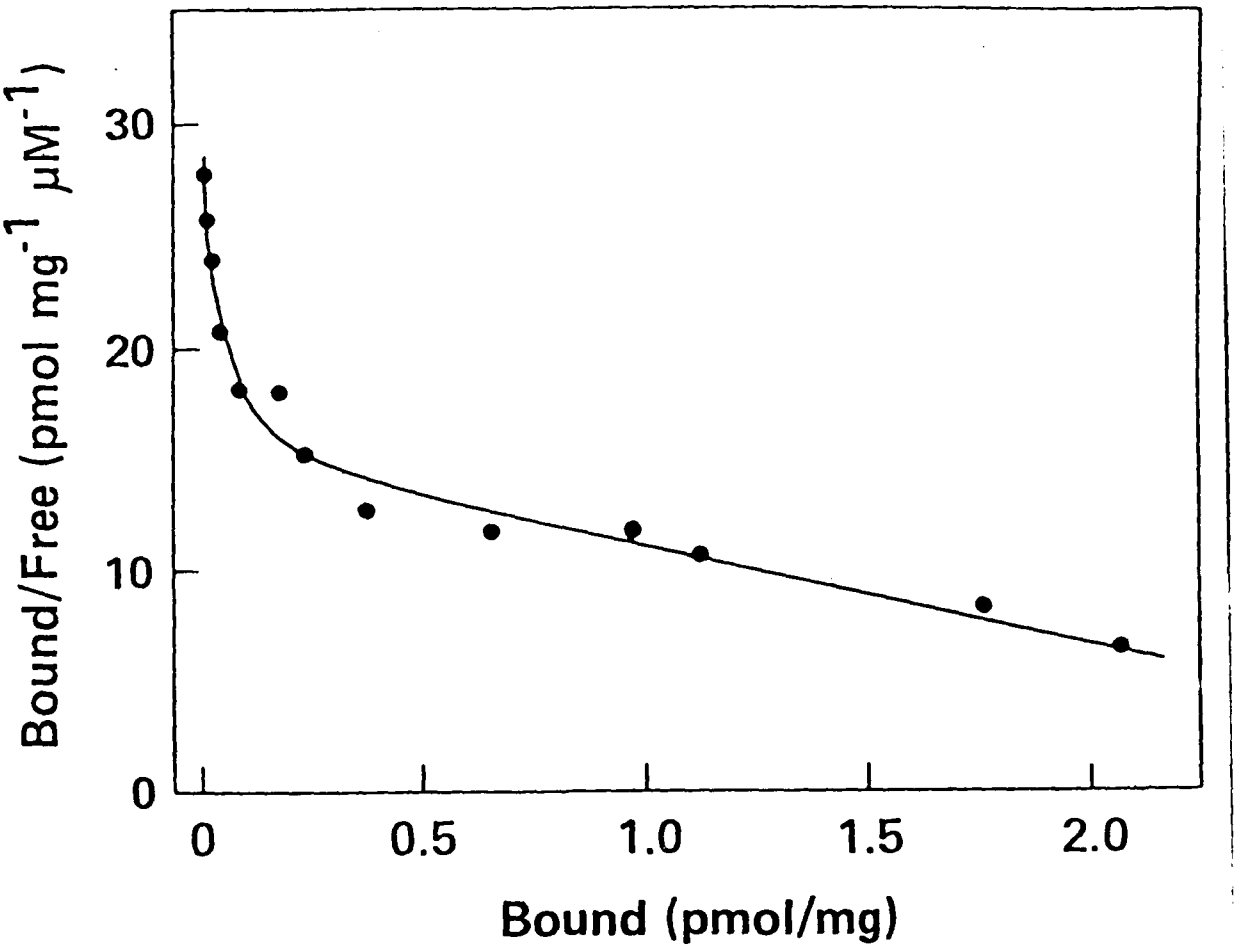

Fig. 12. Scatchard plot of specific [^{35}S]TBPS binding to membranes from cultured neurons. TBPS binding was determined as described under "Methods." The solid line represents the theoretical curve of the best fit based on nonlinear regression analysis (24) based on two binding sites. Each point is the mean of triplicate determinations.

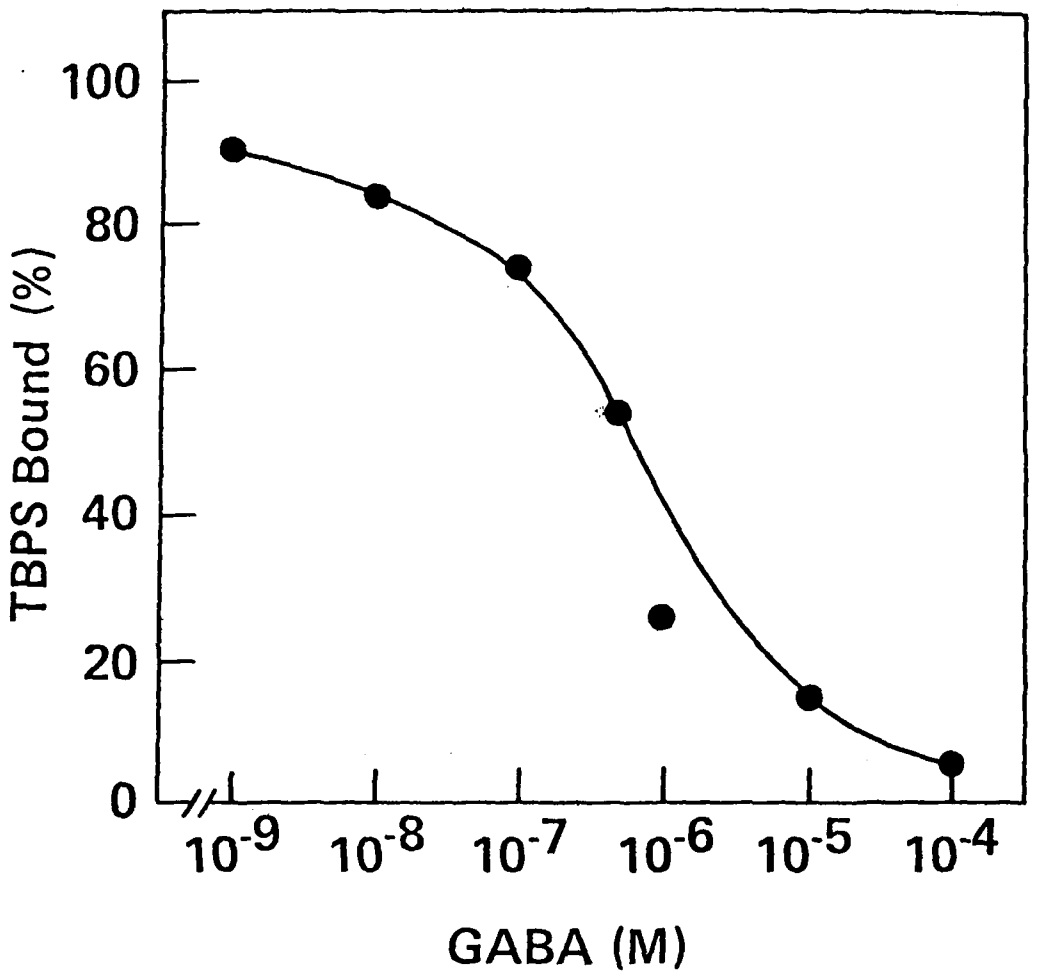

Fig. 13. Displacement of TBPS binding to neuronal membranes by GABA. Assays were carried out as described under "Methods" except that the concentration of TBPS was 25 nM and GABA was added as indicated. Each point is the mean of triplicate determinations.

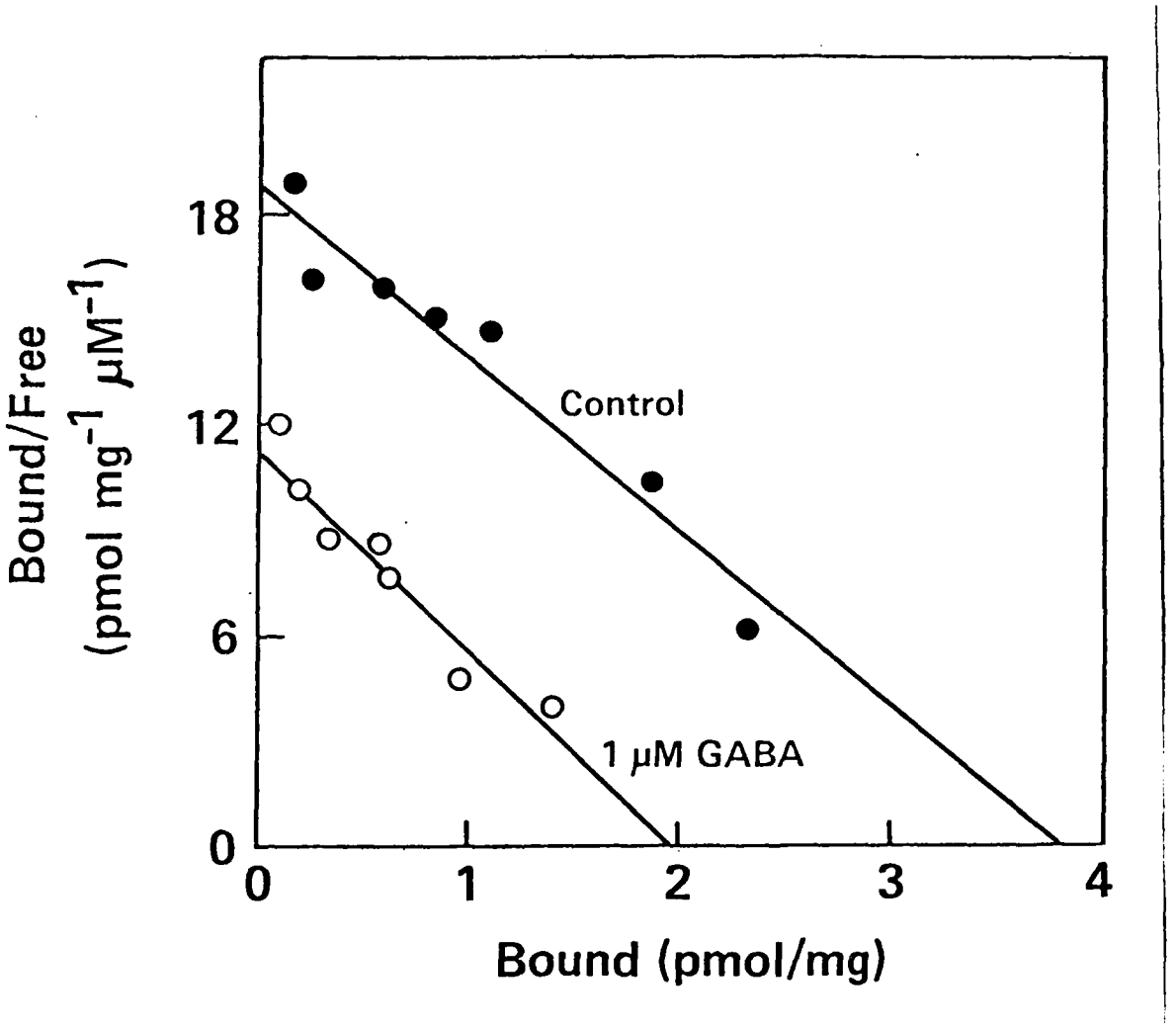

Fig. 14. Scatchard analysis of GABA displacement of TBPS from low affinity sites on neuronal membranes. Assays were carried out as described under "Methods" except that TBPS concentrations from 10 to 500 nM were employed and GABA was added as indicated. (●), Controls. Linear regression analysis gave $r = 0.95$; (○), 1 μM GABA ($r = 0.96$).

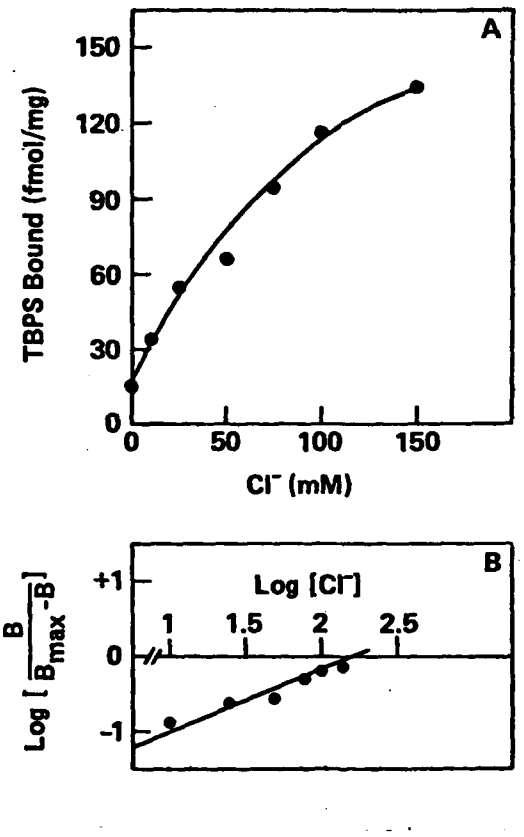

Fig. 15. Effects of Cl^- on TBPS binding to the low affinity site on neuronal membranes. Assays were carried out as described under "Methods" except that the TBPS concentration was 25 nM and NaCl at the concentrations shown (Panel A) replaced NaBr. Ionic strength was kept constant at 150 mM by addition of Na gluconate. The data of Panel A were analyzed on a Hill plot (Panel B). The abcissa scale of Panel B refers to the log of the mM Cl^- concentration. The B_{max} value was obtained from a double reciprocal plot (not shown), using the four highest Cl^- concentrations. Linear regression analysis gave $r = 0.98$.

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