Biochemical Pharmacology, Vol. 37, No. 11, pp. 2195–2200, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

NONEQUILIBRIUM MODULATION OF ^{35}S-TBPS BINDING BY BENZODIAZEPINE AGONISTS AND ANTAGONISTS

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(*Received* 19 *Debember* 1986; *accepted* 7 *December* 1987)

Abstract—Specific binding of ^{35}S-t-butylbicyclophosphorothionate (TBPS) was studied in synaptosomal membranes of rat cerebral cortex under nonequilibrium conditions. TBPS binding proved to be suitable for the characterization of not only the efficacy but also the potency of benzodiazepine (BZ) receptor ligands *in vitro*. Five BZ agonists accelerated the kinetics of TBPS binding in a concentration-dependent manner. The EC_{50} values of acceleration correlated with displacing potencies at BZ receptors suggesting the involvement of high affinity central BZ binding sites. The binding enhancement by 0.3 μM oxazepam could be antagonized *in vitro* by Ro-15-1788 (IC_{50} = 43 nM) and by oxazepam α,α-Me_2-β-phenyl-propionate ester (IC_{50} = 2–3 μM), a weak BZ antagonist. Antagonism might be attributed to the acyl moiety since a 3-*O*-ether derivative appeared to be a partial agonist. Structural requirements of the conversion of BZs from agonists into antagonists are discussed.

The postsynaptic γ-aminobutyric acid ($GABA_A$) receptor–chloride ionophore complex contains several binding sites which are subject to mutual allosteric regulation, e.g. the binding site for picrotoxin-like cage convulsants such as t-butyl-bicyclophosphorothionate (TBPS) is modulated by some ligands of benzodiazepine (BZ) receptors [1–9]. Ligands of BZ receptors have been classified as follows [1, 3, 4]: (a) anxiolytic, anticonvulsant BZ drugs called agonists such as flunitrazepam and oxazepam which appeared to enhance the specific binding of ^{35}S-TBPS [6]; (b) anxiogenic, convulsant ligands called inverse agonists, having typically β-carboline ring-structures, which seemed to decrease ^{35}S-TBPS binding [5]; and (c) antagonists such as Ro-15-1788 [10] which essentially lack any pharmacological effect of their own and do not affect ^{35}S-TBPS binding [7]. We have recently described that the *in vitro* effects of BZ receptor ligands on ^{35}S-TBPS binding can only be demonstrated under nonequilibrium conditions [11]. These effects can be attributed to the acceleration (by BZ agonists) or deceleration (by inverse agonists) of ^{35}S-TBPS binding [11]. The effect of BZs could be characterized by a kinetic modulatory factor [11]. Previous studies have demonstrated a correlation between the efficacies of BZs and their maximal effects on ^{35}S-TBPS binding [1–6]. However, most of these studies were restricted to the examination of high saturating concentrations of BZ receptor ligands and did not allow the evaluation of the potency of BZs *in vitro*. Deliberately applying nonequilibrium incubation, we wish to show now that the concentration dependence of the effect of BZs on TBPS binding can characterize both efficacies and potencies.

Ester derivatives of some 3-hydroxy-BZs investigated earlier as prodrugs have been demonstrated to possess weak affinities to BZ receptors *in vitro* [12, 13]. The decrease in receptor affinity of some aliphatic esters of oxazepam correlated with the steric parameters of the acyl moiety [14]. Furthermore, some phenyl-substituted aliphatic esters of oxazepam appeared to antagonize the protective effect of oxazepam against pentetrazol convulsions [13, 15]. A given degree of antipentetrazol effect requires a certain brain concentration of BZ agonists [16], due to the occupancy of central BZ receptors [4, 17]. However, increased brain levels of oxazepam were required to elicit the same anticonvulsant effect when certain β-phenyl-propionic esters of oxazepam were present in the brain of mice [13, 15]. Now we demonstrate that the above *in vivo* activities of oxazepam derivatives can be attributed to antagonism at the GABA receptor complex as revealed by the interaction of BZ receptors with ^{35}S-TBPS binding sites.

MATERIALS AND METHODS

Materials. Oxazepam and its derivatives were prepared as described [12]. Ro-15-1788 and flunitrazepam originated from Hoffman-LaRoche (Nutley, NJ) and picrotoxin from Sigma. ^{35}S-TBPS (20–80 Ci/mmol) was obtained from New England Nuclear and ^{3}H-flunitrazepam (80 Ci/mmol) from Amersham.

Membrane preparation. Crude synaptosomal membranes of rat cerebral cortex were prepared according to previously described methods [6, 18]. Briefly, brains of male Wistar rats were rapidly dissected and cortices were homogenized in 20 vol. of 5 mM Tris HCl (pH = 7.4) for 20 sec by Ultra-Turrax. Following 30 min of incubation at 4°, the suspension was centrifuged at 48,000 *g* for 10 min. The pellet was washed with 50 mM Tris HCl at least four times by similar centrifugations. Then it was suspended in 50 mM Tris citrate (pH = 7.1) and frozen. On the day of the binding assay the suspension was thawed and washed by centrifugation in 50 mM Tris HCl at 48,000 *g* for 10 min. For the

comparison with ^{3}H-flunitrazepam binding, a freeze-thawing cycle was included which can decrease esterase activity of the membrane fraction [14] and make the effect of BZ receptor ligands on TBPS binding more reproducible [5]. After the second washing, the membrane suspension was frozen, thawed, washed by two more centrifugations and frozen.

^{35}S-TBPS binding. Membrane suspensions (1–2 mg protein/ml) in 50 mM Tris HCl (pH = 7.4) containing 300 mM NaCl were incubated with 1.5 nM ^{35}S-TBPS and BZ receptor ligands at 22°. For nonspecific binding 30 μM picrotoxin was coincubated. At various times of incubation aliquots of 0.45 ml were filtered on Whatman GF/B filters under vacuum in duplicates and washed with 3 × 3 ml of cold buffer. Radioactivity of the filters was measured in a benzyl alcohol-based scintillation cocktail. Apparent association rate constants of ^{35}S-TBPS binding were determined by plotting ln $B_e/(B_e - B_t)$ against time as described [18]. B_t and B_e refer to specific binding at various times and in equilibrium (180 min), respectively.

The potency of BZs to affect TBPS binding. For BZ agonists, specific ^{35}S-TBPS binding was determined after 20 min and 180 min of incubation, in the presence of various concentrations of benzodiazepines. Ln $B_e/(B_e - B_t)$ values were determined for t = 20 min and the ratio of BZ modulated/control binding gave the kinetic modulatory factor a. The EC_{50} values giving half-maximal acceleration of TBPS binding were calculated by regression of the a values against log c of BZ agonists. Simultaneously, the EC_{50} values giving half-maximal enhancements of TBPS binding were also determined: the ratios $(B_t' - B_t)/B_t$ for BZ modulated (B_t') and control (B_t) TBPS binding were calculated for t = 20 min and a correlation of these ratios against log c of BZ agonists gave another EC_{50} value. This latter value was smaller by only 5–8% than that calculated by the former method. For the examination of the potency of BZ antagonists ^{35}S-TBPS binding was measured in the presence of 0.3 μM oxazepam and various concentrations of Ro-15-1788 or oxazepam α,α-Me$_2$-β-phenyl propionate. Data were processed as above to determine the IC_{50} value of the antagonists which decreases the enhancing effect of 0.3 μM oxazepam by 50%.

^{3}H-Flunitrazepam binding. The membrane suspension in 50 mM Tris HCl containing 300 mM NaCl was incubated with 0.5 nM ^{3}H-flunitrazepam and displacing concentrations of oxazepam derivatives at 22° for 15 min. Triplicate aliquots were filtered, the filters were washed with 3 × 3 ml cold buffer. For nonspecific binding 10^{-5} M clonazepam was applied.

RESULTS

Specific binding of ^{35}S-TBPS was slow in the presence of 300 mM NaCl and it reached equilibrium after 3 hr at 22°. Figure 1a demonstrates the initial period of association. The presence of 0.3 μM oxazepam increased the association rate but the equilibrium of binding was not affected (Fig. 1a). Association data can be linearized according to Fig. 1b. The slope of the straight line gives the apparent rate constant of association. This rate constant was increased by oxazepam from 0.0192 ± 0.0025 min^{-1} (control) to 0.0287 ± 0.0035 min^{-1} (Fig. 1b). The ratio of these values corresponds to a kinetic modulatory factor a = 1.49.

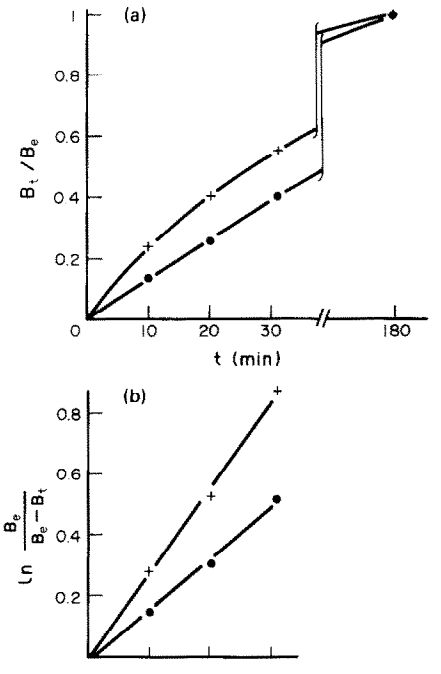

Fig. 1. The association rate of 1.5 nM ^{35}S-TBPS in control synaptosomal membranes (●) and in the presence of 0.3 μM oxazepam(+). (a) Time-dependence of the initial part of association. Specific binding at time t (B_t) is related to binding in control at 180 min, B_e. Note that the points at 180 min are identical. Specific binding was 2.71 ± 0.33 and 2.73 ± 0.36 pmol/g cortex in the absence and presence of 0.3 μM oxazepam, respectively. Specific binding did not significantly increase after 180 min. (b) Linearization of the same data by a semilog plot. The points are averages of three experiments which varied by less than 7%.

Parallel with the progress of association of TBPS, the relative effect of BZ receptor ligands on TBPS binding decreases (Fig. 1a and [11]). An incubation time of 20 min was found to be optimal for further studies on BZs. Figure 2 shows the effect of different concentrations of flunitrazepam and oxazepam. The enhancing effects increase up to 53 ± 2% over control of TBPS binding in the concentration range of 10^{-7}–10^{-6} M for both BZ agonists (Fig. 2). Kinetic modulatory factors (a) were determined for various concentrations of the BZ agonists. A correlation of a values against log c resulted in the EC_{50} values of 25 ± 13 nM for flunitrazepam and 46 ± 1 nM for oxazepam. Three less potent BZ agonists: chlordiazepoxide, medazepam and (+)dihydrodiazepam (DHD) were also examined. DHD is a saturated precursor of diazepam [19]. These BZs also caused concentration-dependent enhancements of TBPS binding with similar maximal enhancements (not shown). Table 1 summarizes the EC_{50} values of enhancements. It also contains ^{3}H-diazepam displacing potencies at 0° available from the literature [19, 20]. Log EC_{50} values correlated fairly well (r = 0.96) with log IC_{50} values, in spite of the temperature differences.

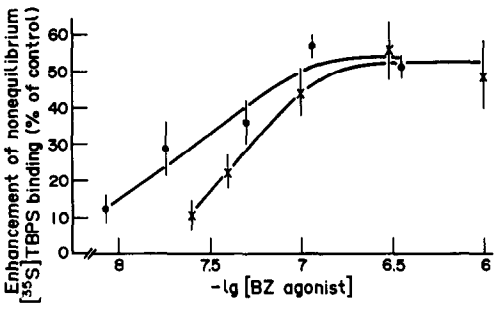

Fig. 2. The effect of BZ receptor agonists on the non-equilibrium binding of ^{35}S-TBPS. Specific binding of 1.5 nM ^{35}S-TBPS was determined for 20 min of incubation at 22° in the presence of various concentrations of flunitrazepam (●) or oxazepam (×) and related to that in control (without BZs). Data are % enhancements over control. Points are mean ± SD of 3–5 experiments.

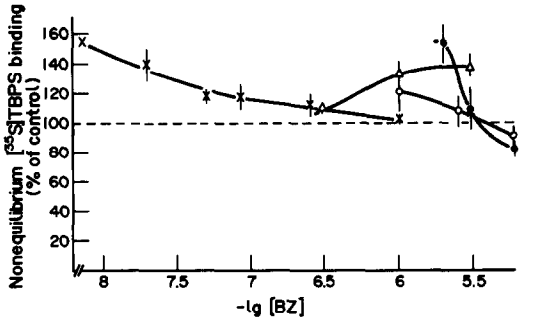

Fig. 3. The effect of BZ receptor antagonists on non-equilibrium ^{35}S-TBPS binding. Specific binding of 1.5 nM ^{35}S-TBPS was determined for 20 min of incubation in the presence of 0.3 μM oxazepam and various concentrations of Ro 15-1788 (×) or oxazepam α,α-Me$_2$-β-Phe-propionate (●) and related to that in control (without BZs). Empty symbols represent the effects of oxazepam α,α-Me$_2$-β-Phe-propionate (○) and its δ-Phe-butyl ether (△) without the addition of oxazepam. The points are mean ± SD of 2–4 experiments.

A maximally enhancing concentration, 0.3 μM of oxazepam was used to investigate the effect of BZ antagonists. Figure 3 shows that the enhancing effect of oxazepam can be antagonized by the α,α-Me$_2$-β-Phe-propionic ester of oxazepam in a concentration-dependent manner. The IC_{50} value of this ester abolishing 50% of the enhancement by oxazepam was about 2–3 μM. This ester *per se* displayed a small enhancing effect at lower concentrations. The highest ester concentration examined both alone and in the presence of oxazepam slightly but significantly decreased the binding of TBPS (Fig. 3). For comparison, the effect of a potent BZ antagonist Ro-15-1788 [10] was also examined. This antagonism required smaller concentrations of Ro-15-1788 but a much broader concentration range (Fig. 3). The IC_{50} value of Ro-15-1788 was 43 ± 11 nM.

Three 3-*O*-derivatives of oxazepam were compared in equimolar concentrations (3 μM) in combination with 0.3 μM oxazepam (Table 2). The structures of the 3-substituents are included in Table 2. The 54% enhancing effect of oxazepam was decreased significantly only by the ester derivatives. A 3-*O*-alkyl derivative with a similar phenyl substituent, the δ-Phe-butyl ether of oxazepam slightly decreased the enhancement by oxazepam (Table 2). This ether derivative alone caused a concentration-dependent enhancement (Fig. 3) the maximal value of which remained below the level by full BZ agonists (Fig. 2).

The displacing potencies of oxazepam derivatives for ^{3}H-flunitrazepam binding were also determined. The same concentration of oxazepam derivatives which partially antagonized the enhancing effect of oxazepam, also partially displaced ^{3}H-flunitrazepam binding (Table 2). However, the rank orders of potencies were not identical. Oxazepam (−)α-Me-β-Phe-propionate was most potent in both tests. The δ-Phe-butyl ether derivative was relatively more potent as a displacer of ^{3}H-flunitrazepam binding, while the α,α-Me$_2$-β-Phe-propionic ester was a stronger antagonist.

DISCUSSION

The demonstration of the effects of BZ receptor ligands on TBPS binding sites required non-equilibrium conditions of TBPS binding [11]. Accordingly, oxazepam did not affect the binding equilibrium of ^{35}S-TBPS measured at 180 min of incubation but accelerated the approach to equilibrium. The trend seen in Fig. 1 could be reproduced by model calculations [18]. The enhancing effect can

Table 1. Half-maximal enhancing concentrations (EC_{50}) of benzodiazepines on ^{35}S-TBPS binding and their ^{3}H-diazepam displacing potencies.

	EC_{50} on ^{35}S-TBPS (nM)	IC_{50} for ^{3}H-diazepam (nM)*
Flunitrazepam	25 ± 13	5
Oxazepam	46 ± 1	38
(+)4,5-Dihydrodiazepam	690 ± 380	360†
Chlordiazepoxide	1220 ± 340	640
Medazepam	950 ± 15	1600

Specific binding of 1.5 nM ^{35}S-TBPS was determined for 20 min of incubation at 22° in the presence of 5–6 concentrations of BZs and related to that in control. Data are mean (±SD) of 4–5 experiments.

* Displacing potencies at 0°, taken from Ref. 20.

† Taken from Ref. 19.

Table 2. The effect of oxazepam and its derivatives on the nonequilibrium binding of ^{35}S-TBPS and the displacing potencies of oxazepam derivatives on ^{3}H-flunitrazepam binding

Oxazepam derivatives	3-Substituents	^{35}S-TBPS*	^{3}H-Flunitrazepam
		(% of control)	
0.3 μM oxazepam (19)	OH	154 ± 9	22 ± 1
3 μM oxazepam (−)α-Me-β-Phe-propionate (4)	O—CO—CH(CH$_3$)CH$_2$Phe	112 ± 6†	33 ± 4
3 μM oxazepam α,α-Me$_2$-β-Phe-propionate (3)	O—CO—C(CH$_3$)$_2$CH$_2$Phe	118 ± 12†	71 ± 12
3 μM oxazepam δ-Phe-butyl ether (8)	O—CH$_2$CH$_2$CH$_2$CH$_2$Phe	142 ± 12	39 ± 8

Specific bindings of 1.5 nM ^{35}S-TBPS and 0.5 nM ^{3}H-flunitrazepam were determined at 22° in double freeze-thawed cortical membrane suspensions in 50 mM Tris HCl containing 300 mM NaCl. Data are means ± SD of the number of experiments indicated in parentheses.

* In the presence of 0.3 μM oxazepam.

† Significantly different from the effect of oxazepam alone ($P < 0.05$).

be attributed to the kinetic modulation of convulsant sites [11]. BZ agonists increase in equal extent the association and dissociation rate constants of TBPS binding so that binding equilibrium remains unaffected. This kinetic modulation can be expressed by a factor (a) whose value is greater than one for accelerating BZ agonists and smaller than one for decelerating inverse agonists [11]. This kinetic modulatory factor is a ratio of modulated/control rate constants, thus dimensionless and independent of the time of incubation, expressing the upper limit of the time-dependent enhancement of binding at $t = 0$ [11]. Acceleration and deceleration might represent opening and closing, respectively, of the attached chloride ionophore [18], possibly via cooperation with GABA receptors [9]. The a value was 1.49 for 0.3 μM oxazepam. This *in vitro* factor is dependent on the occupancy of the modulating BZ receptors, i.e. on the concentration of the BZ. We can characterize the potency of BZs by studying this concentration dependence. BZ concentrations were correlated both with the experimentally observable enhancements and with the calculated kinetic modulatory factors (a). These correlations resulted in EC_{50} values which were not significantly different. Therefore the type of presentation in Fig. 2 can be used in practice to evaluate the *in vitro* potency of BZs. Since the % enhancements also depend on time, in order to study the concentration-dependence of the enhancement, the effect of time-dependence should be excluded by keeping the time of incubation constant. In our system 20 min of incubation was found to be optimal when specific ^{35}S-TBPS binding reached about 25% of the equilibrium value (Fig. 1a); it sufficiently surpassed nonspecific binding and BZ modulated binding substantially exceeded the control. In previous studies 60 or 90 min of incubation were applied [1–6, 8]. These studies did not extend to the determination of EC_{50} values of BZs for the modulation of TBPS binding, partly because high, saturating concentrations of BZ receptor ligands were applied only. In addition, in some studies binding was too close to equilibrium and the maximal effect of BZs amounted only to 10–20% [7, 9], therefore EC_{50} values could not be accurately determined.

Five BZ agonists caused similar maximal enhancements of TBPS binding (Fig. 2) in spite of the great differences in their potencies (Table 1). EC_{50} values correlated with the IC_{50} values. Consequently, the effects of BZs on TBPS binding appear to be mediated via the central type BZ receptors of the $GABA_A$ receptor complex. Moreover, nonequilibrium enhancements of TBPS binding *in vitro* can sufficiently characterize the potency of BZ agonists to modulate the convulsant site.

Anticonvulsant effects of BZ agonists correlated with their brain concentrations [16], e.g. 50% protection of mice from pentetrazol convulsions has been associated with a constant brain level of 0.33 μM for oxazepam, irrespective of the time of BZ pretreatment [13, 15, 16]. This correlation reflects a certain occupancy of central BZ receptors [4, 17]. The potencies of BZ agonists to affect TBPS binding correlate with the antipentetrazol activities of the compounds [7]. Both effects can be prevented by the potent BZ antagonist Ro-15-1788 [6, 8, 10]. The antipentetrazol effect of oxazepam appeared to be antagonized by its phenyl-propionic esters since increasingly higher brain levels of oxazepam were required to elicit the same 50% protection from convulsions when the brain concentrations of these esters varied from 0.3 to 3 μM [13, 15]. These findings led us to examine the antagonist effects of oxazepam derivatives in comparison with Ro-15-1788 through their influence on TBPS binding.

Synaptosomal membranes also possess esterase activity for oxazepam esters [14] and enzymatic formation of oxazepam during incubation at 22° could result in the overestimation of the displacing potency of oxazepam esters [14]. However, displacing potencies of oxazepam derivatives were very similar at 22° and 0° (data not shown). Consequently, displacing potencies of oxazepam esters at BZ receptors appear to be intrinsic such as that of the ether derivative which cannot form oxazepam *in vitro*.

The 0.3 micromolar concentration of oxazepam applied for enhancement corresponded to its antipentetrazol brain level *in vivo* [13, 15] and resulted in a maximal acceleration of TBPS binding *in vitro* (Fig. 2). The effect of oxazepam was antagonized by its α,α-Me$_2$-β-Phe-propionic ester in a concen-

tration-dependent manner (Fig. 3). The antagonist IC_{50} value of this ester is close to the brain concentrations [13, 15], but it is almost two orders of magnitude less potent than Ro-15-1788. The slopes of the concentration-dependent antagonism are strikingly different which might indicate different interactions with the receptor complex for these antagonists. The *in vitro* effect of the ester alone was bidirectional (Fig. 3): submicromolar concentrations enhanced TBPS binding while the highest concentration examined decreased it slightly but significantly. It is similar to the effects of quinolines PK 8165 and PK 9084 which have been explained by their concentration dependent dual activities as BZ agonists and inverse agonists [9]. High ester concentrations both in the absence and presence of oxazepam slightly decelerated the binding of TBPS. This might reflect a slight partial inverse agonist efficacy of the ester similarly to β-carboline-3-carboxylic acid ethyl ester [3]. In contrast, Ro-15-1788 is known to have a slight BZ agonist activity [3]. This might explain that Ro-15-1788 could not completely abolish the enhancing effect of oxazepam in the concentration range of 10^{-7} M–10^{-6} M. The above minor partial agonist and inverse agonist properties might distort in an opposite way the curves in Fig. 3 for the antagonist effect of Ro-15-1788 and the oxazepam ester and result in the remarkable difference in the slopes.

The antagonist activities of 3-*O*-substituted oxazepam derivatives were compared to their ^{3}H-flunitrazepam displacing potencies in equimolar concentrations relevant *in vivo* (Table 2). The same concentration of oxazepam derivatives which antagonized the effect of oxazepam both *in vivo* [13, 15] and *in vitro*, also displaced ^{3}H-flunitrazepam binding (Table 2). Furthermore, the dimethyl ester was less potent than the (−)monomethyl in all *in vitro* (Table 2) and *in vivo* tests [13, 15]. These suggest the involvement of BZ receptors in the effects of oxazepam derivatives. However, a comparison of the two types of *in vitro* activities of Table 2 also reveals dissimilarities. The δ-Phe-butyl ether is less potent as an antagonist than as a displacer of ^{3}H-flunitrazepam. The ether enhanced TBPS binding to the same intermediate level (Fig. 3) to which it decreased the enhancement by oxazepam (Table 2). Consequently, the δ-Phe-butyl ether appears to be a partial agonist and antagonism can be attributed to the 3-*O*-acyl group rather than the ω-phenyl substituent.

Analogous binding mode of Ro-15-1788 and β-carboline inverse agonists to BZ receptors has recently been suggested by the crystallographic comparison of their structures [21]. The ester groups of β-carbolines and Ro-15-1788 have been supposed to attach to the same area of BZ receptors [21, 22]. The flexible ester groups can bind differently and result in mixed agonist/antagonist properties [22]. Since this binding area is also available for the 3-*O*-acyl groups of oxazepam derivatives, it is tempting to speculate that they might analogously bind and elicit dual activities. Bulky 3-alkyl-phenyl substituents decrease not only the potency but also the agonist efficacy of BZs such as for the ether derivative in Fig. 3. Further, in the presence of the 3-*O*-acyl group antagonism becomes dominant.

BZ agonists have been shown to antagonize the inhibitory effect of a protein called GABA modulin on ^{3}H-GABA binding [23]. Some of the above oxazepam esters were shown to attenuate such an antagonism by oxazepam (unpublished observations). It also supports the conclusion that the antagonism by these oxazepam derivatives against the antipentetrazol activity of BZ agonists is exerted via the GABA/BZ receptor complex.

Although the above oxazepam derivatives are unfavourable test compounds due to their biotransformation and weak potency, still they might contribute to our understanding of the structural requirements for a transition from BZ agonists to antagonists. Further, the kinetic modulation of the convulsant TBPS binding by agonists and antagonists of BZ receptors offers a suitable *in vitro* system to characterize not only the efficacy but also the potency of these agents to modulate the GABA receptor Cl^- ionophore complex.

Acknowledgements—Technical assistance of Miss A. Martinek is acknowledged. The authors are grateful to Dr M. Nielsen (Roskilde, Denmark) for the supply of ^{35}S-TBPS and to Dr I. Lukovits (Budapest) for regression analysis. The 4-Phe-butyl-ester of oxazepam, (+)dihydrodiazepam and medazepam were kindly donated by Dr Zs. Tegyey. This study was partly supported by a grant of the Hungarian Academy of Sciences no. AKA-1-3-86-340.

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