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RADIATION INACTIVATION OF BRAIN [^{35}S]*t*-BUTYLBICYCLOPHOSPHOROTHIONATE BINDING SITES REVEALS COMPLICATED MOLECULAR ARRANGEMENTS OF THE GABA/BENZODIAZEPINE RECEPTOR CHLORIDE CHANNEL COMPLEX

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Abstract—[^{35}S]*t*-Butylbicyclophosphorothionate ([^{35}S]TBPS), a bicyclic cage convulsant, binds to the anion gating mechanism of the GABA/benzodiazepine receptor chloride channel complex. Using a carefully calibrated radiation inactivation technique, the molecular weight of [^{35}S]TBPS binding complexes from frozen rat cerebral cortex was estimated to be 137,000 daltons. The GABA agonist muscimol reduced [^{35}S]TBPS binding to 0–10% of the control value, in a way which is independent of the radiation dose. This shows that the GABA receptor (Mw = 55,000 daltons) is included in the 137,000-dalton [^{35}S]-TBPS binding complex; the [^{35}S]TBPS binding protein alone accounts for 137,000–55,000 = 82,000 daltons. The pyrazolopyridazine etazolate (SQ 20.009) and etomidate in appropriate concentrations both reduced specific binding of [^{35}S]TBPS. The ability of SQ 20.009 and etomidate to reduce [^{35}S]TBPS binding was greatly reduced by exposure to low radiation doses, suggesting that SQ 20.009 and etomidate reduce [^{35}S]TBPS binding by an allosteric mechanism requiring a molecular structure of 450,000–500,000 daltons. Benzodiazepine agonists (ethyl 4-methoxymethyl-6-benzyloxy-β-carboline-3-carboxylate, ZK 93423) and inverse agonists (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, DMCM) enhance and reduce [^{35}S]TBPS binding, respectively, in repeatedly frozen and washed membrane preparations. The effects of ZK 93423 and DMCM on [^{35}S]TBPS binding disappeared upon exposure of membranes to low radiation doses. This suggests that the benzodiazepine receptor site interacts allosterically with the [^{35}S]TBPS binding site, requiring a molecular complex of at least *c.* 400,000 daltons. The [^{35}S]TBPS site alone in these latter conditions of membrane preparation (repeatedly frozen/washed) revealed a molecular weight of 221,000 daltons (TBPS-site + GABA receptor + unknown structures). The number of binding sites for [^{35}S]TBPS (145 pmol/g tissue) was only slightly higher than for [^{3}H]flunitrazepam (130 pmol/g tissue) in cerebral cortex. These results are all consonant with the conclusion that the GABA/BZ receptor chloride channel complex is composed of highly integrated multimeric subunits, tentatively accounted for by a tetramic complex of molecular weight 548,000 daltons.

Receptors for GABA and benzodiazepines (BZ) are tightly coupled in the plasma membrane of neurons in the central nervous system of higher vertebrates [for reviews see 1–3]. GABA agonists enhance the membrane conductance for chloride and certain other anions. The benzodiazepine receptor is a modulatory unit which allosterically regulates the gain in the GABAergic neurotransmission system. When benzodiazepine receptor ligands with positive efficacy (agonists) are present at the BZ receptor, there is an enhancement of the GABA-stimulated chloride conductance. Conversely, GABA-stimulated chloride conductance is reduced when benzodiazepine receptor ligands with negative efficacy (inverse agonists) are present at the BZ receptor (further discussion [4]). The molecular weight of single GABA receptor proteins (subunit) and of single BZ receptor proteins (subunit) have each been determined to be *c.* 50,000 daltons [5, 6] (Fig. 1). Preliminary experiments suggest that the molecular size of the chloride gating mechanism is 137,000 daltons [7].

The chloride gating mechanism can be detected by the radioligands [^{3}H]picrotoxinin and [^{35}S]TBPS [8, 9]. In the present study we have applied the radiation inactivation technique [10] for determining the functional molecular weight of the chloride gating mechanism as detected by the [^{35}S]TBPS binding. We also investigated whether the regulation of [^{35}S]TBPS site by GABA agonists, by benzodiazepine receptor ligands, and by SQ 20.009 and etomidate was sensitive to irradiation. The latter studies revealed unexpected insight into the molecular organization of the GABA/BZ receptor chloride channel complex.

METHODS

Irradiation. Tissue samples, either frozen whole tissue or frozen brain tissue homogenates (64 mg original tissue in 400 μl buffer) in 15 ml glass test

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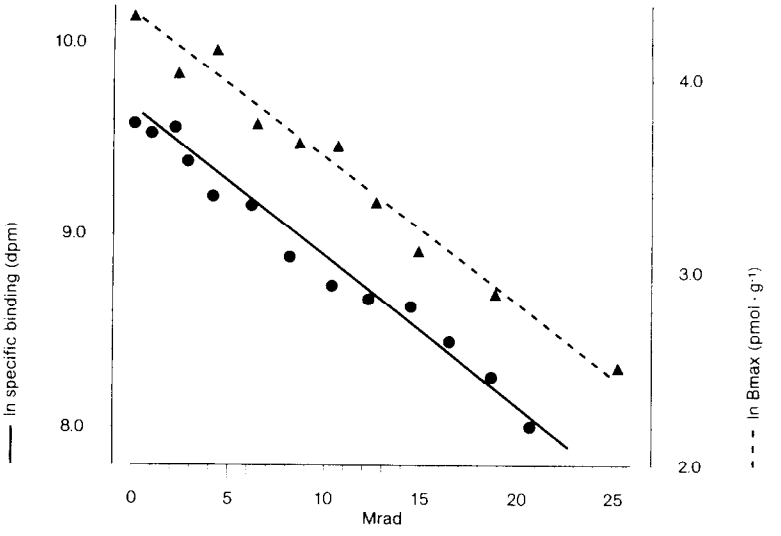


Fig. 1. Radiation inactivation of high affinity GABA-A receptors in rat brain. ▲—▲, Scatchard plot of [^{3}H]muscimol binding were done on cortex exposed to various doses (0–25 Mrad) of radiation. After radiation membranes were treated with Ag^+ (see Methods). Upon irradiation the affinity constant, K_D, decreased (K_D was 22 nM, 9 nM and 6 nM at 0, 6 and 12 Mrad, respectively). The figure is representative of four experiments showing a molecular weight of 54,800 ± 5200 daltons (mean ± S.D., N = 4). ●—●, [^{3}H]GABA (10 nM) binding in cerebellum exposed to irradiation. After radiation cerebellum was homogenized and treated with 0.05% Triton X-100 (see Methods). The affinity constant, K_D, was not changed upon radiation (K_D = 13, 16 and 16 nM at 0, 10 and 18 Mrad, respectively). The figure is representative of three experiments showing a molecular weight of 55,200 ± 2300 daltons (mean ± S.D., N = 3).

tubes were irradiated in the presence of atmospheric air. We prefer to irradiate frozen whole tissue because this represents the most unperturbed form of tissue available. In some experiments, however, elaborate manipulations (repeated freezing/thawing and washings) were required before the final binding assay (Figs. 6–8); for the irradiation on these preparations we preferred to reduce variation due to manipulation by freezing and washing prior to irradiation.

The samples were cooled to −10° to −15° prior to exposure to radiation which was delivered in doses of 0.5–2 Mrad. In between exposures, samples were cooled to −15° for at least 2 min to ascertain that they remained completely frozen during the irradiation process. Radiation was delivered as 10 MeV electrons at the linear accelerator at Risø, Denmark. The total dose of radiation was determined using a calibrated thermo dosimeter (water).

Calibration. Molecular weights of unknowns were obtained by comparison with enzymes of known molecular weight, either native brain enzymes (cholinesterase, glutamic acid decarboxylase, lactate dehydrogenase and pyruvate kinase) or enzymes which were injected into the brain tissue (yeast alcohol dehydrogenase, horse liver dehydrogenase and *E. coli* β-galactosidase). Calibration was not performed in each radiation experiment. Experience from weekly irradiations over a period of two years indicated that day-to-day variation in the radiation output was negligible; one single calibration factor, [K daltons × Mrad], was therefore used in these experiments. The K value was determined in the following way. Several enzymes were exposed to 10–14 radiation doses at weekly intervals. For each enzyme the K value was determined from the expression $K = Mw/k$, where Mw is the molecular weight of the enzyme and k is the radiation inactivation constant determined experimentally from the slope of the logarithmic transcription of the decay curve: $A = A_0 \times e^{-kD}$, where A is the activity in enzyme units after exposure to a dose of D Mrad; A_0 is the enzyme activity at $D = 0$. In frozen whole cortex the radiation inactivation constant, k Mrad^{-1}, was estimated for (Mw, daltons; reference): yeast alcohol dehydrogenase (148,000; [11]), $k = 0.202$; *E. coli* β-galactosidase (116,248; [12]), $k = 0.157$; pyruvate kinase (114,000; the subunit in, e.g., bovine skeletal muscle is 57,000 daltons [13]), $k = 0.152$; horse liver alcohol dehydrogenase (73,000; [14]), $k = 0.095$; lactate dehydrogenase (70,000; the subunit is 35,000 daltons [15]), $k = 0.108$; cholinesterase (70,000; [16]), $k = 0.087$; glutamic acid decarboxylase (67,000; [17]), $k = 0.098$. A mean calibration factor of K = 730,000 daltons × Mrad was determined as the mean for all enzymes. The K value of 730,000 daltons × Mrad is closely similar to a value calculated from Kempner's [18] data: 640,000 × 1.2 = 768,000 daltons × Mrad (1.2 is the temperature factor at −10° [18]). Apparent molecular weights in the present study were calculated according to $Mw = 730{,}000 \times k$ daltons.

The enzymatic activities of yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, *E. coli* β-galactosidase and cholinesterase were estimated essentially as previously described [19]. The activities of pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.28) were determined in

aliquots of the supernatant after centrifugation of brain homogenate. The Boehringer Mannheim test combination 126047 was used for estimation of pyruvate kinase while lactate dehydrogenase was estimated as described in [20]. The activity of glutamic acid decarboxylase (EC 4.1.1.15) was determined in crude brain homogenate according to [21]. The measured radiation inactivation constants of pyruvate kinase and lactate dehydrogenase are best correlated with molecular weights assuming that these enzymes in frozen whole cortex are inactivated as dimers.

Tissue preparation. Cerebral cortices (*c.* 250 mg) from one hemisphere of rats were frozen at −20° in a test tube and irradiation was delivered 18 hr to 5 days later. Tissues were homogenized 24–144 hr after irradiation in 2 × 5 ml ice-cold 50 mM Tris-citrate pH 7.1 by an Ultra Turrax homogenizer. The membranes were washed three times by centrifugation at 30,000 *g* for 10 min at 0° and rehomogenization as before. After the final (3rd) centrifugation, the pellet was rehomogenized in 100 ml/g original tissue in a 50 mM Tris-citrate buffer, pH 7.1, including NaCl in various concentrations (100–1000 mM).

Initially we observed that the effects of benzodiazepine receptor agonists and inverse agonists on [^{35}S]TBPS binding were more reproducible in a triple-frozen/washed membrane preparation, therefore, in these experiments brain tissue was triple-frozen/washed before irradiation: Frozen tissue (−50°) was thawed, homogenized in 2 × 5 ml ice-cold 50 mM Tris-citrate, pH 7.1, by an Ultra Turrax homogenizer and centrifuged at 30,000 *g* for 10 min at 0°. The membranes were washed twice by rehomogenization and centrifugation as before. The resulting pellet, which had been frozen (−20°) overnight, was thawed and washed twice by rehomogenization and centrifugation. The pellet was frozen (−50°) and stored for two days at −20° before thawing and another two washes by rehomogenization and centrifugation. The final pellet was resuspended in 50 mM Tris-citrate pH 7.1 (6.25 vol/g original tissue) and aliquots of 400 μl were stored at −20° overnight before irradiation. The irradiated samples were stored at −20° for 1–3 days before thawing and dilution in 50 mM Tris-citrate buffer, pH 7.1 containing 100–1000 mM NaCl (100 vol/g original tissue) and used for binding assays.

[^{35}S]*TBPS binding assays.* Aliquots of 0.5 ml tissue homogenate was incubated for 60–180 min at 25° with 25 μl [^{35}S]TBPS working solution (Butyl bicyclophosphorothionate, tertiary-[^{35}S]-; 15–29 Ci/mmol, NEN, Boston; prepared fresh in water) to give a final concentration of 0.9 nM. Nonspecific binding, identified as binding in the presence of picrotoxinin (Sigma), 10 μg/ml, was always subtracted from total binding to give specific binding. Test substances were added in a volume of 25 μl aqueous solutions prior to the radioligand. After incubation, 10 ml ice-cold 50 mM Tris-citrate pH 7.1, was added, and the sample was vacuum filtered immediately through Whatman GF/C glass fibre filters followed by another 10 ml wash. Radioactivity on filters was measured by conventional scintillation counting at *c.* 95% efficiency.

A 60 min incubation period is not sufficient to reach equilibrium, depending on the membrane preparation (slower in triple-frozen) and the concentration of NaCl (slower at higher concentrations). In experiments designed to show allosteric interactions, pre-equilibrium incubation times (normally 60 min) were chosen; for Scatchard analyses, equilibrium conditions were used as described in legend to figures.

[^{3}H]*GABA binding assays.* Frozen non-irradiated or irradiated tissue was homogenized in 10 ml (approx. 40 vols) Tris-citrate 50 mM, pH 7.1 by an Ultra Turrax homogenizer. The homogenate was centrifuged at 30,000 *g* for 10 min and the pellet was resuspended in 20 vols (w/v) of Tris-citrate 50 mM, pH 7.1, containing 0.05% Triton X-100. Following incubation at 0° for 30 min the suspension was centrifuged for 10 min at 30,000 *g*. The pellet was washed twice in Tris-citrate by homogenization and centrifugation. The final pellet was resuspended in Tris-citrate 50 mM, pH 7.1 (10 mg original tissue/ml). Aliquots of 1 ml were added 25 μl [^{3}H]GABA (38.5 Ci/mmol, NEN) final concentration 10 nM, incubated at 0° for 30 min, and filtered through Whatman GF/C glassfiber filters. The filters were washed with 2 × 10 ml ice-cold Tris-citrate 50 mM, pH 7.1. Non-specific binding was obtained by adding GABA (10^{-4} M final concentration) to separate samples. All assays were performed in duplicate. [^{3}H]GABA concentrations in the range 1–85 nM were used for Scatchard analyses.

[^{3}H]*muscimol binding assays.* Tissue (non-irradiated or irradiated) was homogenized in 10 ml (approx. 40 vols) Tris-citrate 50 mM, pH 7.1, by an Ultra Turrax homogenizer and centrifuged at 30,000 *g* for 10 min at 0°. The pellet was resuspended in 50 vol (w/v) of Tris-citrate 50 mM, pH 7.1, containing 0.10 mM $AgNO_3$, incubated at 0° for 30 min and centrifuged for 10 min at 30,000 *g*. The pellet was washed twice in Tris-citrate buffer and the final pellet was resuspended in Tris-citrate 50 mM, pH 7.1, at a concentration of 20 mg original tissue/ml. Aliquots of 0.5 ml were used for binding of [^{3}H]muscimol (20.6 Ci/mmol NEN) as described for [^{3}H]GABA binding. The concentrations of [^{3}H]-muscimol were in the range 1–55 nM for Scatchard analysis.

RESULTS

Binding of [^{35}S]*TBPS.* In preliminary experiments we found that specific binding of [^{35}S]TBPS was greatly enhanced when tissues or homogenates were frozen before assay. In frozen triple-washed tissues we obtained a high level of specific binding in the presence of 1000 mM NaCl (*c.* 3000 cpm per assay at 0.9 nM [^{35}S]TBPS) and low nonspecific binding (10–20% of total binding). A saturation analysis showed that the number of [^{35}S]TBPS sites was 145 pmol/g (equilibrium conditions, 3 hr incubation) similar to the value for [^{3}H]FNM 130 pmol/g. The K_D values were 12 nM (TBPS) and 1.2 nM (FNM) (Fig. 2).

Irradiation. Figure 3 shows that the apparent radiation inactivation constant for the [^{35}S]TBPS site, *k*, is dependent on the concentration of chloride in the assay medium. At 1000 mM NaCl we found a *k* value

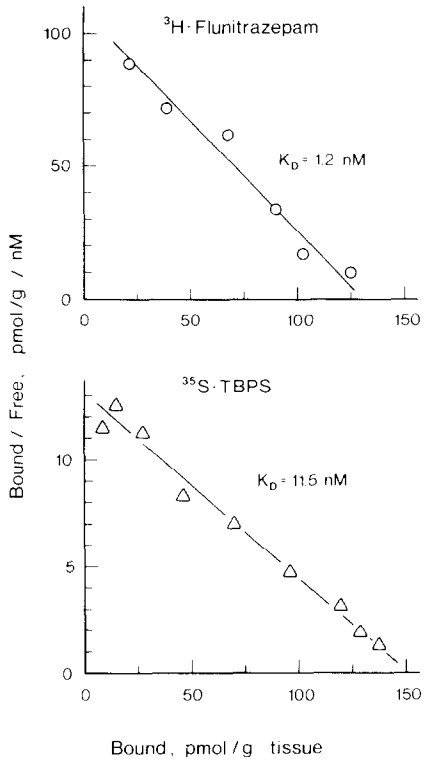


Fig. 2. Scatchard plot of [^{35}S]TBPS binding (2–70 nM) to once-frozen triple-washed rat cortex membranes; incubation time 3 hr in the presence of 1000 mM NaCl. Also for comparison is the [^{3}H]flunitrazepam binding ([^{3}H]FNM, 76.9 Ci/mmol, Amersham) (0.24–12.3 nM); assay as for [^{35}S]TBPS except incubation time (which was 60 min), temperature (0°), and definition of non-specific binding (clonazepam, 0.3 μM).

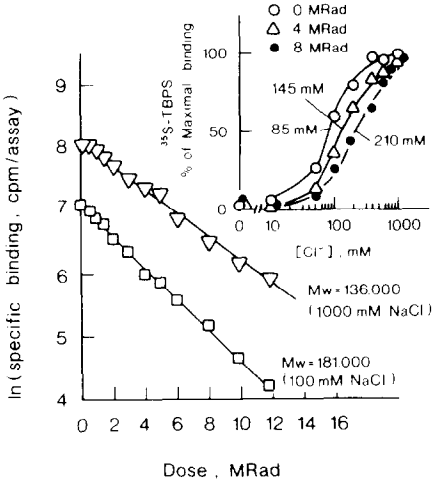


Fig. 3. Radiation inactivation of [^{35}S]TBPS (0.9 nM) binding sites in the presence of 100 mM or 1000 mM NaCl. Once-frozen, triple-washed rat cortex tissue was incubated for 60 min. Inset, dose response for NaCl in rat cortices exposed to 0, 4 and 8 Mrad. EC_{50} values for Cl^- are shown.

Table 1. Irradiation of frozen whole cortex does not change the affinity of [^{35}S]TBPS binding

Dose of radiation (Mrad)	Affinity of [^{35}S]TBPS binding (K_D, nM)	Inhibition by IPTBO (IC_{50}, nM)
0	12.5 ± 1.5 N = 4	195, 270
2	12, 10	205
4	14, 12	255
6	15, 14	220, 320
8	15, 15	260
10	15, 15	255

Frozen whole cortex was irradiated. Scatchard plot of [^{35}S]TBPS binding (concentrations within 0.5–40 nM) and inhibition of [^{35}S]TBPS (0.9 nM) binding by isopropylbicylophosphate (IPTBO) were assayed in a thrice-washed membrane suspension (final pellet in Tris-citrate, 50 mM, pH 7.1, containing 1 M NaCl, incubation at 25° for 3 hr). IC_{50} values for inhibition of [^{35}S]TBPS (0.9 nM) binding by GABA were: 0 Mrad, 8.7 ± 5.5 μM (N = 5); 3 Mrad, 11 ± 5 μM (N = 4) and 6 Mrad, 18 ± 10 μM (N = 4) (mean ± S.D.). Hill coefficients were 1.04, 0.94 and 0.89 at 0, 3 and 6 Mrad, respectively.

of 0.188 Mrad^{-1}, corresponding to Mw = 136,000 daltons. An average value, Mw = 137,000 ± 18,000 daltons (mean ± S.D., nine values) was determined from values including both 60 min incubation (Fig. 3) and 3 hr incubation (equilibrium, data not shown). This value corresponds to loss of binding sites, not to a change in affinity for the cage convulsant in the course of radiation. This was shown by determining the affinity constant, K_D, for [^{35}S]TBPS binding and the IC_{50} for the close TBPS analogue, isopropylbicyclophosphate (IPTBO). Various doses of radiation (0, 2, 4, 6, 8 and 10 Mrad) induced no marked changes either in K_D values or in IC_{50} values (Table 1). At 100 mM of NaCl, we found a k value of 0.249 Mrad^{-1}, corresponding to Mw = 181,000 daltons. This value is probably artifactal, because the chloride affinity was reduced by radiation (Fig. 3 inset). Thus the apparent inactivation observed in the presence of 100 mM NaCl is a combination of disappearance of [^{35}S]TBPS binding molecules and a concerted decrease in the binding affinity of [^{35}S]-TBPS. Figure 3 inset shows that chloride must be present at more than 700 mM to obtain near to maximal stimulation of [^{35}S]TBPS binding after various doses of radiation.

Muscimol and other GABA agonists reduce [^{35}S]-TBPS binding to low values ([9] see also Fig. 4 and Table 1) by an allosteric mechanism (bicuculline antagonizes muscimol, not shown). Irradiation did not impair the inhibitory effect of muscimol or GABA on [^{35}S]TBPS binding, except for a slight (1.5–2 fold) increase in the EC_{50} for GABA and muscimol. Independently of radiation dose muscimol reduced binding of [^{35}S]TBPS to less than 10% of the control value (similar results were obtained with GABA, THIP and piperidine-4-sulphonic acid (data not shown)). This shows that the [^{35}S]TBPS binding molecular entity, which disappears with a rate corresponding to Mw = 137,000 daltons, still carries the muscimol recognition site (the GABA receptor).

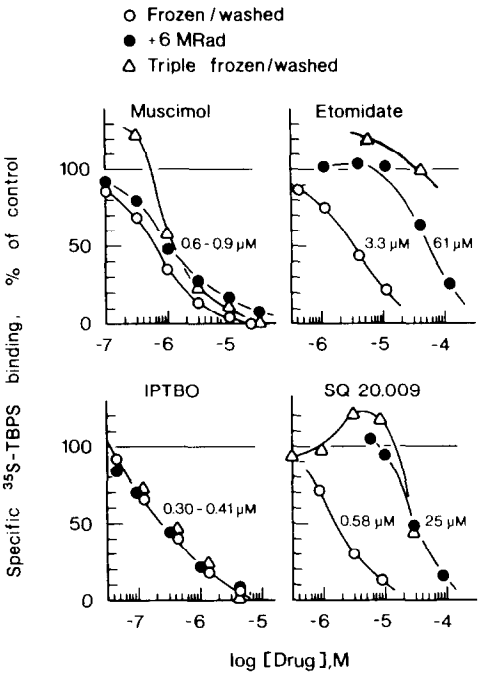


Fig. 4. Inhibition of specific [^{35}S]TBPS binding in three different membrane preparations, by muscimol, IPTBO, etomidate and SQ 20.009. Shown are IC_{50} values. Incubation time 60 min. Frozen whole cortex non-irradiated (○) or irradiated with 6 Mrad (●) were triple washed after radiation and used for binding assays. A triple-frozen/washed membrane preparation was irradiated (△) and after radiation used directly for binding assays.

The molecular weight of the GABA receptor was 54,800 daltons and 55,200 daltons using [^{3}H]muscimol and [^{3}H]GABA, respectively, as radioligands (Fig. 1). If the GABA receptor had been inactivated independently of the [^{35}S]TBPS site there should have been a 36% loss of GABA receptors after 6 Mrad ($A = a_0 \times e^{-0.074 \times 6} = 0.64\,A_0$), and consequently there should have been more than 36% of the [^{35}S]TBPS sites which had lost their recognition site for muscimol; this was not the case.

Binding of [^{35}S]TBPS is also reduced by low concentrations SQ 20.009 and etomidate ([9] see also Fig. 4). These agents are believed to interact with the chloride gating mechanism of the GABA/BZ receptor chloride channel complex [2, 22, 23].

In contrast to the results obtained for IBTBO and muscimol, it appeared that inhibition of [^{35}S]TBPS binding by SQ 20.009 and etomidate was highly affected by irradiation. After a dose of 6 Mrad (Fig. 4), there was a 20–40 fold reduction in the affinity of SQ 20.009 and of etomidate. There was no further reduction in the affinity of SQ 20.009 or etomidate after irradiation with 14 Mrad where only 9% of the original [^{35}S]TBPS sites remained (data not shown). High concentrations of SQ 20.009 (5 μM) reduced the B_{max} value for [^{35}S]TBPS binding to 40% of control and enhanced the apparent K_D value from 9.3 to 18.9 nM (data not shown); the inhibition is mixed competitive/noncompetitive. The loss of affinity of SQ 20.009 by radiation, paired with the result that complete inhibition at high concentration is preserved, indicates that the SQ 20.009 recognition site resides within the 137,000-dalton [^{35}S]TBPS site, but that this site shifts conformation from a high to a low affinity form when a large molecular weight structure deteriorates. This situation would be parallel to the situation where BZ receptor agonists lose their affinity for BZ receptors by u.v./flunitrazepam exposure (24–26).

We attempted to obtain information about the size of the allosteric complex responsible for the high affinity SQ 20.009 sites by following the irradiation-dependent disappearance of the inhibitory effects of SQ 20.009 (2.3 μM) on [^{35}S]TBPS binding. Figure 5 shows that SQ 20.009 at 2.3 μM rapidly loses its ability to inhibit [^{35}S]TBPS binding. After 4 Mrad, SQ 20.009 (2.3 μM) inhibits [^{35}S]TBPS binding by only 12% as compared to 77% before irradiation. The size of the allosteric unit can be approximated from the slope of the decay curve (Appendix). The

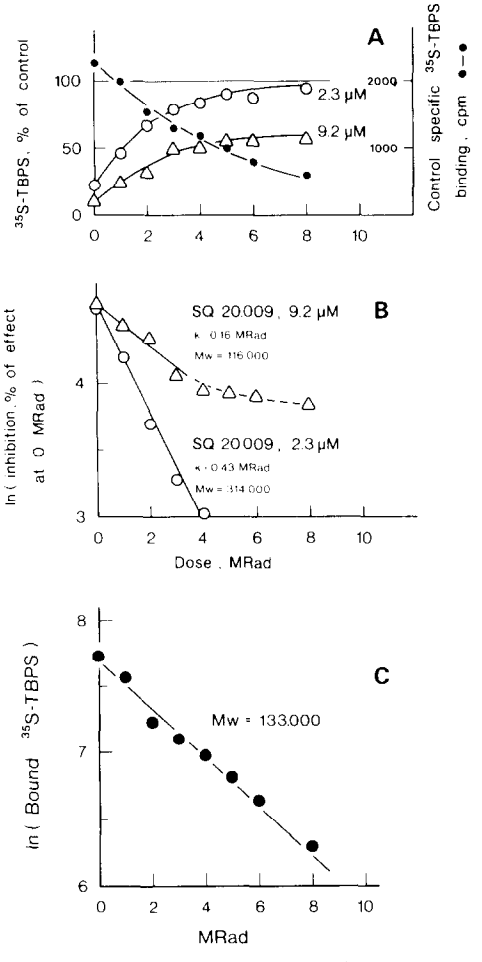


Fig. 5. (A) Radiation inactivation of [^{35}S]TBPS binding sites; loss of inhibition by SQ 20.009. Frozen whole cortex from rat brain was irradiated; triple-washed membranes were incubated for 60 min in the presence of 1000 M NaCl. Control is specific binding of [^{35}S]TBPS in the absence of SQ 20.009. (B) Semi-logarithmic plot of the percentage effect of SQ 20.009; data from (A). (C) Semi-logarithmic plot of the decay of [^{35}S]TBPS binding; data from (A).

radiation inactivation constant $k = 0.43$ Mrad^{-1} suggests a *Mw* of *c.* 314,000 daltons for the allosteric unit. A second identical experiment yielded a value of 321,000 daltons. Note that the [^{35}S]TBPS binding entity (133,000 daltons in this experiment, Fig. 5C) is not included in the value for the allosteric unit. The total functional size of the complex, which is a minimum value, is *c.* 314,000 + 133,000 = 447,000 daltons.

The actual value obtained for the allosteric unit is subject to several artifactual interactions. For example, high concentrations of SQ 20.009 affect [^{35}S]-TBPS binding in additional ways than by competitive interaction. At high SQ 20.009 concentration, inhibition of [^{35}S]TBPS binding persists after irradiation; apparently this inhibition is not dependent on a high molecular weight structure, and consequently a low molecular weight for the allosteric complex will be obtained. For example in the presence of 9.2 μM of SQ 20.009, the molecular weight corresponding to the loss of SQ 20.009 effect was determined to be only 116,000 daltons, with a curvilinear decay function (Fig. 5). Values obtained at 4.6 μM SQ 20.009 were intermediate between the values obtained at 2.3 and 9.2 μM (data not shown). In addition, the determination of the size of the allosteric unit will be sensitive to changes in the affinity for [^{35}S]TBPS which are not due to SQ 20.009. Chloride ions, for example, will have an effect (see also Fig. 3). When the experiment depicted in Fig. 5 was conducted in 200 mM or 100 mM NaCl, progressively increasing, apparently artifactal, values for the molecular weight allosteric unit were obtained due to the gradual loss in chloride stimulation of [^{35}S]TBPS binding (data not shown). The value obtained for the allosteric unit (314,000 daltons) therefore indicates the order of magnitude rather than an exact value.

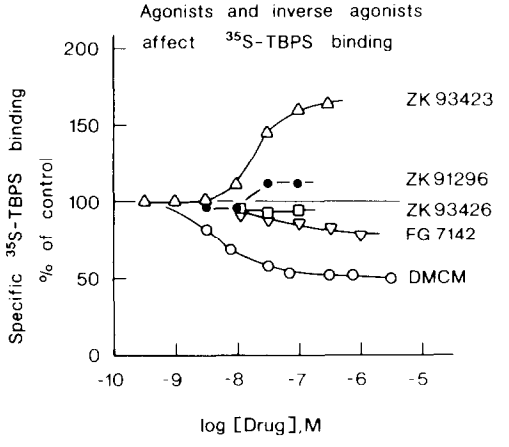


Fig. 6. Inhibition and enhancement of [^{35}S]TBPS binding to triple-frozen/washed rat cortex tissue by a benzodiazepine receptor agonist (ZK 93423, ethyl 4-methoxymethyl-6-benzyloxy-β-carboline-3-carboxylate), a partial agonist (ZK 91296, ethyl 4-methoxymethyl-5-benzyloxy-β-carboline-3-carboxylate), a benzodiazepine receptor antagonist (ZK 93426, ethyl 4-methyl-5-isopropoxy-β-carboline-3-carboxylate), a partial inverse agonist (FG 7142, *N*-methyl β-carboline-3-carboxamide) and an inverse agonist (DMCM, methyl 4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate). Incubation time 60 min.

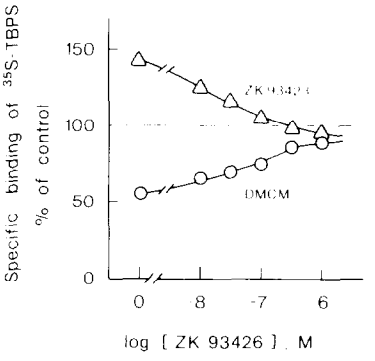


Fig. 7. The benzodiazepine receptor antagonist ZK 93426 antagonizes the effects of ZK 93423 (100 nM) and DMCM (100 nM) on [^{35}S]TBPS binding; control [^{35}S]TBPS binding was 2316 cpm, non-specific binding was 250 cpm. See legend to Fig. 6 for further details.

Benzodiazepine agonists enhance and benzodiazepine inverse agonists (such as DMCM) reduce [^{35}S]TBPS binding [27]. We observed that this effect was less reproducible in frozen thrice-washed cortex than in triple-frozen/washed cortex tissue. The effects on [^{35}S]TBPS binding of five β-carboline derivatives representing agonists, antagonists and inverse agonists of BZ receptors are shown in Fig. 6. Figure 7 shows that the benzodiazepine receptor antagonist, ZK 93426, antagonizes both the BZ receptor agonist, ZK 93423, and the inverse agonist, DMCM. The enhancement or reduction by ZK 93423 or DMCM of [^{35}S]TBPS binding is caused by an effect on both association rates and on dissociation rates of [^{35}S]TBPS (not shown), so that the effect is negligible at equilibrium. In addition we observed that [^{35}S]TBPS binding in triple-frozen/washed tissue was less susceptible to inhibition by etomidate and SQ 20.009; in fact, SQ 20.009, etomidate and muscimol first enhanced (at low concentrations) and then reduced (at high concentrations) [^{35}S]TBPS binding in triple-frozen/washed tissue (Fig. 4; see also ref. [37]).

When homogenates of triple-frozen/washed membranes were subjected to irradiation, the capability of DMCM and of ZK 93423 to reduce and enhance [^{35}S]TBPS binding, respectively, gradually disappeared. After a dose of 4 Mrad, DMCM reduced [^{35}S]TBPS binding by only 23% as compared to 49% in non-irradiated membranes (Fig. 8A). As described for the experiments with SQ 20.009, we obtained information about the apparent molecular weight of the allosteric benzodiazepine receptor complex by calculating the inactivation constant for the effect of ZK 93423 and of DMCM on [^{35}S]TBPS. Figure 8B indicates that the order of magnitude of the allosteric components for DMCM was 146,000 daltons and for ZK 93423, 263,000 daltons. Average values from three independent experiments were 177,000 ± 38,000 daltons and 282,000 ± 24,000 (mean ± S.D., N = 3) for the DMCM and ZK 93423 capability, respectively, to affect [^{35}S]TBPS binding. Again the values do not include the size of the [^{35}S]-

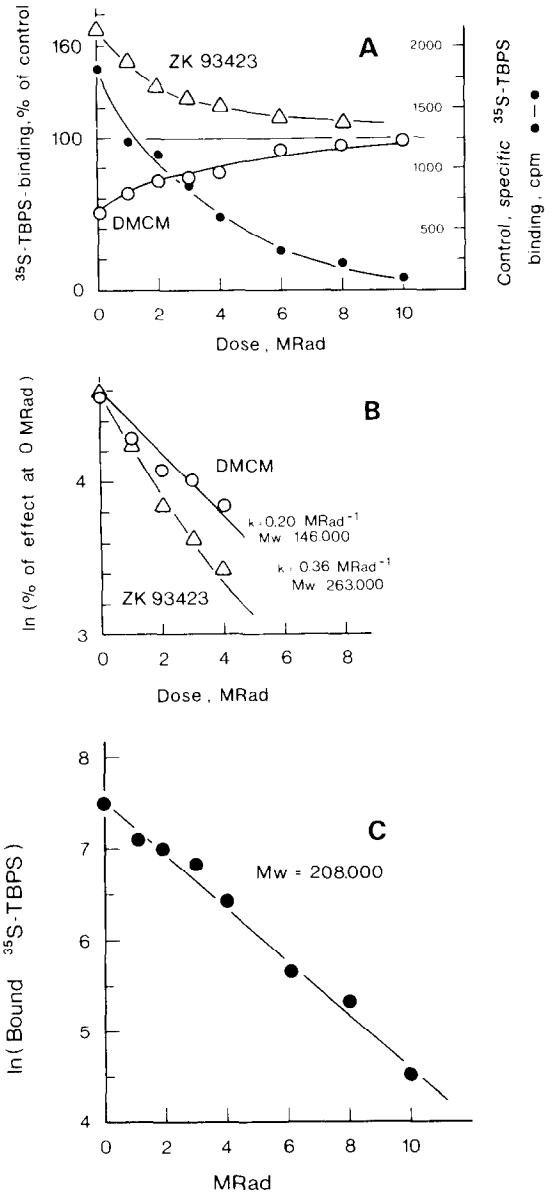


Fig. 8. (A) Radiation inactivation of [^{35}S]TBPS binding; loss of enhancement by ZK 93423 (100 nM) and decrement by DMCM (100 nM). Samples of triple-frozen/washed tissue was used, incubation time 60 min, at 25° in the presence of 1000 mM NaCl. Control is specific [^{35}S]TBPS binding in the absence of DMCM or ZK 93423. (B) Semi-logarithmic plot of the percentage effect (see Appendix); data from (A). (C) Semi-logarithmic plot of the decay of [^{35}S]TBPS binding; data from (A).

TBPS site, which in these membrane preparations (Fig. 8C) yielded 221,000 ± 11,000 daltons (mean ± S.D., N = 3) (determined both at 60 min incubation and at equilibrium, 5 hr incubation), which is above the value found in frozen, thrice-washed cortex tissue. The minimal size of the [^{35}S]-TBPS binding DMCM regulatory unit would then be 177,000 + 221,000 = 398,000 daltons.

It should be noted that calibration of the irradiation setup was done using frozen cortex tissue. A separate calibration was not done for the BZ-ligand experiments, where tissue was irradiated in form of frozen membrane preparations; the absolute values for molecular sizes are therefore less accurate in these latter experiments.

DISCUSSION

The radiation inactivation theory is based on the assumption that the chance of "hitting" a molecule with a package of energy, for example from a linear accelerator, is dependent on the mass of the molecule. In addition, it is assumed that the biological function of the molecule is completely destroyed by one single "hit" (energy transfer). These assumptions have been validated for a number of enzyme activities and also for some receptors [see 28–31].

The theory of the radiation inactivation technique is poorly understood. The precise mechanism is not known by which one of the high energy electrons transfers a package of energy to a protein molecule with the consequence that number of peptide bonds are destroyed. Furthermore, it is not known to what extent the energy absorbed in one peptide chain can be transferred to tightly, but not covalently attached, adjacent peptide chains. Likewise, it is not clear to what extent energy is transferred via S—S bridges.

We have corroborated the validity of the irradiation technique in our experimental setup by calibrating the system with a number of enzymes with known molecular weight.

In our conditions, there is a tight correlation between the radiation inactivation constant, k, and the molecular weight for a given protein (see Methods).

We have taken care to avoid known artifact in the determination of the molecular weight. Samples remained frozen for at least 24 hr after irradiation to reduce inactivation of receptor by free radicals. Tissues were irradiated in concentrated form (whole cortex or low dilutions).

The advantage of the radiation inactivation technique is that the *functional* mass (molecular weight) of a given protein can be determined without isolation of the protein from its membraneous and other environments. By functional mass [32] we mean the sum of all single proteins and other entities, either covalently bound (for example S—S bridges), or by other attachments, which are necessary for a given measurable function to be expressed. To illustrate this idea, consider the two proteins in Fig. 9; their

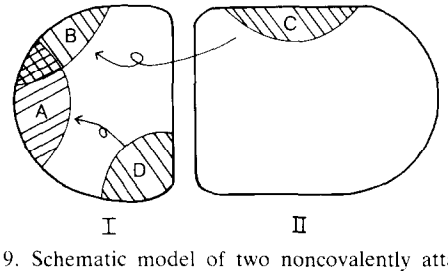


Fig. 9. Schematic model of two noncovalently attached proteins, I and II, which carry binding sites for A, B, C and D. See Discussion for a description.

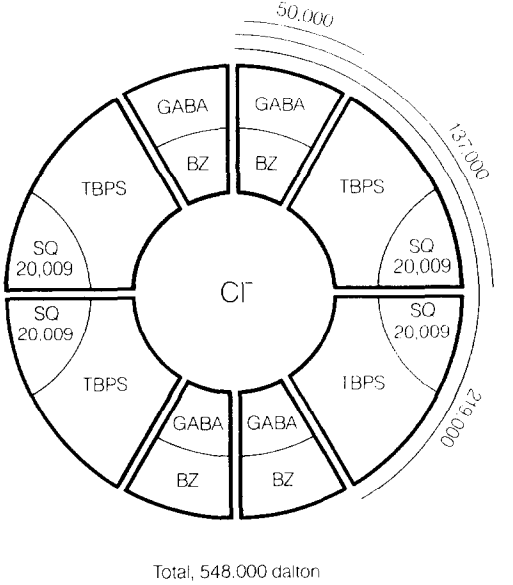
molecular weight is 50,000 daltons for I and 75,000 daltons for II. Protein I carries binding sites for radioligand [^{3}H]A and [^{3}H]B; in addition protein I and II carry binding sites for C and D. Suppose that I and II are non-covalently attached, and that irradiation will destroy only one protein by each hit; the rate of disappearance of [^{3}H]A binding activity after irradiation will correspond to a molecular weight of [^{3}H]A sites of 50,000 daltons. Suppose that [^{3}H]B requires the presence of protein II to shape protein I in the correct conformation for binding of [^{3}H]B. Binding of B to protein I is dependent in this way on the presence of protein II. The functional molecular size of [^{3}H]B binding will therefore be 125,000 daltons, because destruction of either I or II will preclude binding of [^{3}H]B. Thus, even though the "functional size" of [^{3}H]B binding is 125,000 daltons, only one protein decomposes by each hit. If both subunits were destroyed by one single hit, which may occur for some S—S linked proteins, then both [^{3}H]A and [^{3}H]B binding will exhibit masses of 125,000 daltons. The example above illustrates the term "functional size"; note that two ligands may bind to the same proteins and yet exhibit different decay rates (functional masses); this fact has often been overlooked, for example also in heat inactivation studies where differences in heat inactivation rates have been interpreted as showing the existence of different binding proteins.

Figure 9 furthermore illustrates consequences of allosteric interaction via a separate protein. Suppose that occupation of a site in protein II by "C" reduces or enhances the affinity of B for the receptor on protein I. In this case, binding of [^{3}H]B will still disappear corresponding to a size of 50,000 daltons; however, the capability of C to reduce (or enhance) [^{3}H]B binding will disappear even faster, with a decay corresponding to 75,000 daltons. However, an agent, such as "D", which allosterically reduces [^{3}H]A binding by interacting with a site located within protein I, will not lose its capability to reduce [^{3}H]A binding upon radiation; whatever the number of molecules of I still being intact, they will all carry D-sites.

The present results show that the basal, lowest-obtained, molecular size of [^{35}S]TBPS binding was 137,000 daltons. High NaCl concentrations (1000 mM) were used to exclude artifact due to changes in the chloride affinity during irradiation. It was also shown that a loss of binding sites, not a change in affinity, corresponds to this molecular weight. Interestingly, it will be clear that the value of 137,000 daltons includes the GABA receptor, because the GABA agonist muscimol retained its capability of reducing [^{35}S]TBPS binding after irradiation. If the GABA receptor was coupled allosterically to a 137,000 daltons [^{35}S]TBPS binding protein, we would have expected that muscimol had lost 36% of its effect after 6 Mrad. This was not the case (Fig. 4): muscimol was fully active, also, after irradiation. The molecular weight of the GABA receptor is approximately 55,000 daltons (as determined by irradiation with [^{3}H]muscimol or [^{3}H]-GABA as radioligands (Fig. 1)) and by photoaffinity labelling of GABA receptors by [^{3}H]muscimol followed by SDS electrophoresis [6]. This leaves 82,000 daltons for the [^{35}S]TBPS binding protein. Apparently, the TBPS site cannot bind TBPS when the GABA receptor is destroyed; therefore, the total measured size is 137,000 daltons. Conversely, the GABA receptor can bind muscimol, even when the TBPS site (82,000 daltons) is destroyed (see Fig. 10). The BZ/GABA receptor has been purified by affinity chromatography [33, 34]. Those studies did not reveal a 80,000 protein which might represent the TBPS-binding site. The evidence available cannot exclude the possibility that one more GABA receptor is included in the 82,000-dalton unit, leaving 30,000 daltons for the gating mechanism, this is, however, unlikely due to the observed Hill coefficients of unity.

The TBPS binding protein is probably a part of the chloride gating mechanism [9, 35, 37]. [^{35}S]TBPS binding sites are affected by sedative hypnotics such as barbiturates and etomidate as well as by some pyrazolopyridazines such as SQ 20.009 and cartazolate [9, 36]. It has previously been shown that these agents act on TBPS sites in a different way as compared to presumed competitive inhibitors (IPTBO, picrotoxin) [9, 37]. In the present study we showed that repeated freezing/thawing and washings, a treatment which increases the affinity of GABA receptor for GABA [38], reduced the affinity of etomidate and SQ 20.009 as inhibitors of [^{35}S]-TBPS binding (Fig. 4). A similar loss of affinity for SQ 20.009 and etomidate was observed when membranes were exposed to 6 Mrad (Fig. 4). The data also show that etomidate, muscimol and SQ 20.009 affect [^{35}S]TBPS allosterically.

The capability of low concentrations of SQ 20.009 to reduce [^{35}S]TBPS binding disappeared quite rapidly with radiation dose. Apparently the molecular entity responsible for the high affinity of SQ 20.009 disappeared with a rate corresponding to a molecular weight of 314,000 daltons. A similar value was found



Total, 548,000 dalton

Fig. 10. A diagrammatic representation of a putative GABA/benzodiazepine receptor complex, which accommodates the present and published results. Based on ref. [2].

when etomidate was used as an inhibitor of [^{35}S]-TBPS binding (data not shown). These results suggest that the high affinity binding of SQ 20.009 and etomidate to effect [^{35}S]TBPS binding is dependent on a large complex, and that the minimum molecular weight of this whole allosteric complex is 314,000 + 137,000 = 451,000 daltons. This value relates to the high affinity SQ 20.009/etomidate interaction; at high concentrations these agents still fully inhibit [^{35}S]TBPS binding indicating that the site with which they interact is located on the TBPS binding protein. The SQ 20.009 site, however, attains a low affinity conformation, when the allosteric complex is broken.

It was shown that also the benzodiazepine receptor behaves as an allosteric regulator of [^{35}S]TBPS binding sites [27]. It was demonstrated that benzodiazepine agonists enhanced, and inverse agonists reduced [^{35}S]TBPS binding. We have reproduced these findings in triple frozen/washed membranes (Fig. 6), but not in membranes from frozen tissues once washed; in triple-washed membrane preparation the increase and decrease of benzodiazepine agonist and inverse agonist, respectively, were only observed at short [^{35}S]TBPS incubation time (e.g. 15 min) (data not shown). Benzodiazepine agonists and inverse agonists affect both "on"- and "off"-rates of [^{35}S]TBPS binding (not shown). We observed that the allosteric modulation of [^{35}S]TBPS sites with benzodiazepine receptor ligands disappeared upon radiation (Fig. 8), suggesting that benzodiazepine ligands affect [^{35}S]TBPS allosterically via separate proteins (illustrated by protein II and site "C" in Fig. 9). The effects of DMCM and ZK 93423 are inhibited by a benzodiazepine receptor antagonist (Fig. 7), suggesting that both act via BZ receptors.

The apparent size of [^{35}S]TBPS binding sites changes from 137,000 to a maximum of 221,000 daltons by repeated freezing/thawing as compared to frozen tissue thrice washed (Fig. 8B). Apparently, repeated freezing affects the lipid double layers, and/or the molecular couplings in a way that [^{35}S]TBPS binding becomes dependent on a molecular complex with a size of 221,000 daltons, i.e. a higher coupling state. Several combinations of GABA monomers (55,000 daltons), uncoupled [^{35}S]TBPS sites (82,000 daltons), and other proteins may be reflected in this value (see Fig. 10).

The present results clearly show that the size of the benzodiazepine/GABA receptor chloride channel complex in the most unperturbed form accessible (frozen whole tissue) is appreciably larger than 200,000 daltons. Previously, values of 50,000, 100,000 and 200,000 daltons have been obtained by various methods for mono and multimers of the complex [5, 31, 39–48].

In a few gel filtration experiments values of 350,000–800,000 daltons for detergent solubilized receptor complexes were obtained [49, 50].

The exact composition of the intact complex cannot be deduced from the present experiments. The present study points to a minimum weight of *c.* 500,000 daltons. Apparently, there is one TBPS site for each benzodiazepine receptor site (Fig. 2), and there may be one GABA site for each TBPS site (Hill coefficient of unity for muscimol reduction of [^{35}S]TBPS binding) or benzodiazepine site (Hill coefficient of one for GABA enhancement of [^{3}H] diazepam binding). There seem to be at least two GABA receptors affecting one opening event of the chloride channel, as deduced from the Hill coefficient of two for the GABA-stimulated chloride flux in single neurons [see 51]. It has been suggested that four BZ receptor subunits exist in a complex [52, 53]. Added together these results seem to require the existence of at least four major units each consisting of a GABA receptor, a benzodiazepine receptor and a [^{35}S]TBPS binding site. It is striking that the molecular weight of the GABA receptor and the benzodiazepine receptor have always been estimated to very similar values [31, 49, 50] and that benzodiazepine receptors and GABA receptor copurify [48, 54–56]. These studies may suggest that the GABA and the benzodiazepine receptors might reside on one protein. The present results seem to indicate that benzodiazepine ligands affect [^{35}S] TBPS binding via another, protein than the GABA receptor (Fig. 8). These results, however, may be interpreted in an alternative way where both the GABA and the benzodiazepine receptor reside on the same protein. The receptor for the effect of benzodiazepine ligands on [^{35}S]TBPS binding might reside within the 137,000-dalton value, but the function of this site is dependent on the existence of an interact tetrameric complex ($Mw = 4 \times (50{,}000 + 82{,}000) = 530{,}000$ daltons), whereas the GABA decrement effect on [^{35}S]TBPS binding needs the presence of only one subunit (137,000 daltons). The simplest possible model to accommodate all this information is shown in Fig. 10.

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APPENDIX

The derivation of a mathematical expression for the decay of the ZK 93423 effect on [^{35}S]TBPS binding is based on the following assumption.

The TBPS binding protein T is coupled to an allosteric unit S (TS); both T and TS have the same affinity for TBPS, when ZK 93423 binds to S, the affinity of TS for TBPS changes. Inactivation constants for T is k_2 and for S is k_1.

The number of T and TS sites remaining after a dose D of radiation is:

$$TS_D = TS_0\, e^{-(k_1+k_2)D},$$

$$T_D = (T_0 + TS_0)\, e^{-k_2 D} - TS_0 e^{-(k_1+k_2)D}.$$

Binding of [^{35}S]TBPS in the presence and absence of ZK 93423 can be described by the following expressions

$$B_{+ZK} = \frac{(T_0 + TS_0)\, e^{-k_2 D} - TS_0\, e^{-(k_1+k_2)D}}{KD_1/L + 1} + TS_0 \frac{e^{-(k_1+k_2)D}}{KD_2}/L + 1 \quad \text{(I)}$$

$$B_{-ZK} = \frac{(T_0 + TS_0) e^{-k_2 D} - TS_0\, e^{-(k_1+k_2)D}}{KD_1/L + 1} + TS_0 \frac{e^{-(k_1+k_2)D}}{KD_1}/L + 1 \quad \text{(II)}$$

$T_0 + TS_0 = B_{max}$ (the total number of [^{35}S]TBPS binding sites). KD_1 is the dissociation constant of [^{35}S]TBPS binding to T and TS in the absence of ZK 93423 and KD_2 is the dissociation constant of [^{35}S]TBPS binding to TS in the presence of ZK 93423.

Equations (I) and (II) rearrange to the expression

$$\frac{B_{-ZK} - B_{+ZK}}{B_{-ZK}} = \left(\frac{TS_0}{T_0 + TS_0}\right)\left(1 - \frac{KD_1/L + 1}{KD_2/L + 1}\right) e^{-k_1 D}$$

which gives the logarithmic expression

$$\ln \frac{B_{-ZK} - B_{+ZK}}{B_{-ZK}} = -k_1 D + \text{constant}.$$

From this equation it is seen that the inactivation constant k_1 for the effect of ZK 93423 ($B_{-ZK} - B_{+ZK}/B_{-ZK}$, for example calculated in %) gives the target size of the ZK 93423 regulatory unit, S. Note that this calculation is based on a purely "competitive" interaction of ZK 93423.