Endogenous Effector of the Benzodiazepine Binding Site: Purification and Characterization†

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ABSTRACT: A protein has been isolated from the small intestine and bile duct which inhibits the binding of [^{3}H]diazepam to specific benzodiazepine binding sites on synaptosomal membranes. When ion-exchange chromatography and gel filtration chromatography are used, this protein has been purified to apparent homogeneity. "Nepenthin" has been chosen as a name for this protein, which has an approximate molecular weight of 16 000, as determined by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration chromatography. Purified nepenthin is a competitive inhibitor of [^{3}H]diazepam binding with a $K_i = 4.6 \times 10^{-8}$ M. It does not inhibit the binding of specific ligands to the enkephalin, β-adrenergic, γ-aminobutyrate, or dopamine binding sites in the CNS. Neither γ-aminobutyric acid nor glycine alters the inhibition of [^{3}H]diazepam binding by this protein. Nepenthin can be extensively treated with proteases (trypsin, chymotrypsin, and Pronase), and inhibition of diazepam binding remains stable, indicating that a lower molecular weight fragment retains activity. Antibodies raised against this purified effector have been used in in situ double antibody labeling studies with rat brain slices. These studies indicate that cells containing an immunologically similar material are present in the deep cortical region of the forebrain.

The presence of specific binding sites for benzodiazepines within the brain has been well documented by in vitro studies in both the rat and man (Squires & Braestrup, 1977; Möhler & Okada, 1977a, 1978; Braestrup & Squires, 1977; Bosmann et al., 1977; Braestrup et al., 1977; Speth et al., 1978). In addition, in vivo studies with radiolabeled flunitrazepam[1] (Chang & Snyder, 1978) and diazepam (Williamson et al., 1978a,b; Bosmann et al., 1978) indicate stereospecific binding of diazepam, regional distribution of diazepam binding within the CNS, and subcellular association of benzodiazepine receptors in fractions enriched with synaptosomal membranes. A population of these receptors, associated with membrane fractions and isolated from both rat and human brain, have been shown to have affinities for various benzodiazepines which correlate well with their expected pharmacological potencies as muscle relaxants, anxiolytics, and anticonvulsants (Squires & Braestrup, 1977; Braestrup & Squires, 1977; Möhler & Okada, 1977b; Speth et al., 1978). These data suggest that specific binding sites may reflect intrinsic pharmacological receptors in the CNS which mediate part or all of the clinical spectrum of action exhibited by benzodiazepines.

It is a reasonable assumption that the existence of specific receptors in vivo would also imply the presence of endogenous effectors for those receptors (Squires & Braestrup, 1977; Iversen, 1978). Many compounds have been examined as prospective endogenous ligands, but none has demonstrated a high affinity for the benzodiazepine receptor. Nicotinamide proposed by Möhler et al. (1979) and both of the purines, inosine and hypoxanthine, reported by Skolnick et al. (1978) represent examples of two classes of compounds which compete with relatively low affinity for [^{3}H]diazepam binding in vitro. In addition, a [^{3}H]diazepam displacement activity has been isolated from urine (Braestrup et al., 1980) and has been identified as an ester of β-carbolinecarboxylic acid. However, the origin of this compound in vivo is still in question. Davis & Cohen (1980) have also recently isolated a protease-labile peptide with a molecular weight of 3000 from bovine brain which competitively inhibits the specific binding of [^{3}H]diazepam.

By measuring the displacement of [^{3}H]diazepam from rat synaptosomes, it is possible to search for an endogenous ligand with a high affinity for the benzodiazepine receptor. We now report the isolation and characterization of a protein from rat tissue which, at low concentration, competitively inhibits [^{3}H]diazepam binding to specific CNS receptors. We have assigned the name "nepenthin" to this protein.

Experimental Procedures

[*methyl*-^{3}H]Diazepam (79.9 Ci/mmol), [9,10-$^3H_2$(N)]-triolein (14 Ci/mmol), and phosphatidyl[*methyl*-^{14}C]choline (52 mCi/mmol) were purchased from New England Nuclear and used in all binding studies. ^{125}I-Labeled insulin was a gift from Dr. Steven Jacobs of Wellcome Research Laboratories. Trisma-Base, γ-aminobutyric acid, glycine, trypsin, chymotrypsin, α-*N*-benzoyl-L-arginine ethyl ester, *N*-benzoyltyrosine ethyl ester, bovine serum albumin (BSA), and methemoglobin were obtained from Sigma Biochemicals. Pronase was supplied by Calbiochem. Proteins of known molecular weight were purchased from Pharmacia Fine Chemicals and BDH Chemicals Ltd. and used as markers in acrylamide gel and gel filtration studies. Bio-Gel P-30 (100–200 mesh), P-10, and P-2, sodium dodecyl sulfate, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were products of Bio-Rad. Carboxymethylcellulose (standard grade) was purchased from Schleicher & Schuell. Diazepam was a gift from Hoffmann-La Roche.

Purification of Synaptosomes. Synaptosomes were prepared from rats (Sprague-Dawley), average weight of 180 to 250 g, by homogenization of whole brains in a Potter-Elvejhem unit with a loose-fitting Teflon pestle in 1 volume (w/v) of buffer containing 10 mM Tris-HCl, pH 7.5, containing 0.32

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[1] Abbreviations used: flunitrazepam, 7-chloro-1-[2-(diethylamino)-ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride; diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one; GABA, γ-aminobutyric acid; CNS, central nervous system; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G.

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M sucrose. The homogenate was diluted with 10 volumes of this buffer and centrifuged in a GSA rotor at 1500*g* for 10 min at 4 °C in a Sorvall RC-5 refrigerated centrifuge. The supernatant was removed and recentrifuged for 30 min at 15000*g*. The resulting pellet was resuspended in the original buffer to a final volume of 610 mL and loaded, under static conditions, into a Sorvall SZ-14 reorienting gradient rotor. A linear gradient of sucrose solution ranging from 10 to 55% w/v was pumped into the rotor, displacing the sample. The total volume, including sample and gradient (1380 mL), was uniformly buffered with 10 mM Tris-HCl, pH 7.5 (henceforth designated as buffer A). The rotor was slowly accelerated to allow gradient orientation and then centrifuged at 20500*g* for 3.5 h to permit isopycnic banding of synaptosomes. The rotor was slowed with a brake to 1000 rpm and then without the use of a brake, it was brought to a stop. This permits a gradual reorientation of the gradient. Fractions (9 mL each) were collected by a static unloading procedure, and sucrose concentrations were determined by refractive index with an Abbe-3L refractometer (Bausch and Lomb). Protein concentration was determined by the Coomassie blue technique (Bradford, 1976) with BSA as the standard. The synaptosomal fractions showing maximal [^{3}H]diazepam binding were pooled and stored at −20 °C.

Isolation of Nepenthin. Nepenthin was isolated from bile ducts of male Sprague-Dawley rats (180–250 g). Fresh bile ducts were washed 3 times with 5 volumes (w/v) of buffer A. The washed tissue was then diluted with 2–3 volumes of the same buffer and homogenized in a Polytron unit (Brinkman) at three-fourths speed for 1 min. The homogenate was centrifuged at 17000*g* for 1 h and the supernatant was layered on a (2.5 × 20 cm) carboxymethylcellulose (CM-cellulose) column, equilibrated in 50 mM Tris-HCl, pH 7.5 (buffer B). After the sample was applied, the CM-cellulose column was washed with the equilibration buffer to remove unbound material, and a single peak of activity was eluted by a step in buffer concentration to 0.6 M Tris-HCl, pH 7.5. The peak fractions containing activity were pooled and concentrated with an Amicon filtration unit on a type YM-5 membrane to a final volume of 2–3 mL. This concentrated fraction was layered on a Bio-Gel P-30 column (2.5 × 80 cm) equilibrated in buffer B. The effluent from this column was monitored at λ 279 nm by a Schoeffel Model 770 absorbance monitor, and 4.5-mL fractions were collected. Active fractions were concentrated by Amicon filtration using a YM-5 membrane or by lyophilization.

Binding Assay for [^{3}H]Diazepam Displacement. The assay for [^{3}H]diazepam displacement was that of Squires & Braestrup (1977) with minor modifications. The final assay contained 600–800 μg of synaptosomal protein, 50 mM Tris-HCl (pH 7.5), 1.6 nM [^{3}H]diazepam, and purified effector at an appropriate concentration, in a total volume of 3 mL. Nonspecific binding was determined in the presence of 5 μM unlabeled diazepam. Specific binding was determined by subtracting nonspecifically bound [^{3}H]diazepam from total ligand bound. Typically, nonspecific binding represented 5 to 10% of the level observed for specifically bound material. Membranes and buffer were preincubated at 37 °C for 5 min. [^{3}H]Diazepam was then added along with unlabeled diazepam or effector, and the complete reaction was incubated an additional 15 min at 37 °C. The reaction was then placed in an ice bath for 30 min and subsequently filtered on Whatman GF/C filters to isolate synaptosomal membranes. Immediately prior to filtering, 10 mL of ice-cold buffer B was added to each tube, and the samples were then washed twice on the filter with 5 mL of this buffer. The filter membranes were counted in a Packard Model 3385 liquid scintillation counter using the liquid scintillation fluor, Aquasol II (New England Nuclear). Counting efficiency was 30%.

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels contained 12% acrylamide with 0.3% bis(acrylamide), 45 mM Tris-HCl (pH 8.8), and 1% $NaDodSO_4$. Ammonium persulfate was used as the radical generator. The upper and lower reservoirs contained 5 mM Tris–glycine buffer (pH 8.3) with $NaDodSO_4$ present at 0.1% w/v. Vertical slab gels were preelectrophoresed at 20 mA (constant current) for 2 h before sample loading. Protein samples at low concentration (<0.1 mg/mL) were first precipitated in ice-cold 5% Cl_3CCO_2H and then denatured at 90 °C for 3 min in 3% $NaDodSO_4$ and 1 mM β-mercaptoethanol prior to loading. $NaDodSO_4$ gels were stained in an aqueous 0.1% Coomassie blue R-250 solution containing 50% ethanol and 10% acetic acid and destained in a solution containing 5% ethanol and 7% acetic acid. Urea gels were prepared and stained by the methods of Hoffmann & Chalkley (1976).

Antibodies to the Purified Nepenthin. A 1-mL solution of effector (nepenthin) at 116 μg/mL in 0.9% NaCl was emulsified with 1 mL of Freunds adjuvant. Three New Zealand male rabbits were injected subcutaneously in six places on the back with a total of 60 μL of this immunogen per rabbit. This was repeated after 3 and 6 weeks and 5 days before blood was drawn. Blood was collected from the ear, allowed to clot overnight at 4 °C, and centrifuged at 1000*g* for 15 min. The sera were collected and frozen in 0.5-mL aliquots at −20 °C until used.

In Situ Immunofluorescent Studies in Rat Brain Slices. Sprague-Dawley rats (75–100 g) were injected intracerebroventricularly with colchicine (150 μg/kg) 48 h before sacrifice. The rats were sacrificed by intracardial perfusion of cold phosphate-buffered saline (PBS) containing 4% w/v paraformaldehyde. Brains were removed, postfixed in this fixative at 4 °C for 2 h, and then placed in PBS containing 7% w/v sucrose for 24 h at 4 °C. Brains were then frozen and sliced into 8- to 12-μm sections. Sections were thaw-mounted onto gelatin-coated slides. The slides were washed for 30 s in an acetone/chloroform solution (1:1) to better facilitate antibody movement through the membranes. Sections were stained at 4 °C for 48 h by use of rabbit primary antisera to the purified effector (diluted 1:20 with PBS). The primary antiserum was removed by three 5-min washes with PBS at 25 °C, and the sections were then exposed to a fluorescein-conjugated goat antibody to rabbit IgG (Miles) at a 1:16 dilution with PBS and incubated at 4 °C for 24 h. The sections were then washed 3 times with PBS for 5 min each at 25 °C. Cover slips were mounted and sections were examined under dark-field conditions by using a Leitz Orthoplan microscope equipped with epifluorescence optics.

Results

[^{3}H]Diazepam Specific Binding to Synaptosomal Membranes. Synaptosomes were isolated by sucrose density gradient centrifugation as described under Experimental Procedures. Fractions containing purified synaptosomes were identified enzymatically by having elevated levels of tyrosine hydroxylase activity and structurally by transmission electron microscopy. Specific binding of [^{3}H]diazepam to synaptosomes, isolated by zonal centrifugation, was determined in 3-mL reaction mixtures containing 1 mg of synaptosomal protein. The binding of [^{3}H]diazepam across the sucrose gradient is shown in Figure 1. Specific binding of the ligand is coincident with the elution of peak synaptosomal protein

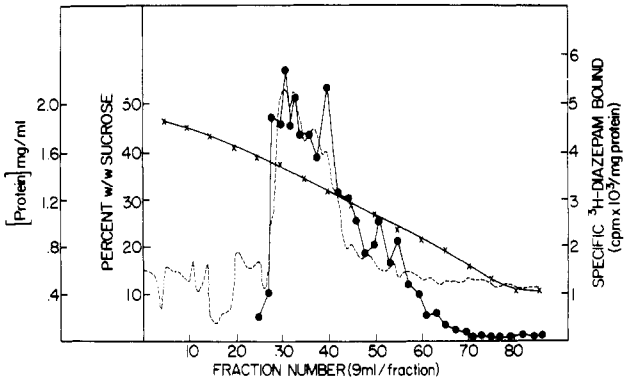

FIGURE 1: Specific binding of [^{3}H]diazepam to synaptosomes. Rat synaptosomal fraction was isolated by zonal centrifugation using a linear sucrose gradient of 10–50% w/w sucrose. Protein (---), sucrose concentration (×), specific [^{3}H]diazepam binding (●). Isopycnic banding of synaptosomes occurs between 34 and 36.5% w/w sucrose at a density of 1.147–1.159.

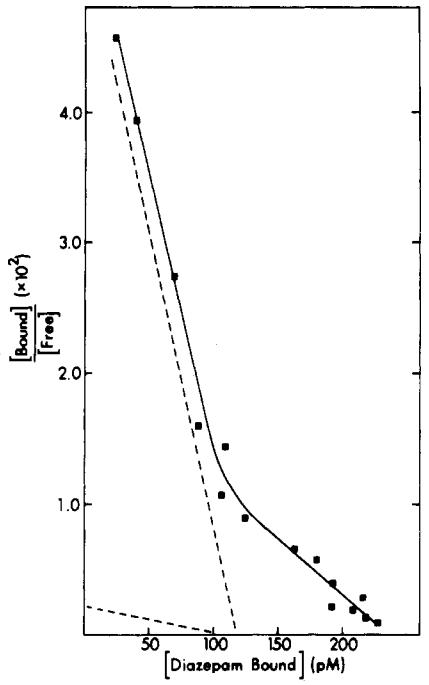

FIGURE 2: Scatchard analysis of specific [^{3}H]diazepam binding to purified synaptosomal membranes. The data indicate that at least two binding sites exist, with the higher affinity site at a K_D = 2.6 ± 0.9 nM and a lower affinity site with a K_D = 49 ± 21 nM. (■) Data as calculated by the method of Scatchard (1949). (---) Computer-corrected data derived by the DUD method of nonlinear least-squares regression analysis (Ralson & Jennrich, 1979) as fitted to a model for the sum of two noninteracting binding sites as defined in the following: with diazepam at <10 nM, $B = (B_{max(1)}F)/(K_{D(1)} + F)$, and with higher concentrations of diazepam, $B = [(B_{max(1)}F)/(K_{D(1)} + F)] + [(B_{max(2)}F)/(K_{D(2)} + F)]$. The DUD method was utilized as part of the statistical analysis system described by Helwig & Council (1979).

fraction. Nonspecific binding was constant (250–300 cpm/assay) throughout the gradient, indicating that most of this type of binding was probably a function of the filtration technique. The fractions with maximal specific binding (28–42) were pooled and used in subsequent assays.

A characterization of synaptosomal benzodiazepine binding sites was determined by a titration of [^{3}H]diazepam from 1 to 10 nM. The data were plotted according to Scatchard (1949) to determine binding parameters. The biphasic plot (Figure 2) suggests that purified synaptosomes contain two noninteracting types of binding sites, a high-affinity site with a K_D = 2.6 ± 0.9 nM and a low-affinity site with a K_D = 49 ± 21 nM. The number of sites present was 487 ± 55 fmol/mg for the high-affinity site and 397 ± 68 fmol/mg of the low-affinity site.

Table I: Tissue Distribution of [^{3}H]Diazepam Binding Inhibitory Activity

tissue type[a]	relative [^{3}H]diazepam displacement act. per wet wt of tissue	tissue type[a]	relative [^{3}H]diazepam displacement act. per wet wt of tissue
brain	1.00	liver	1.95
pituitary	1.00	testes	2.00
heart	1.05	muscle (striated)	2.05
fat (lateral)	1.20	lung	2.05
adrenal	1.20	spleen	2.15
kidney	1.25	small intestine	2.75
pancreas	1.45	bile duct	3.00

[a] Each assay contained the soluble protein equivalent of 67 mg of tissue (wet weight).

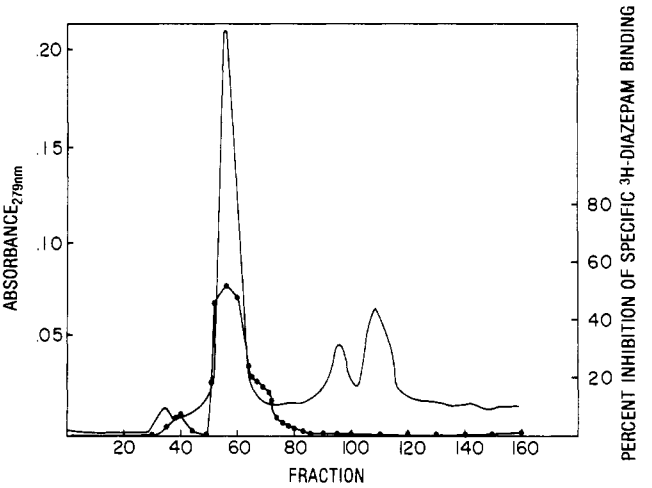

FIGURE 3: Elution profile of nepenthin from Bio-Gel P-30. [^{3}H]-Diazepam binding inhibitory activity (●) was eluted with 50 mM Tris-HCl (pH 7.5) at 1.6V_0.

Tissue Localization and Purification of an Endogenous Ligand. To search for an endogenous ligand for the benzodiazepine receptor, crude soluble protein extracts were secured from various rat tissues. The protein extracts were prepared by homogenization of tissues in 2 volumes (w/v) of buffer A containing 0.32 M sucrose and centrifuged at 15000*g* for 30 min. The supernatants were assayed for activity in the [^{3}H]diazepam displacement assay as previously described. The results shown in Table I indicate that crude extracts from the bile duct and small intestine have relatively high displacement activity for specific [^{3}H]diazepam binding.

Purification of nepenthin was performed by ion-exhcange and gel filtration chromatography as described under Experimental Procedures. Bile ducts (6.15 g) were collected, washed, and suspended in 12.3 mL of buffer A. The suspended tissue was then homogenized with a Polytron unit at three-fourths speed for 1 min. The homogenate was centrifuged at 17000*g* for 30 min, and the resulting supernatant was layered on a CM-cellulose column. The column was washed with buffer B until the absorbance at 279 nm was <0.05 (≅2.5 bed volumes). The activity was then eluted with 0.6 M Tris-HCl (pH 7.5). The CM-cellulose eluate was concentrated by Amicon filtration using a type YM-5 membrane. The concentrated sample was then applied to a Bio-Gel P-30 gel filtration column. The P-30 column was developed with buffer B, and the elution profile from this column is given in Figure 3. The [^{3}H]diazepam displacement activity elutes at 1.6V_0.

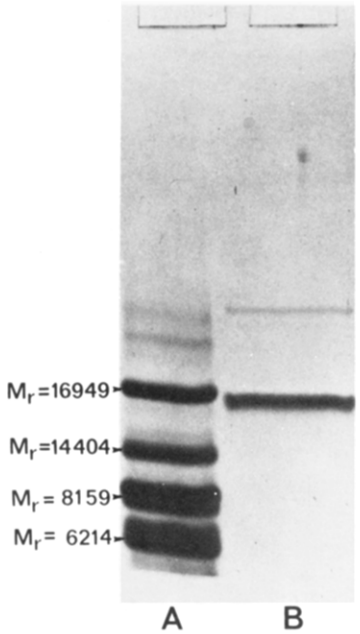

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified nepenthin. Low molecular weight standards (A) represent cyanogen bromide cleaved fragments of horse heart myoglobin secured from BDH Chemicals. A single major protein band (B) is present with M_r = 15 800 ± 800. Acrylamide gel preparation and experimental conditions are described under Experimental Procedures.

This preparation yielded approximately 350 μg of purified nepenthin, which represents 0.1% of the total soluble protein in the crude supernatant.

Molecular Weight Determination of Nepenthin. The molecular weight of the purified effector was determined by gel filtration chromatography on Bio-Gel P-10. With insulin monomer, cytochrome *c*, and myoglobin as standards, the [^{3}H]diazepam displacement activity eluted at a M_r = 16 000 ± 800. $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified effector (Figure 4) showed one major protein band at M_r = 15 800 ± 800 with a minor protein contaminant at a higher value. Additional attempts to remove this higher molecular weight species were unsuccessful. The purity of the effector was also confirmed by polyacrylamide electrophoresis on acid–urea gels. In this system, a single major protein band was observed. In addition, a single, less mobile, minor band, representing <10% of the total observable protein, was present. Nepenthin is stable in buffer B at 37 °C for 24 h, at 50 °C for 15 min, and at room temperature for >48 h. However, it is unstable under acidic conditions, and [^{3}H]diazepam displacement activity could not be recovered from acid–urea gels.

Specificity of the Nepenthin for [^{3}H]Diazepam Binding. The [^{3}H]diazepam displacement activity was determined in a 3-mL reaction mixture containing 800 μg of synaptosomal protein. Displacement was linear from 0.05 to 0.25 μg/mL of effector and appeared to saturate between 1 and 1.5 μg/mL (Figure 5). Displacement reactions performed at a constant nepenthin concentration and varying concentrations of [^{3}H]-diazepam indicate that nepenthin is a competitive inhibitor of [^{3}H]diazepam binding. When these data were plotted by the method of Lineweaver & Burk (1934), assuming M_r = 16 000 for nepenthin, a $K_i = 4.6 \times 10^{-8}$ M was calculated. Approximately 50% displacement of total specific [^{3}H]diazepam bound could be achieved with nepenthin at an apparent saturating concentration. This displacement activity was unaltered by apomorphine at 1 μM, γ-aminobutyric acid (GABA) or glycine at 100 μM, or Met-enkephalin at 50 μM.

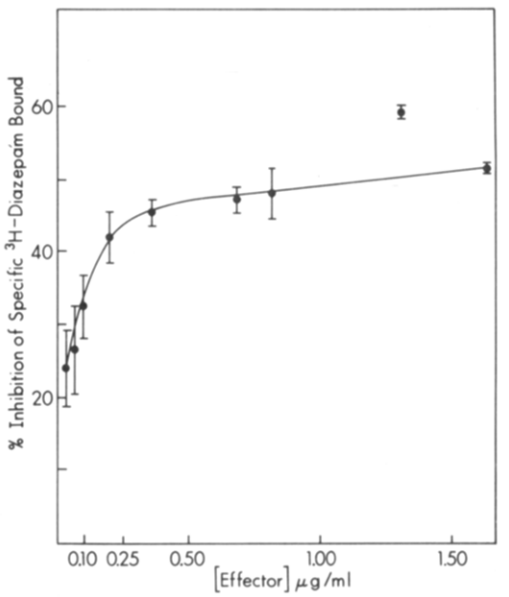

FIGURE 5: Saturation curve for the inhibition of [^{3}H]diazepam binding by purified nepenthin. Increasing concentrations of nepenthin from 0.05 to 1.65 μg/mL were incubated under standard assay conditions as described. Diazepam concentration was constant at 1.6×10^{-9} M.

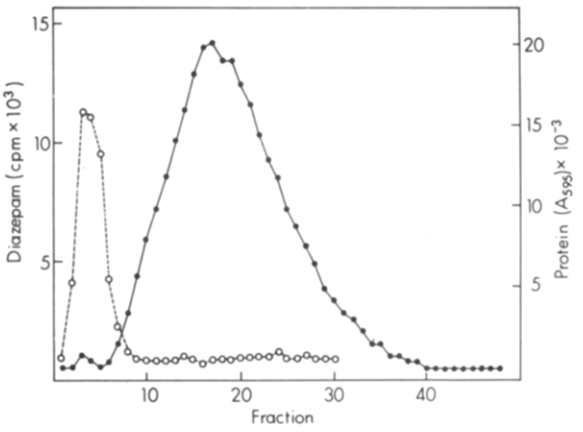

FIGURE 6: Bio-Gel P-2 chromatographic separation of [^{3}H]diazepam incubated with nepenthin. (○) Protein profile was determined by the method of Bradford (1976). (●) [^{3}H]Diazepam elution profile.

Purified nepenthin showed no activity as a competitor in the following binding assays: GABA (Enna & Snyder, 1975), dopamine (Burt et al., 1976), β-adrenergic (Bylund & Snyder, 1976), and enkephalin (Miller et al., 1978). In addition, nepenthin has no activity in the rat anococcygeus muscle contraction assay as described by Leighton et al. (1979), indicating no presynaptic or postsynaptic α-adrenergic, muscarinic, or 5-hydroxytryptamine activity. Also, there was no evidence of a cholinergic response with the protein in isolated guinea pig ileum contraction experiments (Paton & Vizi, 1969).

Nepenthin was incubated with [^{3}H]diazepam to determine whether the observed "displacement" of radiolabeled ligand from synaptosomal membranes was due to a high affinity of the diazepam for the effector protein. This reaction mixture, containing both [^{3}H]diazepam and nepenthin, was first incubated at 37 °C for 15 min and then at 0 °C for 30 min. Nepenthin was then separated from unbound [^{3}H]diazepam on a Bio-Gel P-2 column, and the elution profile from this column is given in Figure 6. These data indicate that under standard assay conditions nepenthin does not selectively bind [^{3}H]diazepam.

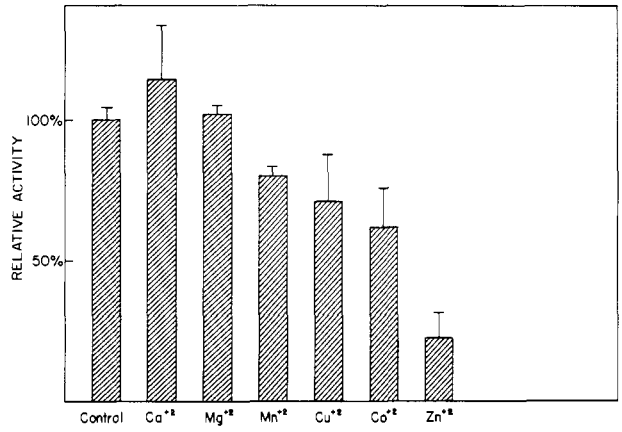

FIGURE 7: Effect of divalent cations on nepenthin activity. Divalent cations were added to the standard assay at 1 mM as the chloride salts. A decrease in relative activity reflects a decrease in the inhibition of [^{3}H]diazepam binding by the effector protein. Error bars represent the standard deviation with $N = 3$.

The effect of divalent cations on the nepenthin inhibition of [^{3}H]diazepam binding is presented in Figure 7. It can be seen that neither Ca^{2+} nor Mg^{2+} at a concentration of 1 mM has any major effect on the inhibition of binding activity. However, other divalent cations tested appeared to decrease nepenthin activity, with Zn^{2+} being the most potent inhibitor.

The Effect of Protease Treatment of Nepenthin on Its Inhibition of [^{3}H]Diazepam Binding Activity. Proteases have been shown to decrease the amount of [^{3}H]diazepam bound to synaptosomes, presumably by either extensive membrane degradation or specific binding site alterations [Möhler & Okada (1977a); J. H. Woolf and J. C. Nixon, unpublished observations]. It was therefore necessary to determine if any contaminating protease activity was present in the purified nepenthin preparation. An assay for tryptic activity, using α-*N*-benzoyl-L-arginine ethyl ester (BAEE) as a substrate, indicated that the purified protein preparation contains 0.7 BAEE unit/μg. Also, an assay for chymotryptic activity, using *N*-benzoyltyrosine ethyl ester, showed that the purified effector has 3.6×10^{-3} BTEE unit/μg. It should be noted that these activities are low when contrasted to either trypsin or chymotrypsin at comparable concentrations and that only microgram amounts of nepenthin are needed to achieve marked displacement of [^{3}H]diazepam binding in the standard assay containing 800 μg of synaptosomes. The standard assay conditions were performed in duplicate by adding 1 μg of nepenthin or 0.1 μg of Pronase to determine if this minor protease activity in the nepenthin preparation could be specifically degrading the receptor. The membranes were first incubated at 37 °C with either nepenthin or Pronase and then washed free of any residual protease by suspension in 10 mL of buffer A for 5 min at 4 °C and centrifugation at 1500*g* for 5 min. This washing procedure was repeated 3 times. The resulting "washed membranes" were then assayed for residual binding sites with [^{3}H]diazepam. Table II indicates that 55% of the specific diazepam binding sites in the control (no nepenthin or Pronase) are lost by the washing procedure alone. Preincubation of membranes with the nepenthin showed no difference in the number of residual binding sites when compared to controls, while treatment with Pronase resulted in an additional 58% decrease in the number of residual binding sites. Also, the addition of 800 μg of ovalbumin or 3 mg of methemoglobin to the standard displacement assay as an alternative substrate for the protease activity resulted in no significant decrease in the inhibition of [^{3}H]diazepam binding activity by nepenthin. Thus, it does not appear that the residual protease activity observed in the nepenthin preparation is responsible for the inhibition of [^{3}H]diazepam binding. Purified nepenthin was also shown not to have any detectable lipase or phospholipase activity as assayed by the methods of Belfrag & Vaughan (1969) and Smith & Silver (1973).

Table II: Effect of Nepenthin and Pronase on [^{3}H]Diazepam Binding Sites on Synaptosomal Membranes

reaction conditions	[^{3}H]diazepam bound (fmol)
control (no additions, no wash, 0 °C)	83.5
control (no additions, 3 times wash, standard assay conditions)	36.4
experimental (1 μg of nepenthin,[a] 3 times wash, standard assay conditions)	34.3
experimental (0.1 μg of Pronase,[a] 3 times wash, standard assay conditions)	15.3

[a] Preincubation reactions are described under Results.

Table III: Inhibition of [^{3}H]Diazepam Binding by Proteolytic Digests of Nepenthin

	binding assay		
proteolytic digestion[a]	[^{3}H]-diazepam bound (fmol)	inhibition (%)	relative nepenthin act.
control (no nepenthin or protease)	23.7		
control (nepenthin, no protease)	17.6	25.7	1.00
trypsin + nepenthin	20.0	15.6	0.86
chymotrypsin + nepenthin	20.3	14.4	0.85
Pronase + nepenthin	11.7	50.6	1.34

[a] Nepenthin was incubated with each protease at at 25:1 concentration ratio for 12 h at 37 °C. Reaction products were combined with the standard binding assay for inhibition studies. Separate controls for protease reactions involved identical incubations of each protease without nepenthin to index autodigestion against residual proteolytic activity. Percent inhibition of [^{3}H]diazepam binding is based on an equivalent amount of 1 μg of nondigested nepenthin.

The results of protease digestion of nepenthin on its inhibition activity are presented in Table III. In each experiment, 2 μg of nepenthin was incubated with 0.08 μg of a specific protease at 37 °C for 12 h in buffer B. Control reactions containing either nepenthin or the proteases alone were also run. These proteolytically digested reactions and their controls were then incorporated into the standard binding assay to index residual inhibitory activity of proteolytically treated nepenthin to [^{3}H]diazepam binding. Under these conditions, very little loss of nepenthin activity was observed, suggesting that the activity may be present on lower molecular weight, proteolytically stable peptide fragments. However, such a fragment has not as yet been isolated. Protease controls in these experiments autodigested and were inactive in the binding assay.

Immunofluorescent experiments with antibodies to nepenthin, prepared in rabbits, were undertaken to examine the presence and localization of immunoreactive material in the CNS. Rats were treated with colchicine and brain tissue prepared as described under Experimental Procedures. Colchicine has been previously used in immunofluorescent work with CNS peptides by Uhl et al. (1979) and did improve the resolution in our tissue preparations. Preliminary evidence indicates that there is specific staining of neurons located in the deep cortical regions of the rat forebrain (Figure 8). Preincubation of the primary antibody with nepenthin prevented this staining, while preincubation of the primary an-

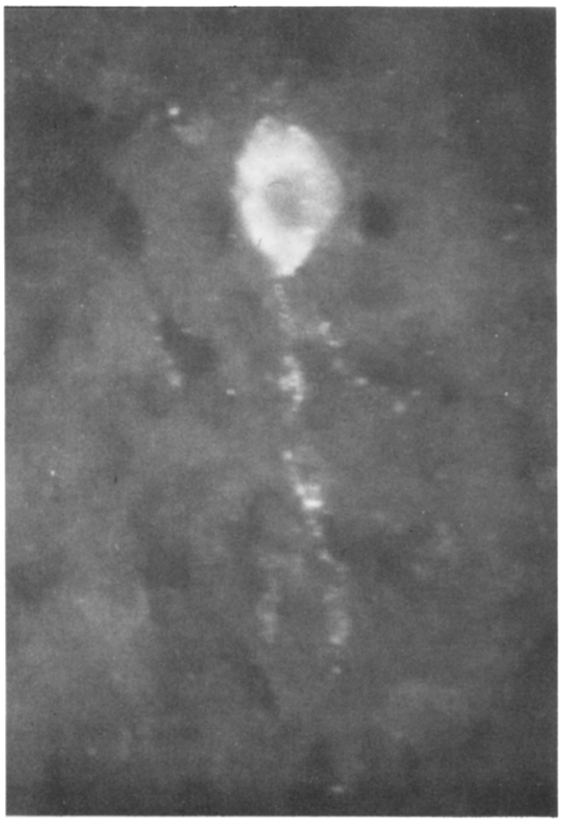

FIGURE 8: Immunohistochemical staining of "nepenthin-like" material in brain. Techniques for rat brain tissue preparation and immunofluorescent staining are described under Experimental Procedures. Pictured is one of a cluster of neurons located in a deep cortical region of the forebrain which stained positively (magnification 237×).

tibody with diazepam at a saturating level did not alter staining. The primary antibody did not bind [^{3}H]diazepam and showed a single immunodiffusion precipitin band when run against nepenthin on immunodiffusion plates.

Discussion

The report of specific sites for benzodiazepine binding in the brain (Squires & Braestrup, 1977) has prompted investigations to search for an endogenous compound which will bind to this site and hopefully elicit pharmacological activity. The types of compounds previously reported which have competitive activity for benzodiazepines in binding assays are structurally varied, and range from purines to peptides. Reports of putative purine ligands for the diazepam receptor include inosine and hypoxanthine (Skolnick et al., 1978). However, the affinity constants of these purines lie in the millimolar range. These concentrations are unlikely to be achieved in brain unless induced by severe ischemia (Kleihues et al., 1974). Möhler et al. (1979) have shown that nicotinamide competes for [^{3}H]diazepam binding with an IC_{50} of 3.9 mM. Nicotinamide also has some pharmacological properties common to the benzodiazopines such as anticonflict, anticonvulsant, hypnotic, and muscle relaxant activities. However, the concentration of nicotinamide in brain is also low when compared to the level necessary to achieve significant inhibition of diazepam binding in vitro. Karobath et al. (1978) reported a low molecular weight factor of less than 500 which inhibits diazepam binding to rat brain membranes. This factor has been partially purified by acetone extraction and gel filtration chromatography and is both stable in acid and resistant to protease activity. In the absence of any analytical data for this factor, it is possible that it could be one of the purines previously reported by others.

A large molecular weight protein isolated from porcine brain has been shown to competitively inhibit [^{3}H]benzodiazepine binding (Colello et al., 1978). This protein (BCF-I) has a M_r = 60 000, and its inhibitory activity is labile to protease treatment. These same authors report a low molecular weight peptide (M_r = 1000–2000) with 20% of the activity of BCF-I. It was postulated that BCF-I is the precursor to the lower molecular weight species. Little data are available on the structure or activities of these endogenous protein ligands, and thus its mechanism of inhibition or origin of the lower molecular weight species is still in question. Davis & Cohen (1980) have recently reported a protein of M_r = 3000 which has been isolated from brain and competitively inhibits diazepam binding. This protein is described as being heat stable but protease labile. Preliminary in vivo investigations with this peptide indicate that it induced a change in EEG similar to that produced by diazepam (L. G. Davis and R. K. Cohen, unpublished observations). Also, in a recent report by Braestrup et al. (1980), a compound, β-carboline-3-carboxylate, has been isolated from brain and urine. This compound is reported to have a high affinity (IC_{50} = 4–7 nM) for the benzodiazepine receptor, but its possible origin and pharmacological activity are as yet unknown.

We have described the isolation and purification of a protein from rat bile duct which has a high affinity for the benzodiazepine binding site. This protein, "nepenthin", has a molecular weight of 16 000, is cationic at neutral pH, and has good thermal stability. Nepenthin competitively inhibits the specific binding of [^{3}H]diazepam to rat synaptosomes with a $K_i = 4.6 \times 10^{-8}$ M. At the concentration of nepenthin used in in vitro studies (Figure 6), the effector appears to compete specifically for the high-affinity binding site described in Figure 2. Nepenthin inhibitory activity is apparently stable to extensive proteolytic digestion, which suggests that a lower molecular weight peptide fragment would retain activity, but this had not as yet been demonstrated. It seems unlikely that a high molecular weight protein would be synthesized peripherally to interact with binding sites in the CNS, and while this peptide has not as yet been isolated in CNS, there appears to be a population of neurons in the deep cortical region of the forebrain which selectively bind antibody to nepenthin. This common immunoreactive material in the CNS may well be an active fragment or native protein identical with nepenthin.

Nepenthin possesses a low level of protease activity. The specific activity of this protease is 4% that of purified trypsin and 7% that of chymotrypsin when determined with specific, known substrates. A measurement of the loss of [^{3}H]diazepam binding sites on synaptosomal membranes after preincubation with nepenthin indicates that it does not decrease residual sites when compared to controls (Table II). Therefore, the protease activity within the nepenthin preparation does not appear to destroy residual diazepam binding sites. Also, when an alternate substrate (ovalbumin) for this protease activity is added to the assay, it does not alter nepenthin's inhibition of [^{3}H]-diazepam binding. We have also determined that nepenthin does not inhibit the binding of enkephalin to its receptor, despite the apparent susceptibility of the enkephalin receptor to protease degradation (Pasternak & Snyder, 1974). These studies strongly suggest that the observed low level of protease activity in the purified nepenthin fraction is not involved in the inhibition of [^{3}H]diazepam binding to synaptosomal membranes. Nepenthin has also been shown not to have any lipase or phospholipase activity. Therefore, the observed inhibition of [^{3}H]diazepam binding does not appear to result

from the degradation of either lipids or proteins associated with the receptor.

Nepenthin does not possess activity in the GABA, β-adrenergic, dopamine, or enkephalin binding assays. Also, no activity was observed when it was added to either the isolated guinea pig ileum or ancoccygeus muscle contraction experiments, indicating no competition for either cholinergic or noradrenergic binding sites. Neither enkephalin, GABA, glycine, inosine, xanthine, nor hypoxanthine altered the inhibition of [^{3}H]diazepam binding by nepenthin.

We therefore conclude that this protein, isolated from a peripheral tissue, has specificity for benzodiazepine binding sites in the CNS and on interacting with these sites results in a decrease in [^{3}H]diazepam binding. Our data suggest that nepenthin may be present in the CNS or it could be a precursor to a lower molecular weight peptide with high affinity for the benzodiazepine binding sites on synaptosomal membranes. Further studies to investigate this possibility are in progress.

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