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Identification, characterization, and purification of a 65,000 dalton protein in rat brain that is photolabeled by nitro-containing benzodiazepines

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IDENTIFICATION, CHARACTERIZATION, AND PURIFICATION OF A 65,000 DALTON PROTEIN IN RAT BRAIN THAT IS PHOTOLABELED BY NITRO-CONTAINING BENZODIAZEPINES

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ABSTRACT

IDENTIFICATION, CHARACTERIZATION, AND PURIFICATION OF A 65,000 DALTON PROTEIN IN RAT BRAIN THAT IS PHOTOLABELED BY NITRO-CONTAINING BENZODIAZEPINES

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Benzodiazepines bind to two well-characterized classes of nanomolar-affinity binding sites, the central and the peripheral types. Although these sites appear to mediate many of the effects of these compounds, they cannot account for all of the biochemical and physiologic effects of the benzodiazepines. In this investigation, a protein that is photolabeled by NO_2-containing benzodiazepines was identified and characterized in rat brain by performing photoaffinity labeling experiments with [^{3}H]-clonazepam and [^{3}H]-flunitrazepam. These experiments demonstrate that this photolabeled protein has a molecular weight of 65,000 daltons. Photolabeling of the protein was saturable, inhibited in a stereoselective manner by benzodiazepine enantiomers, inhibited by therapeutically-relevant concentrations of many different NO_2-containing

benzodiazepines, and was not inhibited by more than 70 non-benzodiazepine compounds. The photolabeled protein is distinct from the central and peripheral sites on the basis of molecular weight, benzodiazepine inhibitory potencies, subcellular localization, and tissue distribution.

The protein was purified to apparent homogeneity by Affi-Gel Blue and Agarose Green column chromatography. This two-column purification scheme resulted in a greater than 2000-fold enrichment of the protein with a yield of 35%. Biochemical studies on the purified preparation established that the protein exists as a monomer in the purified state, contains a high relative abundance of glycine residues, and possesses amino acid sequence homology with the beta chain of hemoglobin.

This newly-characterized protein may mediate some of the effects of the benzodiazepines that are not associated with the central- or peripheral-type sites. It is also possible that the protein is an enzyme that synthesizes or degrades benzodiazepines.

PREFACE

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LIST OF ABBREVIATIONS

B_{max}-maximal binding capacity

CNZ-clonazepam

EGTA-ethyleneglycol-bis-(beta-aminoethylether)-N,N,N',N'-tetraacetic acid

FNZ-flunitrazepam

GABA-gamma-aminobutyric acid

5HT-5-hydroxytryptamine, serotonin

K_D-apparent dissociation constant

MES-2-(N-morpholino)ethanesulfonic acid

Pipes-piperazine-N,N'-bis(2-ethanesulfonic acid)

PMSF-phenylmethylsulfonyl fluoride

SDS-PAGE-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tris-2-amino-2(hydroxymethyl)-1,3-propandiol

I. INTRODUCTION

Benzodiazepines are one of the most widely-administered classes of therapeutic drugs (Greenblatt and Shader, 1974). They possess diverse clinical applications due to their multiple pharmacologic activities, including anticonvulsant, muscle relaxant, hypnotic, and anxiolytic effects (Greenblatt and Shader, 1974). Previous studies have demonstrated that many of the effects of the benzodiazepines are mediated by binding to the central benzodiazepine receptor which is associated with the gamma-aminobutyric acid (GABA) receptor-chloride ionophore complex in neuronal membrane (Squires and Braestrup, 1977; Mohler and Okada, 1977; Tallman et al, 1980; Tallman and Gallager, 1985). Benzodiazepines have also been shown to bind with high affinity to a peripheral benzodiazepine binding site that is present in brain as well as many peripheral tissues (Marangos et al, 1982; Anholt, 1986).

Electrophysiological studies have demonstrated that the benzodiazepines facilitate GABA-mediated inhibition. The major molecular mechanism by which GABA produces its inhibitory effects is the GABA-A receptor, which has been localized to the postsynaptic membrane of GABAergic synapses (Enna, 1983; Simmonds, 1983). GABA binding to the receptor produces an increase in membrane permeability for

anions, primarily chloride ions (Simmonds, 1983; Gallagher and Shinnick-Gallagher, 1983). Evidence supporting an interaction between benzodiazepines and GABA was first obtained more than 20 years ago by electrophysiological studies in which diazepam was found to enhance GABA-mediated presynaptic inhibition of monosynaptic reflexes in the cat spinal cord (Schmidt et al, 1967). Subsequently, benzodiazepines have been shown to facilitate the inhibitory effects of GABA in cultured neurons (MacDonald and Barker, 1982; Study and Barker, 1982) and in neuronal tissue from spinal cord (Stratten and Barnes, 1971; Polc et al, 1974) and many different brain regions, including cerebral cortex (Zakusov et al, 1975; Raabe and Gumnit, 1977), cerebellum (Curtis et al, 1976; Montarolo et al, 1979), and hippocampus (Wolf and Haas, 1977; Tsuchiya and Fukushima, 1978).

The physiologic interaction between GABA and the benzodiazepines has been analyzed electrophysiologically in several systems. In frog spinal cord, the dose-response curve for the GABA inhibitory effect was shifted to the left by flurazepam and midazolam, resulting in an increased apparent potency of GABA (Nistri and Constanti, 1978; Nistri and Berti, 1983). Similarly, in cultured spinal cord cells, the dose-response curve for the GABA-induced increase in membrane conductance was shifted to the left, while the maximal response produced by GABA was not altered

(MacDonald and Barker, 1979; Choi et al, 1981a,b). Fluctuation analysis studies performed on cultured spinal cord cells indicated that diazepam significantly increased the probability of channel opening by GABA but did not alter or only slightly increased open channel lifetime and did not alter single channel conductance (Study and Barker, 1981). These predominant effects on probability of channel opening suggest that benzodiazepines modulate GABA function by increasing the affinity of the GABA-A receptor for GABA or by facilitating the coupling between the GABA-A receptor and the chloride channel.

The relationship between GABA and the benzodiazepines has been clarified by receptor binding studies with radiolabeled benzodiazepines, such as [^{3}H]-diazepam and [^{3}H]-flunitrazepam.. In 1977, binding studies performed with these ligands demonstrated the existence in brain of high-affinity benzodiazepine binding sites, now known as central benzodiazepine receptors (Squires and Braestrup, 1977; Mohler and Okada, 1977). Binding to these sites was selective for benzodiazepines, saturated in the low nanomolar range for most benzodiazepines, and was stereoselective for 3-C enantiomers of benzodiazepines.

Subcellular fractionation studies demonstrated that the central receptor was enriched in the crude synaptosomal fraction (P_2) (Braestrup and Squires, 1977; Mohler and Okada, 1977; Marangos et al, 1982). Subsequent

autoradiographic studies have revealed a synaptic localization for the receptor (Mohler et al, 1980; Richards et al, 1986). In brain regional distribution studies, the affinity of the receptor was similar in all regions investigated, but the density of the receptors was variable, with highest levels in cerebral cortex and cerebellum, intermediate levels in hippocampus, striatum, and thalamus, and lowest levels in pons and medulla (Braestrup et al, 1977; Mohler and Okada, 1978a). High-affinity benzodiazepine binding could be detected in peripheral tissues, but the pharmacologic profile exhibited by this peripheral site was different from that exhibited by the central site (Braestrup and Squires, 1977; Gallager et al, 1981).

Displacement studies were performed with many different benzodiazepines to obtain K_i values which were then correlated with various tests that are predictive of benzodiazepine pharmacologic activity. Potencies for central receptor binding correlated significantly with potencies for many of the pharmacologic activities: muscle relaxation, as measured by muscle relaxation in cats; anticonvulsant effect, as measured by inhibition of pentylenetetrazol-induced seizures in mice; anxiolysis, as measured by a bioassay for human anxiety and inhibition of foot shock-induced fighting in mice (Mohler and Okada,

1977; Squires and Braestrup, 1977; Braestrup and Squires, 1978; Mackerer et al, 1978).

Shortly after the identification of the central benzodiazepine receptor, a functional interaction was discovered between the receptor and GABA. Benzodiazepine binding to the central receptor was found to be enhanced by GABA and GABA analogues (Martin and Candy, 1978; Tallman et al, 1978; Wastek et al, 1978). Scatchard analysis demonstrated that this enhancement of binding was due to an increase in the affinity of the receptor for the benzodiazepines (Martin and Candy, 1978; Tallman et al, 1978; Wastek et al, 1978). The ability of GABA agonists to enhance benzodiazepine binding was stereoselectively inhibited in studies with (-)bicuculline and (+)bicuculline, enantiomeric GABA receptor antagonists (Tallman et al, 1978). In addition, anions, such as chloride, bromide, and iodide, that are able to penetrate the activated inhibitory postsynaptic membrane are also able to produce, like GABA, an increase in the affinity of the central receptor for benzodiazepines (Costa et al, 1979; Martin and Candy, 1980). Subsequent studies have shown that diazepam enhances GABA binding (Skerritt et al, 1982). These findings indicate the presence of an allosteric interaction between GABA binding and central benzodiazepine receptor binding and also provide clues to the molecular mechanism underlying the previously-observed

physiological synergism between GABA and the benzodiazepines.

The functional significance of the central receptor has been established by the identification of two classes of receptor ligands, inverse agonists and antagonists. Beta-carbolines were originally identified as ligands for the central receptor in an investigation of possible endogenous ligands (Nielsen et al, 1979). Beta-carbolines exhibited high affinity for the central receptor (Nielsen and Braestrup, 1980; Braestrup and Nielsen, 1983). However, the behavioral effects of the benzodiazepines were antagonized when the benzodiazepines were administered with beta-carbolines (Oakley and Jones, 1980; Tenen and Hirsch, 1980), while administration of beta-carbolines alone produced effects that were proconvulsant and anxiogenic, opposite to the effects produced by benzodiazepines (Oakley and Jones, 1980; Robertson, 1980; Braestrup et al, 1982; Nutt et al, 1982). This "inverse agonist" activity of the beta-carbolines has been supported both by receptor studies demonstrating decreased beta-carboline affinity in the presence of GABA (Braestrup and Nielsen, 1981; Braestrup et al, 1982) and by physiological studies demonstrating beta-carboline antagonism of the inhibitory effects of GABA (Polc et al, 1981b). Another compound, Ro15-1788, an imidazobenzodiazepinone derivative, was also found to bind to the central receptor with high affinity (Hunkeler et al,

1981; Mohler and Richards, 1981; Mohler et al, 1981) and antagonize the behavioral effects of the benzodiazepines (Hunkeler et al, 1981; Nutt et al, 1982). However, unlike the beta-carbolines, Ro15-1788 exhibited few behavioral effects when given alone (Hunkeler et al, 1981; Darragh et al, 1983). The antagonist activity of Ro15-1788 was apparent in binding and physiologic studies that demonstrated little GABA-induced change in Ro15-1788 binding affinity (Mohler and Richards, 1981) and no significant effect of Ro15-1788 on GABA-mediated inhibition (Polc et al, 1981a). These studies with inverse agonists and antagonists provide further evidence that the central benzodiazepine receptor mediates many of the pharmacologic effects of the benzodiazepines by modulating GABA activity.

In addition to GABA and benzodiazepine agonists, antagonists, and inverse agonists, several other compounds have been shown to interact with the benzodiazepine receptor-GABA receptor complex. In electrophysiological studies, the sedative barbiturates, like the benzodiazepines, enhanced the effects of GABA (MacDonald and Barker, 1979; Olsen et al, 1986). However, unlike the benzodiazepines which primarily increased the probability of chloride channel opening, the barbiturates increased open channel lifetime (Study and Barker, 1981). In binding studies, it has been shown that the barbiturates increase the affinity of benzodiazepines for the central site (Leeb-

Lundberg et al, 1980; Skolnick et al, 1980, 1981; Ticku, 1981a). Barbiturates also increase the enhancement by GABA of benzodiazepine binding (Ticku, 1981; Supavilai et al, 1982). It is believed that the barbiturates produce these effects by interacting with the "picrotoxin site," a site that binds the convulsant picrotoxin and has been studied with [^{3}H]-dihydropicrotoxinin ([^{3}H]-DHP). Barbiturates have been shown to inhibit [^{3}H]-DHP binding to the picrotoxin site (Ticku and Olsen, 1978; Ticku, 1981b). Picrotoxin antagonizes the inhibitory effects of GABA (Simmonds, 1980). Presumably, picrotoxin binding to the site induces an allosteric alteration in the benzodiazepine receptor-GABA receptor complex such that conductance changes are blocked. Barbiturates appear to act at the same site or a site overlapping the picrotoxin site and increase conductance by prolonging the lifetime of the open channel.

During the molecular characterization of the central receptor, it was discovered that benzodiazepines containing a 7-C NO_2 group, such as flunitrazepam and clonazepam, are naturally photoreactive and form a covalent attachment with the central receptor in the presence of ultraviolet irradiation (Battersby et al, 1979; Mohler et al, 1980). It has been hypothesized that ultraviolet light induces the formation of a resonance structure containing a positively charged carbon atom in the 2-C position that is then

subjected to nucleophilic attack by an amino acid residue, possibly by the phenolic group on a tyrosine residue (Sherman-Gold, 1983). This resonance structure is presumably only able to exist in benzodiazepines that contain the 7-C NO_2 group. Photoaffinity labeling of crude synaptosomal fractions with [^{3}H]-flunitrazepam resulted in the specific photolabeling of a protein with a molecular weight of approximately 50,000 daltons (Mohler et al, 1980; Thomas and Tallman, 1981). The K_D value of [^{3}H]-FNZ for photolabeling the protein was similar to that obtained for reversible binding (Mohler et al, 1980; Sieghart et al, 1983), and the potencies of a series of benzodiazepines to inhibit [^{3}H]-FNZ photolabeling of the protein correlated significantly with the potencies of these compounds to inhibit reversible [^{3}H]-FNZ binding to crude synaptosomal membranes (Mohler et al, 1980; Thomas and Tallman, 1981). These findings indicate that the photolabeled protein is the central receptor.

The photoaffinity labeling procedure facilitated the purification of the central receptor. As the initial step in the purification procedure, various detergents have been employed successfully to solubilize the receptor from the synaptosomal membrane (Gavish et al, 1979; Yousufi et al, 1979; Stephenson and Olsen, 1982). A purification scheme has been developed that involves application of the solubilized receptor preparation to a benzodiazepine

affinity column. The retained receptor is then eluted from the column by washing with a high concentration of a water-soluble benzodiazepine, such as clorazepate (Gavish and Snyder, 1981; Martini et al, 1982; Sigel et al, 1983). The purified receptor that is eluted from the column exhibits binding by [^{3}H]-FNZ and [^{3}H]-muscimol.

The benzodiazepine receptor-GABA receptor complex appears to be a tetramer composed of two alpha chains and two beta chains. The alpha chains have molecular weights of approximately 53,000 daltons and contain the benzodiazepine binding domains, while the beta chains exhibit molecular weights of approximately 57,000 daltons and contain the GABA binding sites (Sigel et al, 1983; Sigel and Barnard, 1984; Casalotti et al, 1986; Mamalaki et al, 1987). Recently, cloned cDNAs encoding both the alpha and beta chains have been isolated (Barnard et al, 1987; Schofield et al, 1987). The deduced amino acid sequences for the two subunits possess significant sequence similarity with each other as well as with the subunits of the glycine receptor and the nicotinic acetylcholine receptor, suggesting the existence of a super-family of ligand-gated ion channel receptors.

During the initial characterization of the central receptor, significant levels of high affinity [^{3}H]-FNZ and [^{3}H]-diazepam binding were detected in peripheral tissues (Braestrup and Squires, 1977; Gallager et al, 1981). Like

the central receptor, this peripheral benzodiazepine binding site exhibited saturable, reversible, and selective binding by the benzodiazepines (Marangos et al, 1982; Schoemaker et al, 1983). However, the peripheral site exhibited a pharmacologic profile very different from that exhibited by the central receptor. Binding to the peripheral site was not stereoselective for 3-C enantiomers (Schoemaker et al, 1983; Wang et al, 1984c). Also, peripheral site binding was not affected by GABA or anions such as chloride (Marangos et al, 1982; Schoemaker et al, 1983), suggesting that the peripheral site was not associated with the GABA receptor-chloride channel complex. Furthermore, many benzodiazepines possessed markedly different relative potencies for the central receptor and the peripheral site. For example, clonazepam exhibited a relatively high affinity for the central receptor and a relatively low affinity for the peripheral site, while Ro5-4864 exhibited a relatively low affinity for the central receptor and a relatively high affinity for the peripheral site (Braestrup and Squires, 1977; Gallager et al, 1981).

Due to its high relative affinity for the peripheral site, [^{3}H]-Ro5-4864 has been employed as the radioligand in many of the binding studies of the peripheral site. [^{3}H]-PK-11195, an isoquinoline carboxamide derivative, also possesses a high relative affinity for the peripheral site and, like [^{3}H]-Ro5-4864, has been employed in the

characterization of the peripheral site (LeFur et al, 1983; Anholt, 1986).

The peripheral site exhibits a brain regional and tissue distribution that is clearly distinct from that of the central receptor. The peripheral site is present in brain, with highest levels of binding detected in the olfactory bulb, choroid plexus, and ependymal linings of the ventricles (DeSouza et al, 1985; Richards et al, 1982; Benavides et al, 1983). Binding to the peripheral site, unlike the central receptor, has been detected in many peripheral tissues: highest levels have been measured in the adrenal gland, salivary gland, and lung; intermediate levels in the kidney, heart, and spleen; lowest levels in the liver, pancreas, and skeletal muscle (Anholt et al, 1985; DeSouza et al, 1985).

It has not been possible to photoaffinity label the peripheral site with NO_2-containing benzodiazepines. Photolabeling crude synaptosomal fraction with flunitrazepam does not significantly alter levels of peripheral site binding (Thomas and Tallman, 1981; Marangos et al, 1982). It is possible that the photoreactive portion of the benzodiazepine molecule is not oriented in a favorable position for covalent attachment or the binding domain of the peripheral site does not contain the particular amino acid residue necessary for covalent attachment. Recently, an isothiocyanate derivative of Ro5-

4864 has been synthesized that appears to bind irreversibly to the peripheral site (Lueddens et al, 1986). This compound, AHN 086, may facilitate the molecular characterization of the site.

It is not known whether the peripheral site represents a physiologically-relevant benzodiazepine receptor or merely a benzodiazepine acceptor protein. The relative binding potencies for the peripheral site do not correlate well with potencies for any of the pharmacologic test systems (Braestrup and Squires, 1977; Tallman et al, 1980; Marangos et al, 1982; Schoemaker et al, 1983). However, benzodiazepines affect growth and differentiation in many different cell types, including melanoma cells (Matthew et al, 1981), thymoma cells (Wang et al, 1984a), PC12 cells (Curran and Morgan, 1985; Morgan et al, 1985), HL-60 cells (Ishiguro et al, 1987), and Friend erythroleukemia cells (Clarke and Ryan, 1980; Wang et al, 1984b). Although no definitive correlations have yet been established, it is possible that the peripheral site mediates some of these effects.

Recent studies investigating the possible functional role of the peripheral site have examined the site's subcellular distribution (Anholt, 1986; Anholt et al, 1986). In these studies, it was found that the peripheral site was enriched in the mitochondrial fraction. Further subfractionation of the mitochondrial membrane with

digitonin treatment demonstrated that peripheral site binding was released along with monoamine oxidase, a marker enzyme for the mitochondrial outer membrane. In the mitochondrial outer membrane, the peripheral site exhibited a very high binding capacity of 175 pmol/mg protein. This finding indicates that the binding protein is a major protein of the mitochondrial outer membrane since a density of 175 pmol/mg represents 2-10% of the outer membrane protein for a protein with a molecular weight of 20,000-100,000 daltons (Anholt, 1986). It has been hypothesized that the peripheral site may be porin, a major mitochondrial protein that may allow transfer of large anions across the membrane and serve to anchor hexokinase to the membrane (Anholt, 1986). Benzodiazepine binding to porin could modulate cellular metabolism and perhaps ultimately affect cell growth and differentiation.

In spite of the identification of the central and peripheral sites, binding to these two sites cannot account for all of the effects of the benzodiazepines. In addition to their interaction with the GABA system, the benzodiazepines have been shown to exhibit several other biochemical and physiologic actions that are not clearly associated with either the central receptor or the peripheral site.

Benzodiazepines alter neurotransmitter release and calcium uptake in brain by mechanisms that are independent

of the central receptor. Benzodiazepines at a concentration of approximately 1 uM facilitate depolarization-dependent uptake of calcium into rat brain synaptosomes (Paul and Skolnick, 1982; Paul et al., 1982). Similar concentrations stimulate depolarization-dependent release of dopamine from rat striatal tissue (Mitchell and Martin, 1978; Martin and Mitchell, 1979) and GABA from rat cortical tissue (Mitchell and Martin, 1980). Concentrations of 100 uM inhibit depolarization-dependent calcium uptake into rat and mouse brain synaptosomes (Taft and DeLorenzo, 1984; Leslie et al., 1986) and depolarization-dependent release of GABA from rat cortical tissue (Mitchell and Martin, 1980) and 5-hydroxytryptamine (5HT) from rat hippocampal synaptosomes (Balfour, 1980).

GABA-independent actions of the benzodiazepines on several putative neurotransmitters and modulators have been described. Benzodiazepines antagonize the central and peripheral effects of cholecystokinin (Brandwejn and DeMontigny, 1984; Kubota et al., 1985a-d; Meldrum et al., 1986) and reduce the turnover rate of 5HT (Wise et al., 1972; Lister and File, 1983; Nishikawa and Scatton, 1986), a neurotransmitter that has been implicated in the control of anxiety (Johnston and File, 1986). Benzodiazepines inhibit depolarization that is induced by excitatory amino acids in spinal cord neurons (Evans et al., 1977; Davies and Polc, 1978), cerebral cortex (Assumpcao et al., 1979),

and hippocampus (DeBonnell and DeMontigny, 1983). In the 0.1-10 uM concentration range, benzodiazepines inhibit adenosine uptake in rat brain slices and synaptosomes (Phillis et al., 1980; Wu et al., 1981; Phillis et al., 1981; Bruns et al., 1983a; Morgan and Stone, 1986). Benzodiazepines at a concentration of 10-100 uM have been shown to prolong and increase the magnitude of the stimulation of N-acetyltransferase by norepinephrine (Matthew et al., 1984).

In addition to potentiating GABA-mediated inhibition, benzodiazepines produce other physiologic effects. In cultured mouse spinal neurons, benzodiazepines at 0.1-10 nM depress electrical excitability by increasing membrane conductance and by elevating the threshold for action potential activity (MacDonald and Barker, 1982; Study and Barker, 1982; Barker and Owen, 1986). In the same system, high nanomolar concentrations of benzodiazepines have been shown to inhibit sustained repetitive firing (MacDonald and Barker, 1982; Skerritt et al, 1984; MacDonald and McLean, 1986). Low nanomolar concentrations have been shown to augment Ca^{2+}-mediated K^{+}-conductance in hippocampal neurons (Carlen et al, 1983a,b).

In terms of pharmacologic test systems, relative benzodiazepine binding potencies for the central receptor do not correlate with relative benzodiazepine potencies for inhibition of maximal electric shock (MES)-induced seizures

in mice, a test system for anticonvulsant activity (Mohler and Okada, 1978a,b). Relative binding potencies for the central receptor do correlate with relative potencies for benzodiazepine inhibition of pentylenetetrazol (PTZ)-induced seizures in mice, another anticonvulsant test system (Mohler and Okada, 1977; Squires and Braestrup, 1977; Mackerer et al, 1978). The PTZ-induced seizure model has been associated with the ability of benzodiazepines to inhibit generalized absence seizures in man; the MES-induced seizure system has been associated with benzodiazepine inhibition of generalized tonic-clonic seizures and status epilepticus in man (MacDonald and McLean, 1986). It has been proposed that compounds acting on the GABA system, such as benzodiazepines and phenobarbital, also inhibit PTZ-induced seizures in mice and generalized absence seizures in man, while compounds that inhibit sustained repetitive firing in cultured neurons, such as benzodiazepines, phenytoin, carbamazepine, valproic acid, and phenobarbital, also inhibit MES-induced seizures in mice and generalized tonic-clonic seizures and status epilepticus in man (MacDonald and McLean, 1986). The molecular mechanism underlying the inhibition of sustained repetitive firing has not been identified. It is possible that there is a common molecular mechanism, independent of the benzodiazepine receptor-GABA receptor complex, by which benzodiazepines and other anticonvulsants

inhibit sustained repetitive firing, MES-induced seizures in animals, and generalized tonic-clonic seizures and status epilepticus in man.

Benzodiazepines affect growth and differentiation in several cell types by mechanisms that are not clearly associated with the central or peripheral sites. In PC12 cells, benzodiazepines stimulate the induction of ornithine decarboxylase activity and inhibit nerve growth factor-induced neurite outgrowth (Curran and Morgan, 1985; Morgan et al., 1985). Benzodiazepines stimulate differentiation in HL-60 cells (Ishiguro et al., 1987) and induce the synthesis of hemoglobin in Friend erythroleukemia cells (Clarke and Ryan, 1980; Wang et al., 1984b).

Thus, a wide range of studies indicate that it is possible that another distinct class of binding sites exists that represents the molecular mechanism by which benzodiazepines produce some of their effects. The objective of this investigation was to attempt to identify a novel class of benzodiazepine-binding sites. Photoaffinity labeling studies were performed with [^{3}H]-clonazepam ([^{3}H]-CNZ) and [^{3}H]-flunitrazepam ([^{3}H]-FNZ), photoactivated benzodiazepines that contain a NO_2 group in the 7-C position and are potent in most pharmacologic test systems. These studies led to the identification of a previously unidentified protein in brain that exhibits specific photolabeling by NO_2-containing benzodiazepines.

Photolabeling of this protein was characterized. Subsequent studies were undertaken in which the protein was purified to apparent homogeneity and the biochemical properties of the purified protein were investigated. Results of this work have been published (Bowling and DeLorenzo, 1986; Bowling and DeLorenzo, 1987a,b).

II. METHODS

A. Materials. [^{3}H]-CNZ (43 Ci/mmole) and all unlabeled benzodiazepines were kindly provided by Dr. M. Drzyzga of Hoffman-LaRoche (Nutley, NJ). [^{3}H]-FNZ (85 Ci/mmole) was obtained from Amersham (Arlington Hts., IL). Formula 963 was obtained from New England Nuclear (Boston, MA). Column chromatography media included Affi-Gel Blue (100-200 mesh) from Bio-Rad (Richmond, CA), Reactive Green 19-Agarose from Sigma (St. Louis, MO), and Sephadex G-100 from Pharmacia (Piscataway,NJ). All other reagents were from Sigma (St. Louis, MO).

B. Tissue Preparation. Female Sprague-Dawley rats (100-150 g) were decapitated, and whole brains were quickly excised and homogenized. For brain region studies, the brains were dissected and homogenized within 10 min of decapitation. For tissue distribution studies, the appropriate organs were minced finely and homogenized within 10 min of decapitation. Routinely, the brains were homogenized at 4° C in 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$ (pH 7.4) (100/2/1 Buffer) containing 0.3 mM phenylmethylsulfonyl fluoride (PMSF). A volume of 1.5 ml of 100/2/1 Buffer was employed for each brain homogenized. The homogenate was centrifuged at 100,000xg for 60 min to obtain crude membrane (pellet) and cytosolic (supernatant) fractions. The cytosolic

fractions were employed in standard photolabeling reactions. For boiled samples, cytosol at a concentration of 2 mg protein/ml was placed in a boiling water bath for 10 min prior to photolabeling. For pH studies, the cytosolic fraction at approximately 20 mg/ml was diluted to 2 mg/ml with an aliquot of 100/2/1 Buffer that had been adjusted to the appropriate pH. There were no significant pH changes after addition of the cytosolic fraction to the aliquot of buffer. For subcellular fractionation studies, the brains were homogenized at 4° C in 0.32 M sucrose, 20 mM Pipes (pH 7.4) containing 0.3 mM PMSF. The homogenate was centrifuged at 1200xg for 10 min to yield a crude nuclear pellet (P_1). A crude synaptosomal pellet (P_2) was obtained from the resulting supernatant (S_1) by centrifugation at 17,000xg for 12 min. The supernatant (S_2) was further fractionated by centrifuging at 100,000xg for 60 min to obtain a crude microsomal pellet (P_3) and a cytosolic fraction (S_3). A protein concentration of 2-3 mg/ml was routinely employed for photolabeling reactions by diluting the cytosolic fraction with 100/2/1 Buffer. Protein concentration was determined by the method of Bradford (Bradford, 1977) using bovine gamma-globulin as a standard. Employing the same standard, similar results were obtained by the method of Lowry (Lowry et al., 1951).

C. Photoaffinity Labeling. Wells on microtiter plates were employed for photolabeling. The radioligand ([^{3}H]-CNZ or

[^{3}H]-FNZ) and ethanol (total photolabeling) or 50 uM CNZ (nonspecific photolabeling) were added to the wells. A final radioligand concentration of 600 nM was employed in most studies in order to obtain sufficient radioactive incorporation for accurate quantitation. For saturation studies, a wide range of radioligand concentrations was obtained by diluting the stock solution with ethanol or concentrating it by adding unlabeled compound or by evaporation under a stream of N_2. Reactions were initiated by adding the appropriate fraction and mixing immediately with a micropipet. The final volume of routine reaction mixtures was 100 uL. The final concentration of ethanol was generally 5-10%, a concentration that did not significantly alter specific photolabeling. Samples were incubated in the dark at 4° C for 30 min and then exposed to shortwave ultraviolet irradiation (Mineralight C81) at 4° C for 60 min. It was not possible to photolabel at a constant, controlled temperature other than 4° C due to excessive heating of the reaction mixture during the irradiation time period. For selectivity studies, the irradiation time was reduced to 15 min to increase the probability of detecting the effect of a non-photoactivated, reversibly-bound compound. Photolabeling was terminated by adding sodium dodecyl sulfate (SDS) stop solution to the samples (DeLorenzo et al., 1977). For protease-treated samples, reaction mixtures were incubated

with trypsin (4.5 U/100 uL), pepsin (4.15 U/100 uL), or proteinase K (0.73 U/100 uL) for 5 min at 37° C prior to adding SDS stop solution. Control samples for the protease studies were incubated under the same conditions in the absence of protease.

D. Quantitation of ^3H Incorporation. Following photoaffinity labeling, proteins from each sample were separated by electrophoresis on 25 cm long, 8.5% SDS-polyacrylamide slab gels (DeLorenzo et al., 1977). Gels were stained with Coomassie blue and then dried and autoradiographed or sliced. For autoradiographs, ^3H incorporation was determined by computer-assisted densitometry of digitized images. For gel slicing, 1-2 mm wide slices were oxidized in scintillation vials by adding 250 uL of hydrogen peroxide and incubating at 80° C for 3 hours. Four ml of Formula 963 were added. Liquid scintillation counting of samples was performed in a Beckman LS 2800 at 62% efficiency. Similar results were obtained by the autoradiographic and gel slicing methods.

E. Calculations. For calculations, nonspecific photolabeling was subtracted from total photolabeling to obtain specific photolabeling (Bennett, 1978; Williams and Lefkowitz, 1978). Inhibition constants (K_i values) were determined by plotting the displacement data according to the method of Hill (Bennett, 1978; Williams and Lefkowitz, 1978). IC_{50} values (x intercepts) were obtained from the

Hill plots and converted to K_i values by the equation: $K_i = IC_{50}/(1+[L]/K_D)$, where [L] is the radioligand concentration and K_D is the apparent dissociation constant of the radioligand determined by Scatchard analysis (Cheng and Prusoff, 1973). For potency correlations, the K_i values determined with [^{3}H]-CNZ were correlated with drug potency data from previous studies (Zbinden and Randall, 1967; Randall et al., 1974) by linear regression analysis (Braestrup and Squires, 1977; Mohler and Okada, 1977).

F. Affi-Gel Blue Column Chromatography. Cytosol from whole rat brain was prepared as described for photolabeling studies except that the buffer concentration was reduced in order to decrease the ionic strength of the medium. The resulting homogenization medium was 20 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$ (pH 7.4) (20/2/1 Buffer) containing 0.3 mM PMSF. Routinely, 7.5 ml of undiluted cytosol (approximately 20 mg protein/ml) was applied to a 1.6 cm-diameter column containing 15 ml of Affi-Gel Blue that had been washed with three bed volumes of 20/2/1 Buffer. For all purification procedures, it was essential to use polypropylene tubes since it was found that the binding protein adsorbed strongly to borosilicate and polyallomer. Affi-Gel Blue column chromatography, as well as all other column chromatography, was performed at 4° C. Cytosol was loaded onto the Affi-Gel Blue column at a flow rate of approximately 18 ml/hr; all subsequent column washings and

elutions were performed at a flow rate of approximately 30 ml/hr. After loading the cytosol, the column was washed with the 20/2/1 Buffer until the absorbance at 280 nm reached baseline (generally about three bed volumes) and then sequentially eluted with 20 mM NaCl in 20/2/1 Buffer, 50 mM NaCl in 20/2/1 Buffer, and 500 mM NaCl in 20/2/1 Buffer. For analysis of protein patterns, proteins from column fractions were separated by SDS-PAGE and silver stained. Approximately 10 ml from the initial portion of the 50 mM NaCl peak of absorbance at 280 nm contained the 65,000 dalton photolabeled protein. These fractions were combined to form the "Blue Fraction."

G. Reactive Green 19-Agarose Column Chromatography. Reactive Green 19-Agarose was prepared by swelling the agarose in 20/2/1 Buffer containing 50 mM NaCl (20/2/1+50 Buffer) at 4° C for 60 min. To remove any residual lactose, the agarose was then washed with approximately 50 bed volumes of 20/2/1+50 Buffer on a sintered glass funnel. The agarose was then poured into a 1.2 cm-diameter column to a final bed volume of 5 ml and washed with another three bed volumes of 20/2/1+50 Buffer. Loading of sample and all subsequent washings and elutions were performed at a flow rate of approximately 24 ml/hr. The Blue Fraction was applied to the column and washed with 20/2/1+50 Buffer until the absorbance at 280 nm reached baseline (usually between one-half and one bed volume). A NaCl gradient from

50 mM NaCl to 200 mM NaCl (with a constant buffer of 20 mM Pipes, 2 mM EGTA, and 1 mM $MgCl_2$ (pH 7.4)) was then applied to the column in a total volume of 10 ml. A peak of absorbance at 280 nm generally appeared near 125 mM NaCl. This peak corresponded to the photolabeled protein and constituted approximately 5 ml. These peak fractions were combined to form the "Green Fraction."

H. Sephadex G-100 Column Chromatography. Sephadex G-100 resin was swelled and then loaded in a 0.9 cm-diameter column to a final bed volume of 47 ml. The column was equilibrated with 20/2/1 Buffer. Calibration was performed by loading a 500 uL sample containing 50 ug each of phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen a (25,000 daltons), and ribonuclease a (13,700 daltons). A 5 ug sample of Green Fraction was loaded. This sample had been dialyzed against distilled water for 24 hrs, lyophilized, and resuspended with 500 uL of 20/2/1 Buffer. One ml fractions were collected and subjected to SDS-PAGE to identify fractions which contained the 65,000 dalton protein and protein standards.

I. Amino Acid Composition. Green Fraction was dialyzed against distilled water for 24 hrs and then lyophilized and resuspended to 500 uL with distilled water. Approximately 5 ug of this preparation was employed for amino acid analysis. Aliquots were hydrolyzed in sealed tubes with

constantly boiling HCl for 24 hrs at 110° C. Amino acid analyses were performed with a Durrum MBF amino acid analyzer with fluorescence detection using O-phthaldialdehyde. Proline, cysteine, and tryptophan could not be determined by this method. Amino acid analysis was performed in triplicate.

J. N-terminal Amino Acid Sequencing. Green Fraction was dialyzed against distilled water, lyophilized, and resuspended to 500 uL with distilled water. Approximately 1 nmol (65 ug) of this dialyzed and concentrated Green Fraction was subjected to N-terminal amino acid sequencing. Sequencing was performed with an Applied Biosystems (Model 470A) automatic sequencer equipped with on-line phenylthiohydantoin-amino acid analyzer using the standard program. Sequence analysis was performed in duplicate. The N-terminal sequence that was obtained was tested against Release #10 of the National Biomedical Research Foundation-Protein Information Resource (NBRF-PIR) protein sequence data base (NBRF, Washington DC) using a fast sequence comparison algorithm (Lipman and Pearson, 1985). The program (FASTP) employs Dayhoff's mutation matrix (Dayhoff et al., 1978) to score comparisons.

K. Reversible Binding. Reversible binding to the Green Fraction was examined by several different methods, including filtration on polyethyleneimine (PEI)-treated glass-fiber filters (Bruns et al., 1983b), gel filtration

on small (0.5 ml bed volume) Sephadex G-25 columns (Goldstein and Blecher, 1976), precipitation with polyethylene glycol (PEG)-gamma-globulin (Cuatrecasas, 1972; Yousufi et al., 1979), and small-volume (200 ul) equilibrium dialysis. It was not possible to detect reversible binding to the purified protein with any of these assay systems.

III. RESULTS

A. Identification of a Protein That is Photolabeled by NO_2-Benzodiazepines. Initial studies were performed on the homogenate, crude membrane (100,000xg pellet), and cytosolic (100,000xg supernatant) fractions. In addition to central benzodiazepine receptor binding in the crude membrane fraction (Fig. 1), these studies revealed the presence of a single major protein band that exhibited specific [^{3}H]-CNZ and [^{3}H]-FNZ photolabeling in the cytosolic fraction (Fig. 2). This protein exhibits a molecular weight of approximately 65,000 daltons based on relative mobility on SDS-PAGE (Fig. 3). It was clearly separated from rat albumin by 4-5 mm on 25 cm long, 8.5% SDS-polyacrylamide gels (Figs. 2,3). A few minor bands of radioactivity were apparent in the cytosolic fraction, but the photolabeling of these proteins was not displaced under the conditions employed in this investigation (Fig. 2). In the crude membrane fraction, photolabeling of the 65,000 dalton protein was not observed. Thus, unless otherwise indicated, the cytosolic fraction was utilized in all subsequent studies.

An irradiation time course with a saturating concentration (10 uM) of [^{3}H]-CNZ revealed that photolabeling of the 65,000 dalton protein was maximal at

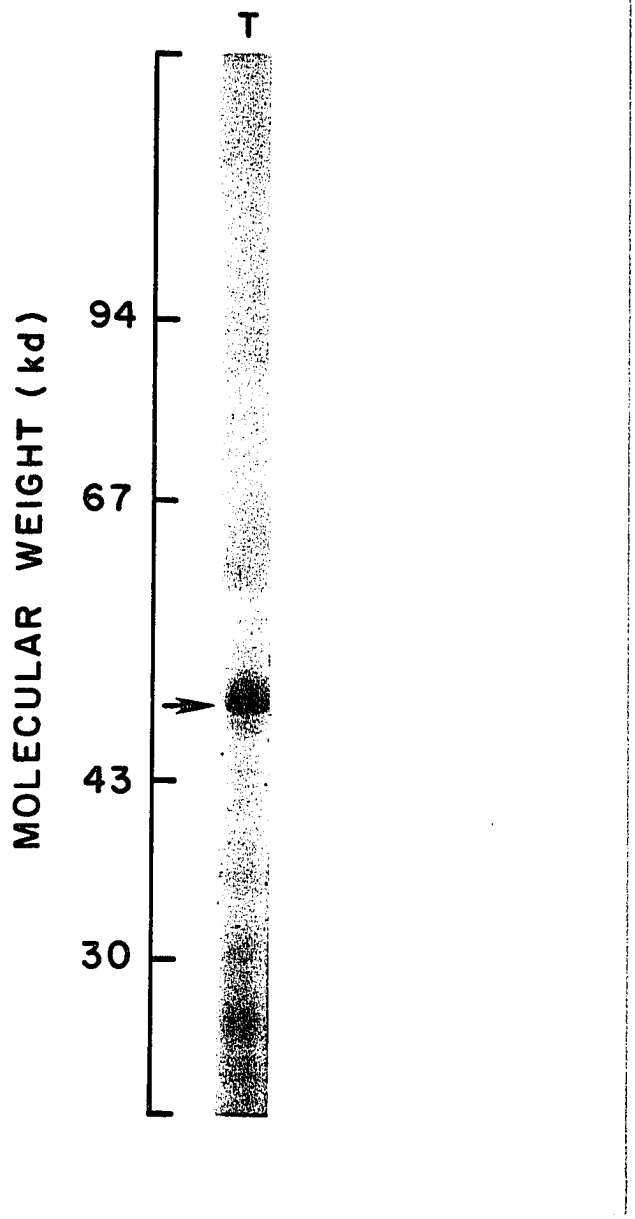


Fig. 1. Autoradiograph of photolabeled crude synaptosomal membrane demonstrating photolabeling of the central receptor (arrow). Samples were incubated with 10 nM [^{3}H]-CNZ at 4° C for 30 min and then irradiated at 4° C for 60 min. The receptor exhibits a relative mobility of approximately 50,000 daltons.

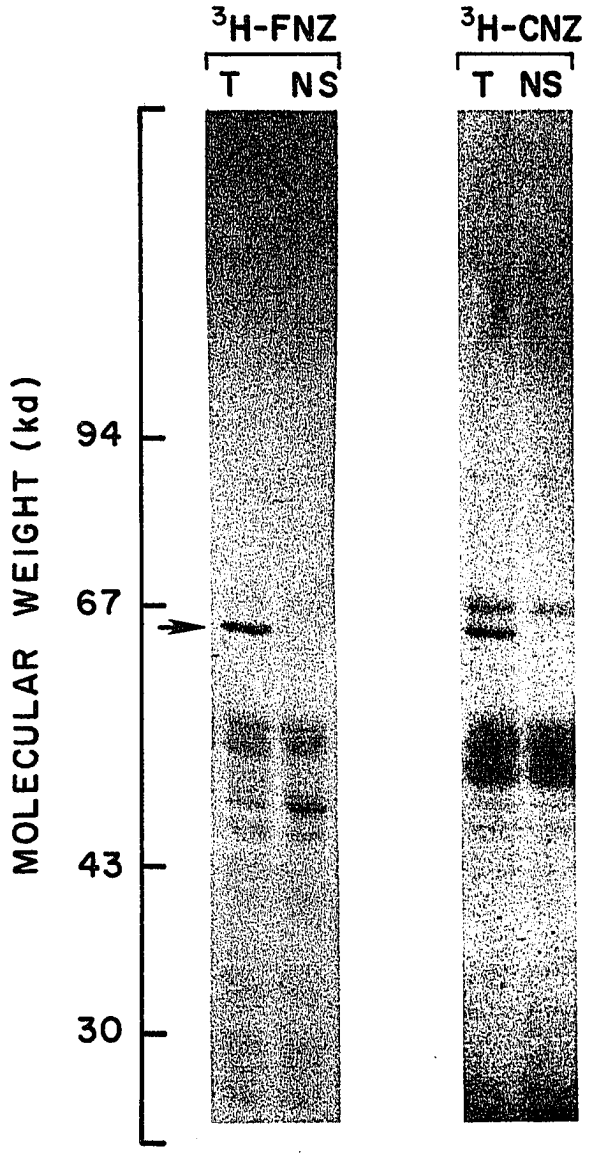


Fig. 2. Autoradiograph of photolabeled cytosolic fraction from rat brain demonstrating displaceable photolabeling of a 65,000 dalton protein (arrow). Samples were incubated with 600 nM [^{3}H]-FNZ or 600 nM [^{3}H]-CNZ in the absence (T, total photolabeling) or presence (NS, nonspecific

photolabeling) of 50 uM unlabeled CNZ. Samples were incubated at 4° C for 30 min and then irradiated with UV light at 4° C for 60 min. Proteins were separated by 8.5% SDS-PAGE on 25 cm long slab gels. The molecular weight markers included phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), and carbonic anhydrase (30,000 daltons).

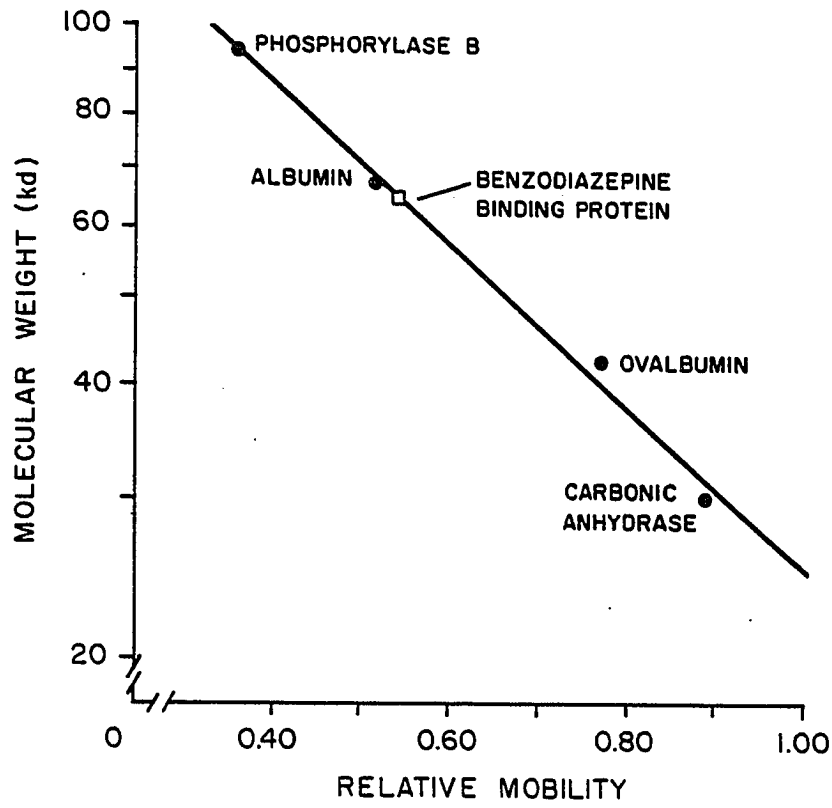


Fig. 3. Molecular weight of the photolabeled protein as determined by relative mobility on SDS-PAGE. The molecular weight determined by linear regression analysis was 65,247 daltons. The value of r for the regression was 0.986.

60 min (Fig. 4). With an irradiation time of 60 min, an incubation time course revealed that specific photolabeling was maximal with a 30 min incubation (Fig. 5). Therefore, experiments were routinely performed with an incubation time of 30 min and an illumination time of 60 min. Photolabeling was linear with protein concentrations up to 6 mg/ml (Fig. 6). A protein concentration of approximately 2 mg/ml was generally utilized. Photolabeling was performed in the presence of many different buffers and ions (Table 1). Labeling was maximal when performed in the presence of 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$ at pH 7.4 (100/2/1 Buffer) (Table 1). Therefore, unless otherwise indicated, this buffer was employed for standard photolabeling reactions. Specific photolabeling was not significantly altered by storage of the cytosolic fractions at -20° C for 30 days or by lyophilization (Fig. 7). Thus, routine binding experiments were performed on cytosolic samples that were stored at -20° C for 30 days or less. Reaction mixtures generally contained 5-10% ethanol. Ethanol concentrations as large as 15% were not found to have a significant effect on specific photolabeling (Fig. 8). Photolabeling data was obtained by autoradiography or gel slicing; similar results were obtained by either method (Fig. 9).

B. Properties of Photolabeled Sites. Many different benzodiazepines were employed in displacement studies.

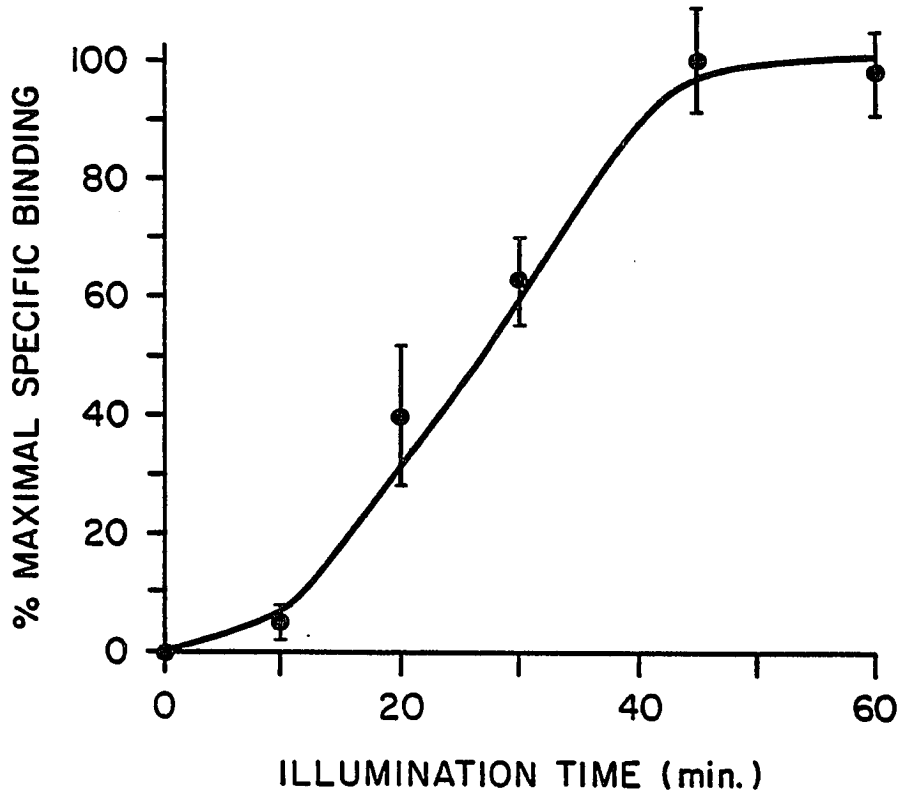


Fig. 4. Illumination time course for specific [^{3}H]-CNZ photolabeling of the 65,000 dalton protein. Specific photolabeling was determined for samples containing the cytosolic fraction and 10 uM [^{3}H]-CNZ. Samples were incubated in the dark at 4° C for 30 min and irradiated for the indicated time. Maximal photolabeling is 0.40 pmol/mg protein. The data are the means (±sem) for four separate determinations.

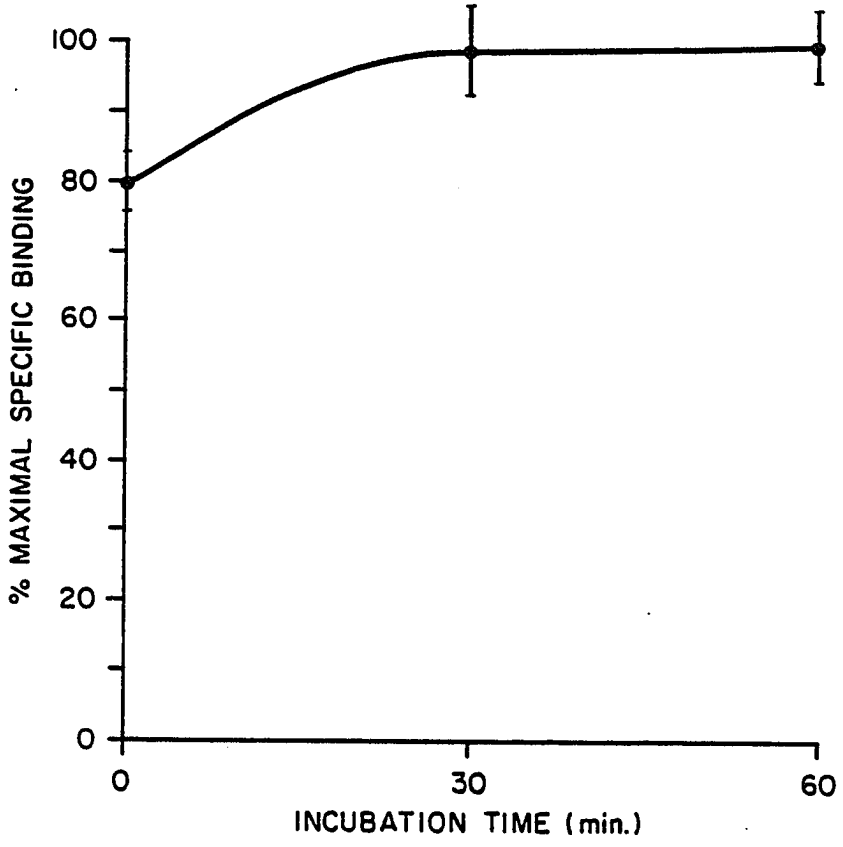


Fig. 5. Incubation time course for specific [^{3}H]-CNZ photolabeling of the 65,000 dalton protein. Samples were incubated in the dark at 4° C for the indicated time and then irradiated for 60 min. Specific photolabeling was determined in the presence of 10 uM [^{3}H]-CNZ. Maximal photolabeling is 0.36 pmol/mg protein. The data are the means (±sem) for four separate determinations.

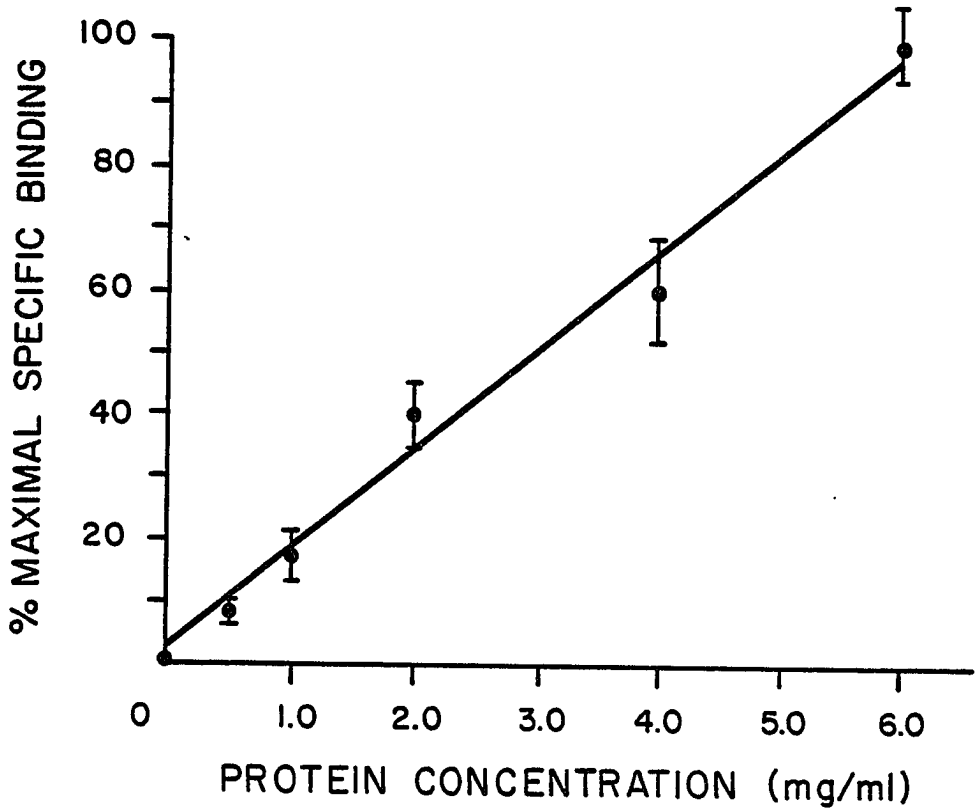


Fig. 6. Dependence of specific [^{3}H]-CNZ photolabeling on protein concentration. The cytosolic fraction at the indicated protein concentration was photolabeled in the presence of 600 nM [^{3}H]-CNZ and ethanol (total photolabeling) or 50 uM CNZ (nonspecific photolabeling). Maximal labeling represents 0.32 pmol/mg protein. The data represent the means (±sem) for three separate determinations.

Table 1. Effect of buffers, ions, boiling, and proteases on specific [^{3}H]-CNZ photolabeling (concentrations in mM).

Pipes	MES	Tris	EGTA	$MgCl_2$	$CaCl_2$	NaCl	Relative Spec. Labeling
100	-	-	2	1	-	-	1.00±0.09
100	-	-	2	-	-	-	0.83±0.07
100	-	-	-	1	-	-	0.92±0.09
100	-	-	-	-	-	-	0.84±0.07
100	-	-	-	-	-	-	0.84±0.06
20	-	-	-	-	-	-	0.44±0.04
-	100	-	-	-	-	-	0.18±0.02
-	-	100	-	-	-	-	0.10±0.01
100	-	-	2	1	-	-	1.00±0.09
100	-	-	20	1	-	-	0.97±0.08
100	-	-	-	1	2	-	0.72±0.06
100	-	-	2	1	-	-	1.00±0.09
100	-	-	2	1	-	150	0.98±0.10
100	-	-	-	1	2	150	0.66±0.05
100	-	-	2	1	-	-	1.00±0.09
100	(no PMSF)		2	1	-	-	0.98±0.10
100	(boiled)		2	1	-	-	0
100	(trypsin)		2	1	-	-	0
100	(pepsin)		2	1	-	-	0
100	(proteinaseK)		2	1	-	-	0

Brains were homogenized in the designated buffers, and cytosolic fractions were prepared. The specific photolabeling in the presence of 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$, and 0.3 mM PMSF was arbitrarily set at 1.00 (actual specific labeling was 0.10 pmol/mg). Unless otherwise indicated, 0.3 mM PMSF was present. Boiled samples were placed in a boiling water bath for 10 min prior to photolabeling. The protease-treated samples were

photolabeled cytosolic fractions that were incubated with trypsin (4.50 U/100 uL), pepsin (4.15 U/100 uL), or proteinase K (0.73 U/100 uL). The values represent the means±sem for at least three separate determinations.

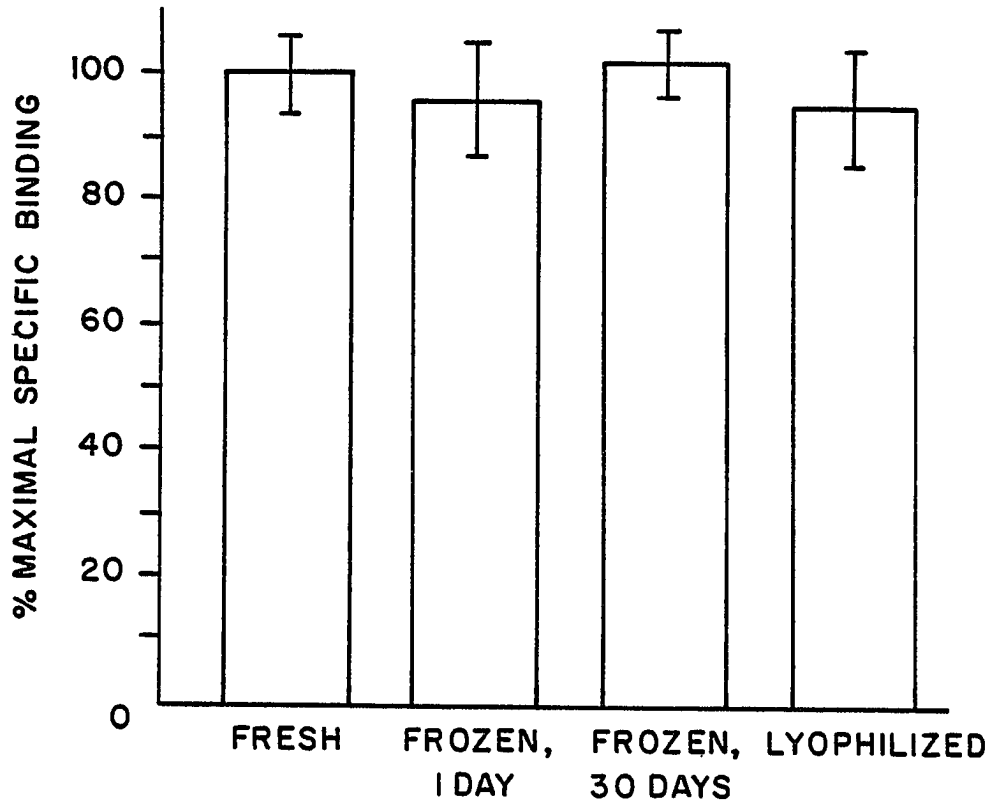


Fig. 7. Effect of storage and lyophilization on specific [^{3}H]-CNZ photolabeling. Cytosolic fractions were either stored for the indicated periods of time at -20° C or lyophilized and resuspended prior to performing photolabeling studies.

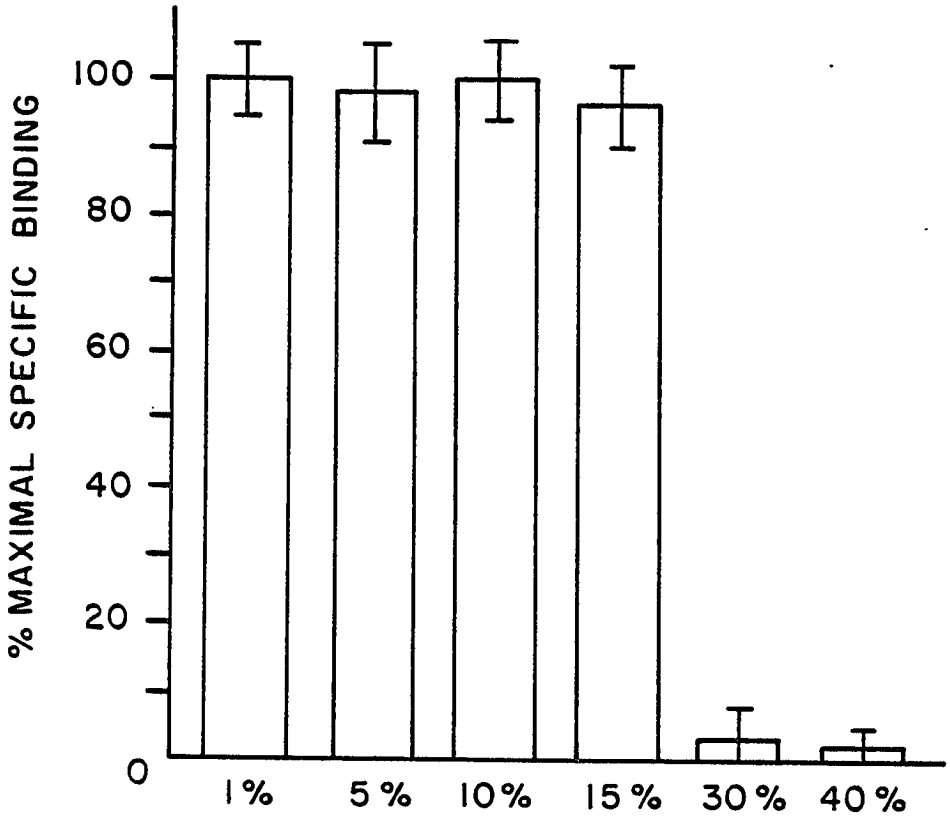


Fig. 8. Effect of ethanol concentration on specific [^{3}H]-CNZ photolabeling. Cytosolic fractions were incubated with the indicated concentration of ethanol at 4° C for 30 min prior to performing photolabeling studies with 600 nM [^{3}H]-CNZ.

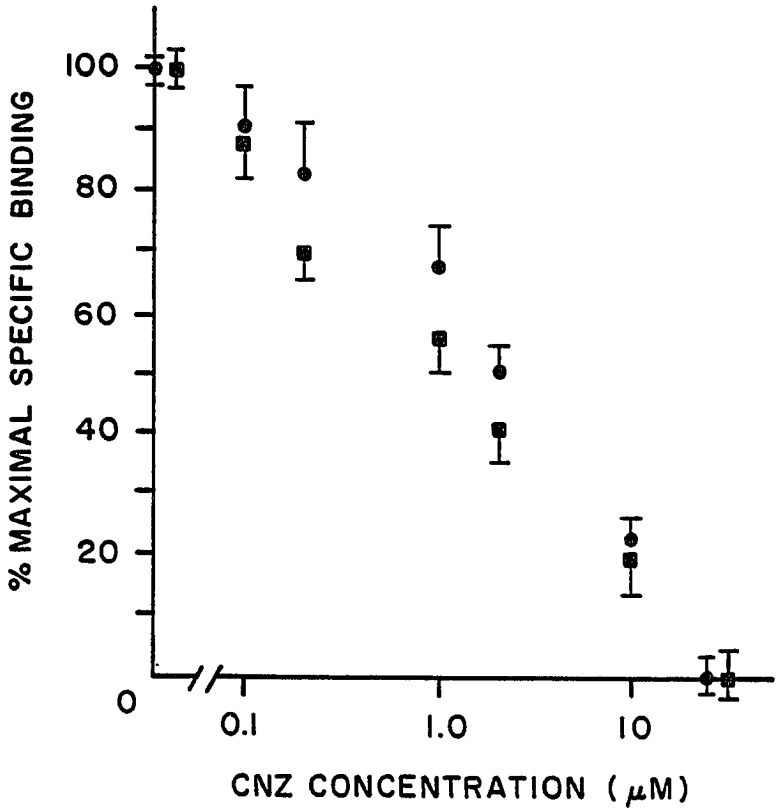


Fig. 9. CNZ displacement of [^{3}H]-CNZ photolabeling as determined by autoradiography and gel slicing. Two samples from the same reaction mixture were separated by SDS-PAGE; photolabeling was then quantitated by autoradiography (filled circles) for one sample and gel slicing (filled squares) for the other sample. The final [^{3}H]-CNZ concentration was 600 nM. Values represent the means (±sem) for at least three separate determinations. For the gel slicing data, maximal specific photolabeling represents 0.10 pmol/mg protein.

Displacement experiments with a wide range of CNZ concentrations were performed. These studies yielded an IC_{50} of 1.47±0.12 uM, a K_i of 0.96±0.08 uM, and a Hill coefficient of 0.91±0.12 (Fig. 10). Stereoselective inhibition of photolabeling was demonstrated by displacement experiments with B9(+) and B9(-), benzodiazepine enantiomers that exhibit markedly different potencies in most pharmacologic test systems (Fig. 11). The K_i value of 0.77 uM calculated for B9(+), the more pharmacologically-active enantiomer, was more than one order of magnitude more potent than that of 8.88 uM determined for B9(-) (Table 2). Displacement experiments were also conducted with many other structurally-different NO_2-containing benzodiazepines. The K_i values and Hill coefficients were determined for these compounds (Table 2). A wide range of K_i values was obtained, and all of the Hill coefficients were close to unity.

Saturation experiments with [^{3}H]-CNZ were conducted using the photolabeling procedure, a technique that has been employed in photolabeling studies of the central benzodiazepine receptor (Sieghart et al, 1983). Significant levels of photolabeling were detectable at concentrations of [^{3}H]-CNZ as low as 100 nM (Fig. 12). Specific photolabeling saturated in the 5-10 uM concentration range. Transformation of the saturation data by the method of Scatchard yielded a linear plot (Fig. 13).

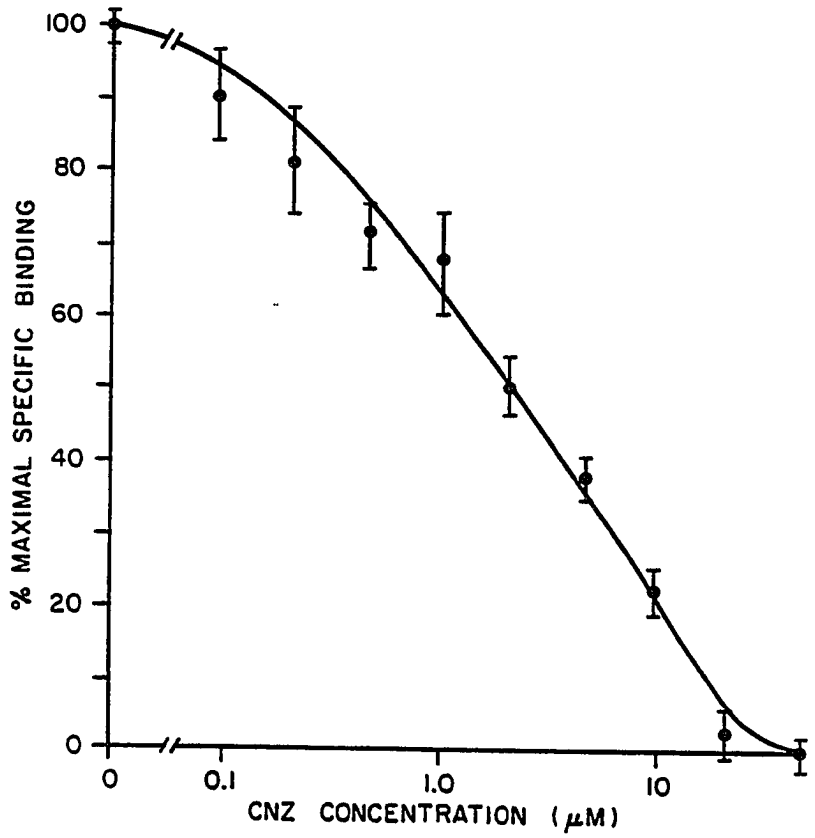


Fig. 10. Displacement by CNZ of [^{3}H]-CNZ photolabeling of the 65,000 dalton protein. The IC_{50} value is 1.47±0.1 uM and the Hill coefficient is 0.91±0.12. Samples were incubated with 600 nM [^{3}H]-CNZ in the presence of the indicated concentration of unlabeled CNZ. Values represent the means (±sem) for six separate determinations. Maximal specific photolabeling (100%) represents 0.10 pmol/mg protein.

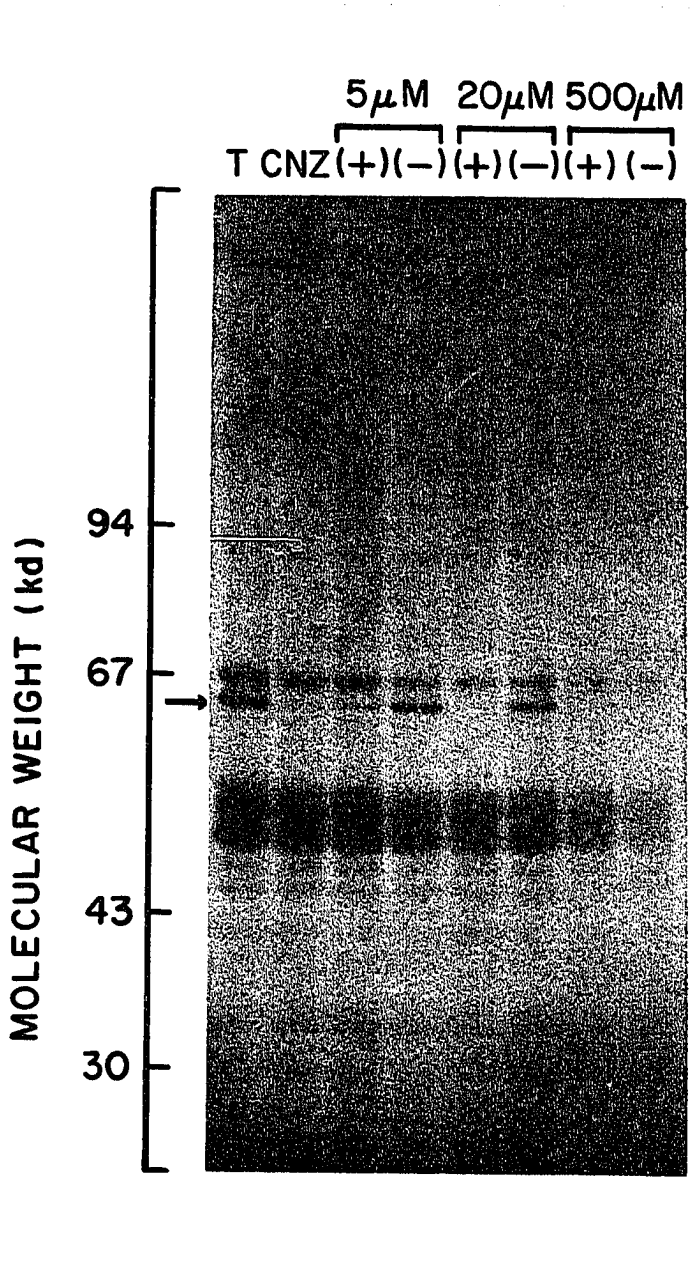
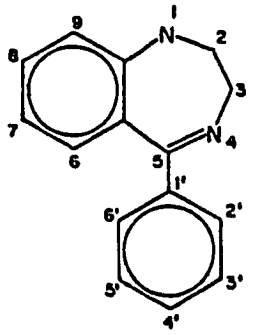


Fig. 11. Autoradiograph of photolabeled cytosolic fraction demonstrating stereoselective inhibition of photolabeling of the 65,000 dalton protein (arrow). Samples were incubated with 600 nM [^{3}H]-CNZ in the presence of ethanol (T, total), 50 uM unlabeled CNZ (NS, nonspecific), or the

indicated concentration of B9(+) or B9(-). Densitometric scanning revealed that specific photolabeling was inhibited 93% by 5 uM of B9(+) and only 25% by 5 uM of B9(-).

Table 2. K_i values and Hill coefficients for nine benzodiazepines.



Compound	7-C	1-N	2-C	3-C	2'-C	Ki(uM)	Hill Coeff.
FNZ	$-NO_2$	$-CH_3$	=O	-H	-F	0.39±0.08	0.87±0.09
B9(+)	$-NO_2$	-H	=O	$-CH_3$	-Cl	0.77±0.09	0.94±0.16
CNZ	$-NO_2$	-H	=O	-H	-Cl	0.96±0.08	0.91±0.12
Nitrazepam	$-NO_2$	-H	=O	-H	-H	1.72±0.11	0.88±0.23
Ro5-3590	$-NO_2$	-H	=O	-H	$-CF_3$	1.75±0.09	1.14±0.22
Ro5-6219	$-NO_2$	*	=O	-H	-H	4.40±1.04	1.00±0.20
B9(-)	$-NO_2$	-H	=O	$-CH_3$	-Cl	8.88±0.80	0.89±0.12
Ro5-3718	$-NO_2$	-H	**	-H	-H	12.17±0.79	0.94±0.10
Ro7-7538	$-NO_2$	***	=O	-H	-H	12.33±0.73	0.83±0.06

* $-CH_2CONHCH_3$
** $-NHCH_3$
*** $-CCHCH_2$

The radioligand employed in these studies was [^{3}H]-CNZ. The values represent the means±sem of six separate determinations.

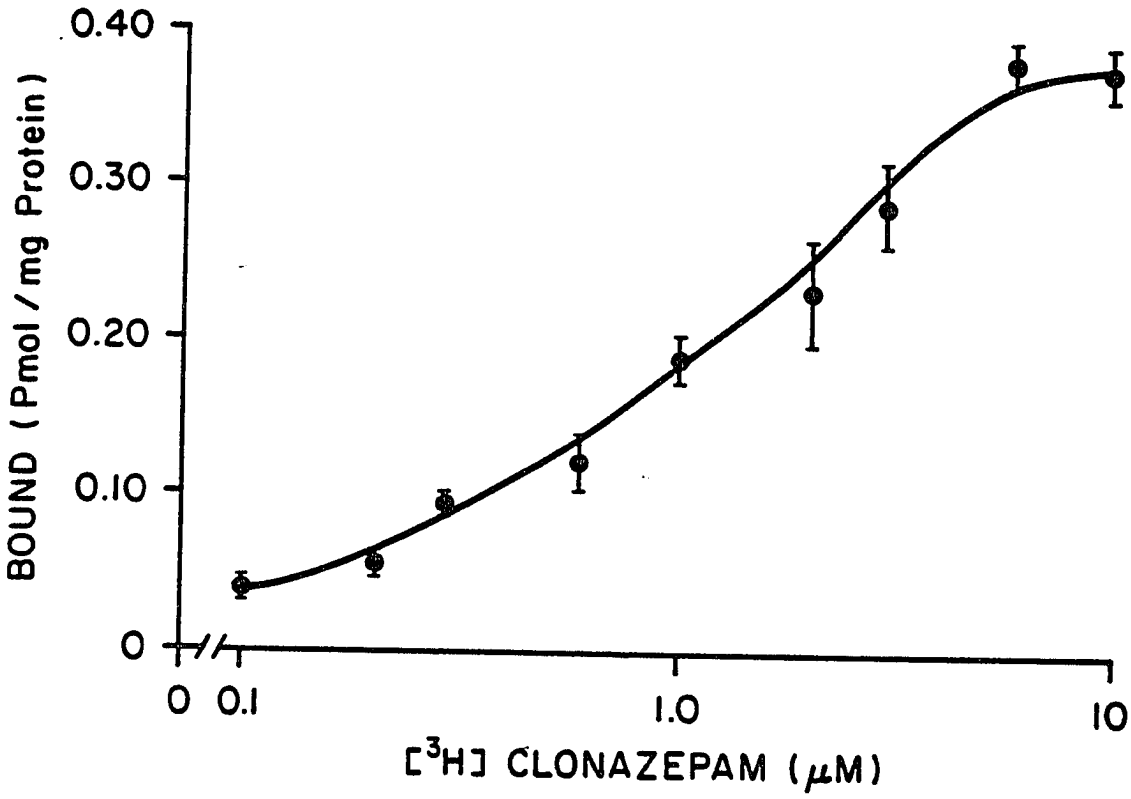


Fig. 12. Saturation curve of specific [^{3}H]-CNZ photolabeling. Samples contained the cytosolic fraction, the indicated [^{3}H]-CNZ concentration, and ethanol (total photolabeling) or 500 uM unlabeled CNZ (nonspecific photolabeling). The data represent the means (±sem) for six separate determinations.

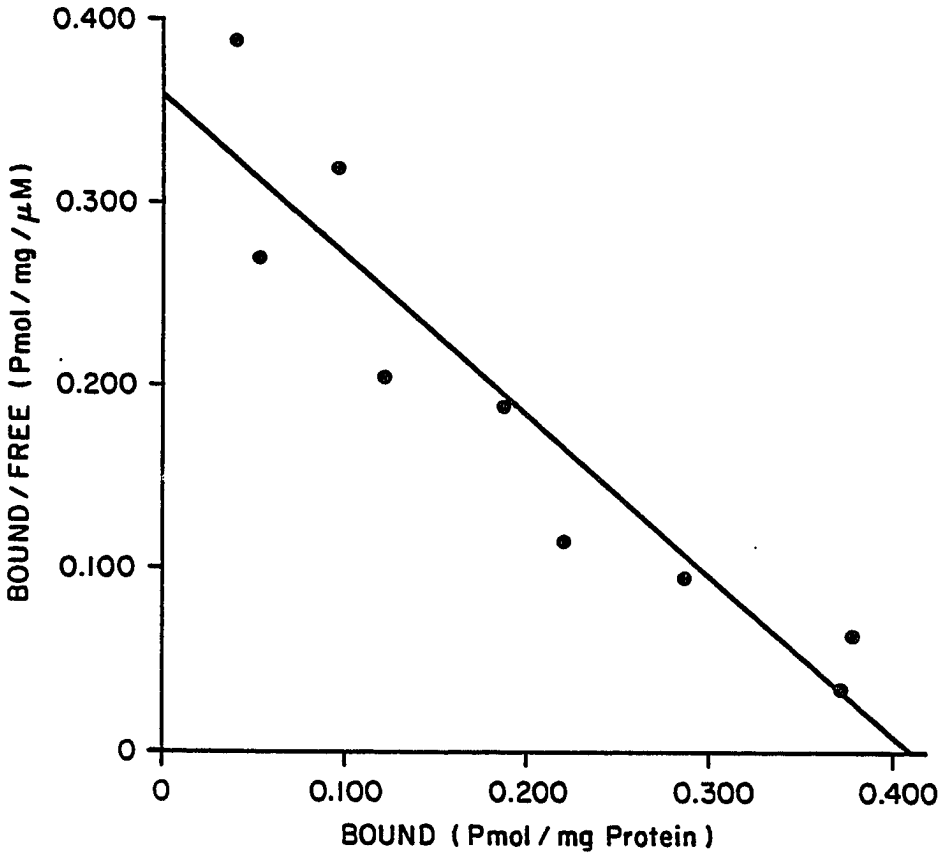


Fig. 13. Scatchard transformation of the saturation data. Statistical analysis of the plot yielded a K_D of 1.14 uM, a B_{max} of 0.41 pmol/mg cytosolic protein, and r=0.942.

This plot was analyzed to obtain an apparent dissociation constant (K_D) of 1.14 uM and a maximal binding capacity (B_{max}) of 0.41 pmol/mg cytosolic protein. Photoaffinity labeling was performed with a wide range of pH values. These studies revealed that specific [^{3}H]-CNZ photolabeling was optimal near pH 7.4 (Fig. 14). Reactions performed at pH 4.0 resulted in photolabeling of a protein with a molecular weight of approximately 59,000 daltons (Figs. 15, 16). Photolabeling of this 59,000 dalton protein at pH 4.0 was displaceable and exhibited an IC_{50} for CNZ of 1.36 uM (Fig. 17), very similar to the IC_{50} of 1.47 uM determined for the 65,000 dalton protein at pH 7.4 (Fig. 10). Protease studies were conducted in which photolabeled cytosolic fractions were treated with trypsin, pepsin, or proteinase K. Treatment with any of these proteases abolished all specific photolabeling of the 65,000 dalton band (Table 1). This finding indicates that the photolabeled site is a protein. Specific photolabeling was also not detectable when cytosolic fractions were boiled prior to performing reactions (Table 1).

C. Subcellular Distribution. Specific photolabeling was determined in various brain subcellular fractions (Table 3). These studies demonstrated that specific photolabeling was enriched in the cytosolic (S_3) fraction. Significant levels of photolabeling were not detected in any of the membrane fractions. Photolabeling of the 65,000 dalton

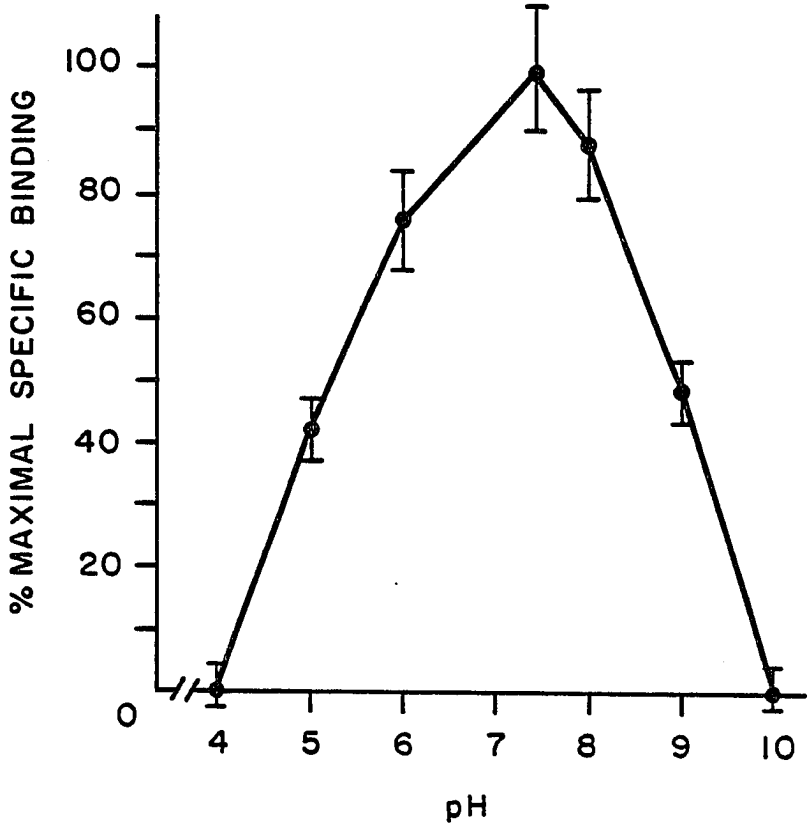


Fig. 14. Dependence of specific [^{3}H]-CNZ photolabeling on pH. Photolabeling was performed on cytosolic fractions that were prepared by diluting a concentrated cytosolic sample with 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$, 0.3 mM PMSF that was adjusted to the proper pH. Maximal photolabeling is 0.12 pmol/mg. The data are the means ($\pm$sem) for three separate determinations.

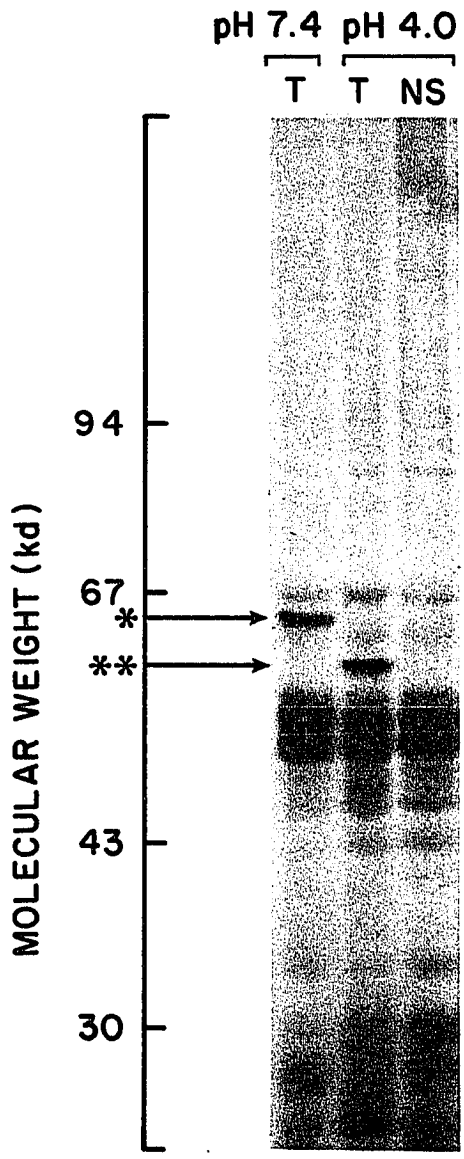


Fig. 15. Autoradiograph demonstrating shift in molecular weight of photolabeled protein with shift in pH from 7.4 to 4.0. Cytosolic fractions were adjusted to the indicated pH values prior to photolabeling in the presence of 600 nM [^{3}H]-CNZ and ethanol (T, total photolabeling) or 50 uM

unlabeled CNZ (NS, nonspecific photolabeling). The molecular weight shift was from approximately 65,000 daltons at pH 7.4 (*) to approximately 59,000 daltons at pH 4.0 (**).

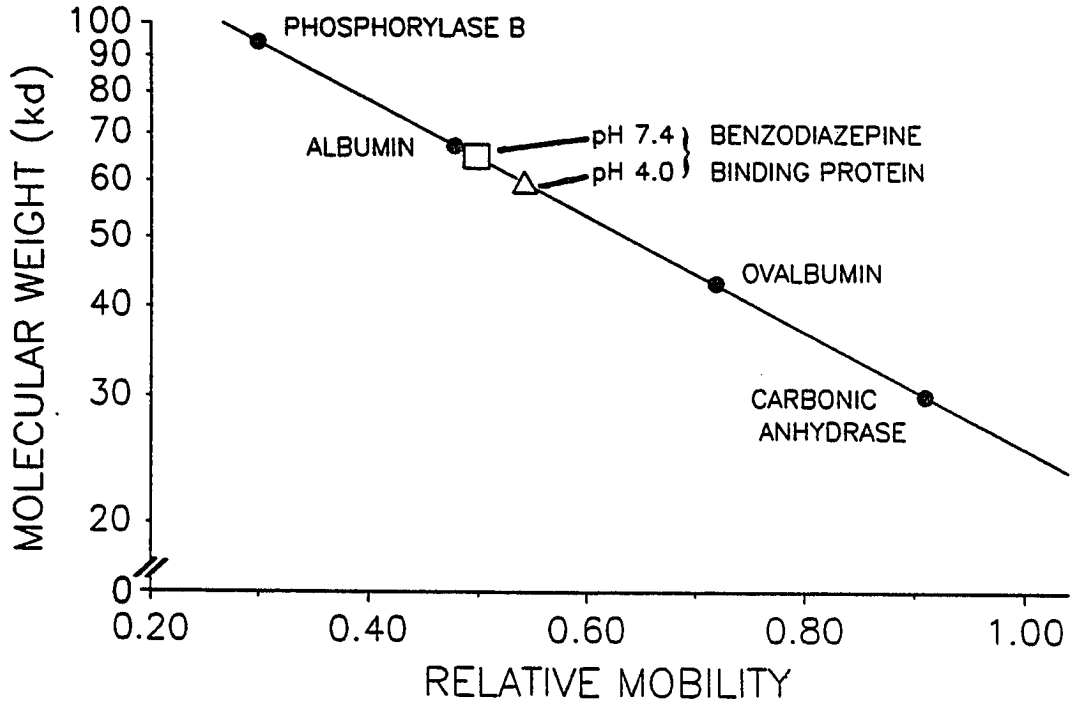


Fig. 16. Molecular weight of photolabeled protein at pH 7.4 and pH 4.0 as determined by relative mobility on SDS-PAGE. The molecular weights determined by linear regression analysis were 65,057 daltons for pH 7.4 and 59,252 daltons for pH 4.0. The value of r for the regression was 0.997.

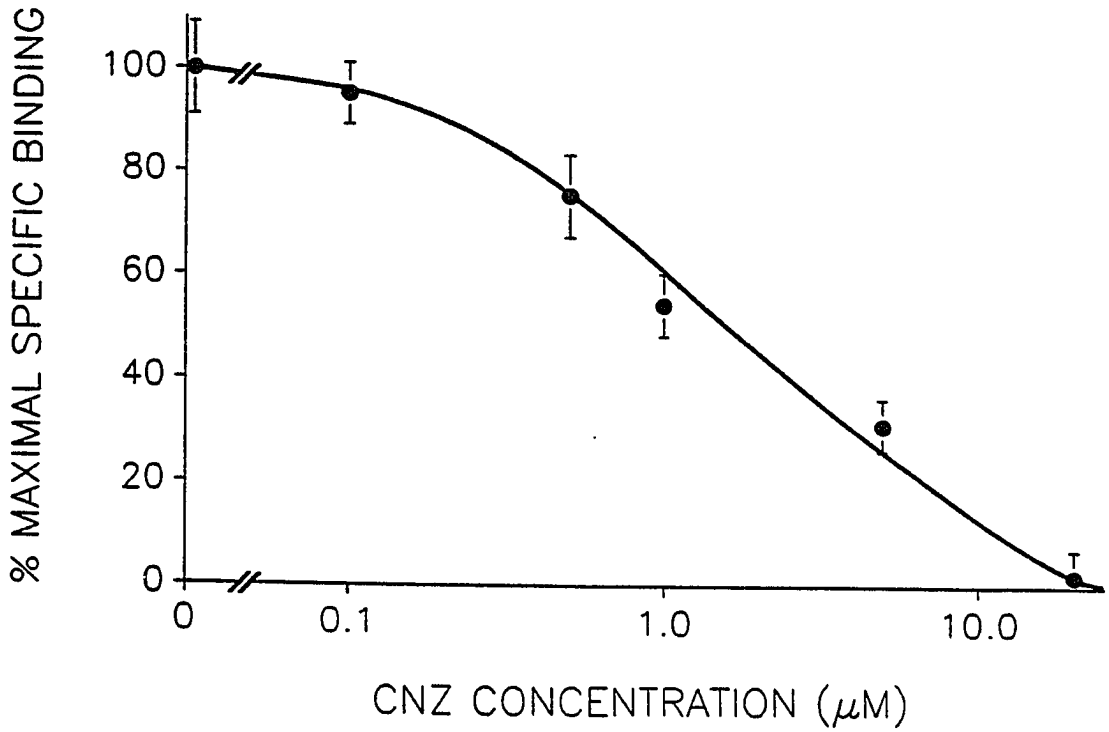


Fig. 17. Displacement by CNZ of [^{3}H]-CNZ photolabeling at pH 4.0. The IC_{50} value is 1.36±0.21 uM and the Hill coefficient is 1.14±0.12. Samples were incubated with 600 nM [^{3}H]-CNZ in the presence of the indicated concentration of unlabeled CNZ. Values represent the means (±sem) for three separate determinations. Maximal specific photolabeling (100%) represents 0.08 pmol/mg protein.

Table 3. Subcellular distribution of specific [^{3}H]-CNZ photolabeling.

Fraction	Relative Spec. Labeling
Homogenate	0.16±0.02
S_1	0.19±0.02
P_1 (crude nuclear)	0
S_2	0.72±0.07
P_2 (crude synaptosomal)	0
S_3 (cytosolic)	1.00±0.10
P_3 (crude microsomal)	0

The values represent the means±sem for four separate determinations. The absolute value of 0.11 pmol/mg protein for specific photolabeling in the cytosolic fraction was arbitrarily set at 1.00.

protein was not obtained in the lysed P_2 fraction or crude membrane fraction (100,000xg pellet) with many different buffers (pH 7.4 for all buffers): 100 mM Pipes; 20 mM Pipes; 100 mM Pipes, 2 mM EGTA; 100 mM Pipes, 1 mM $MgCl_2$; 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$; 100 mM Pipes, 2 mM $CaCl_2$; 100 mM Pipes, 2 mM EGTA, 150 mM NaCl; 100 mM Pipes, 2 mM $CaCl_2$, 150 mM NaCl. Significant photolabeling of the 65,000 dalton protein was not detectable in the lysed P_2 fraction (in 100 mM Pipes, 2 mM EGTA, 1 mM $MgCl_2$ (pH 7.4)) when photolabeling was conducted with a wide range of protein concentrations (0.1-3.0 mg/ml), illumination times (10-60 min), and [^{3}H]-CNZ concentrations (0.1-3.0 uM).

D. Brain Regional and Tissue Distribution. Photolabeling experiments were performed on different brain regions and tissues. Studies with whole brain demonstrated that specific [^{3}H]-CNZ photolabeling was stable for up to 30 min after decapitation (Fig. 18). For brain regional and tissue distribution studies, all tissues were homogenized within 10 min of decapitation. Significant photolabeling of the 65,000 dalton protein was observed in the cytosolic fraction of all brain regions that were examined (Table 4). Highest levels were obtained in the pons-medulla, cerebellum, and midbrain-thalamus. Lowest levels were present in the spinal cord. No specific photolabeling of the protein was detected in the crude membrane fraction (100,000xg pellet) from any of these regions.

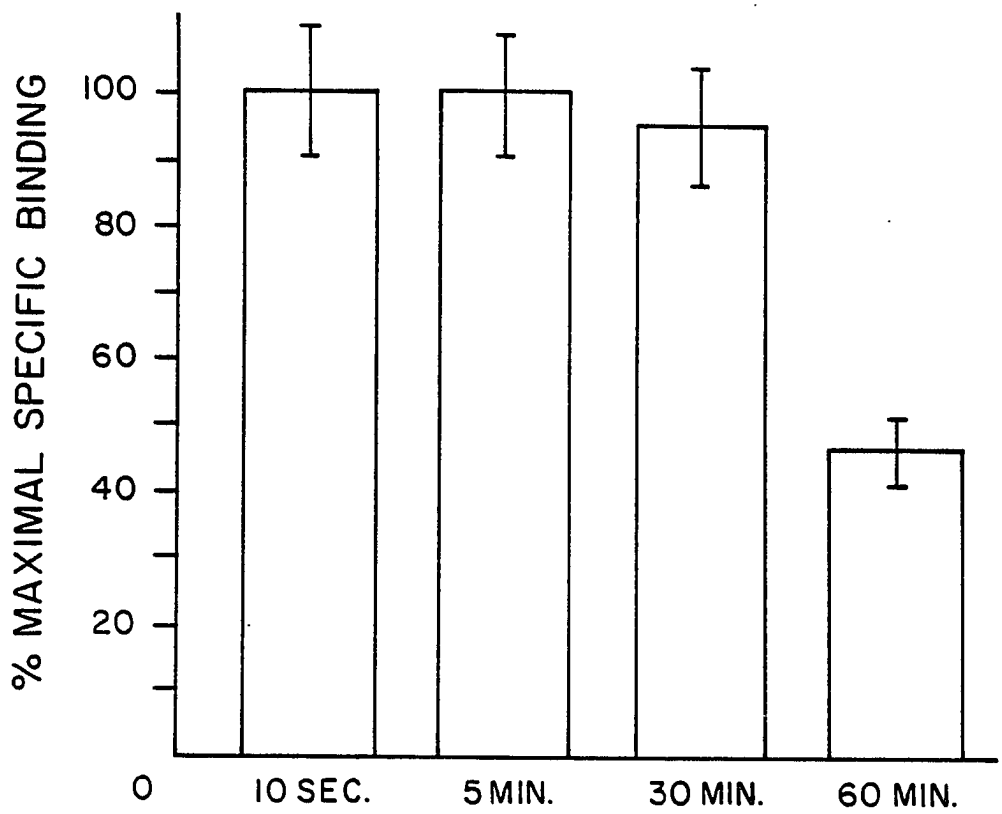


Fig. 18. Effect of time between decapitation and homogenization on specific [^{3}H]-CNZ photolabeling. Animals were decapitated and whole brains were dissected. The indicated time was allowed to elapse before brains were homogenized. Values represent the means (±sem) for three separate determinations. Maximal specific photolabeling (100%) represents 0.11 pmol/mg protein.

Table 4. Brain regional distribution of specific [^{3}H]-CNZ photolabeling.

Region	Relative Spec. Labeling
Pons-medulla	1.00±0.07
Cerebellum	0.93±0.02
Midbrain-thalamus	0.84±0.09
Cortex	0.51±0.06
Striatum	0.43±0.09
Hippocampus	0.42±0.06
Spinal cord	0.23±0.04

The values represent the means±sem for three separate determinations. The absolute value of specific photolabeling in the pons-medulla was 0.14 pmol/mg protein.

Photolabeling of the 65,000 dalton protein was detected in several non-neuronal tissues (Table 5). The photolabeling was highest in liver, but significant levels were also detected in spleen and kidney. No significant photolabeling was detected in serum. Photolabeling of the 65,000 dalton protein was not apparent in the crude membrane fraction of any of these tissues.

E. Selectivity Studies. Binding reactions were performed in the presence of many different non-benzodiazepine compounds to determine the selectivity of the photolabeled site for benzodiazepines (Table 6). To maximize the chance of detecting inhibition of binding by a non-photoactivated, reversibly-bound compound, illumination time was reduced to 15 min and compounds were tested at a concentration of 100 uM. Under these conditions, diazepam, a non-photoactivated, non-NO_2-containing benzodiazepine, exhibits significant inhibition of [^{3}H]-CNZ photolabeling (Fig. 19). However, as expected, the ability of the reversibly-bound diazepam to inhibit binding of the irreversibly-bound [^{3}H]-CNZ was decreased as the illumination time was increased (Fig. 19). For the selectivity studies, photolabeling reactions were performed in the presence of more than 70 compounds, including agonists and antagonists for neurotransmitter receptors, steroids, antidepressants, antipsychotics, autacoids, porphyrins, purines, nucleotides, dinucleotides, and several other classes of

Table 5. Tissue distribution of specific [^{3}H]-CNZ photolabeling.

Tissue	Relative Spec. Labeling
Brain	1.00±0.13
Liver	1.65±0.21
Spleen	0.51±0.05
Kidney	0.37±0.13
Heart	0
Lung	0
Skeletal muscle	0
Blood	0

The values represent the means±sem for three separate determinations. The absolute value of specific photolabeling in brain was 0.09 pmol/mg cytosolic protein.

Table 6. Compounds that do not significantly inhibit [^{3}H]-CNZ photoaffinity labeling.

Acetylcholine	Harmane
Actinomycin D	Hemin
Aldosterone	Histamine
Alpha-bungarotoxin	Hypoxanthine
Androstenedione	Imipramine
ATP	Inosine
Atropine	Isoproterenol
8-azido adenosine	Melatonin
8-azido ADP	Mesoporphyrin IX
8-azido AMP	Mesoporphyrin IX, ester
8-azido ATP	Muscimol
8-azido cyclic AMP	NADH
Beta-estradiol	NADPH
Beta-mercaptoethanol	Naloxone
Carbamazepine	N-ethyl-maleimide
Chloramphenicol	Nitrendipine
2-chloroadenosine	Nitrobenzylthioinosine
Chlorpromazine	Norepinephrine
Cholecalciferol	Norharmane
Clonidine	19-nortestosterone
Corticosterone	Oxotremorine
Cyclic AMP	Phenobarbital
Cyclic GMP	Phenylalanine
Deoxycorticosterone	Phenytoin
Deuteroporphyrin IX, di-HCl	Progesterone
Dexamethasone	Protoporphyrin IX
Dienestrol	Protoporphyrin IX, ester
Diethylstilbestrol	Quinuclidinyl benzilate
Dipyridamole	Serotonin
Dithiothreitol	Strychnine
Dopamine	Testosterone
Epinephrine	D-thyroxine
Ergocalciferol	L-thyroxine
Estrone	Triamcinolone acetonide
Fluorescein isothiocyanate	Trimethadione
Fluorosulfonylbenzoyladenosine	L-tryptophan
GABA	Tyrosine
Glycine	Verapamil
Guanosine	
Harmaline	

For each compound, the maximal concentration tested was 100 uM.

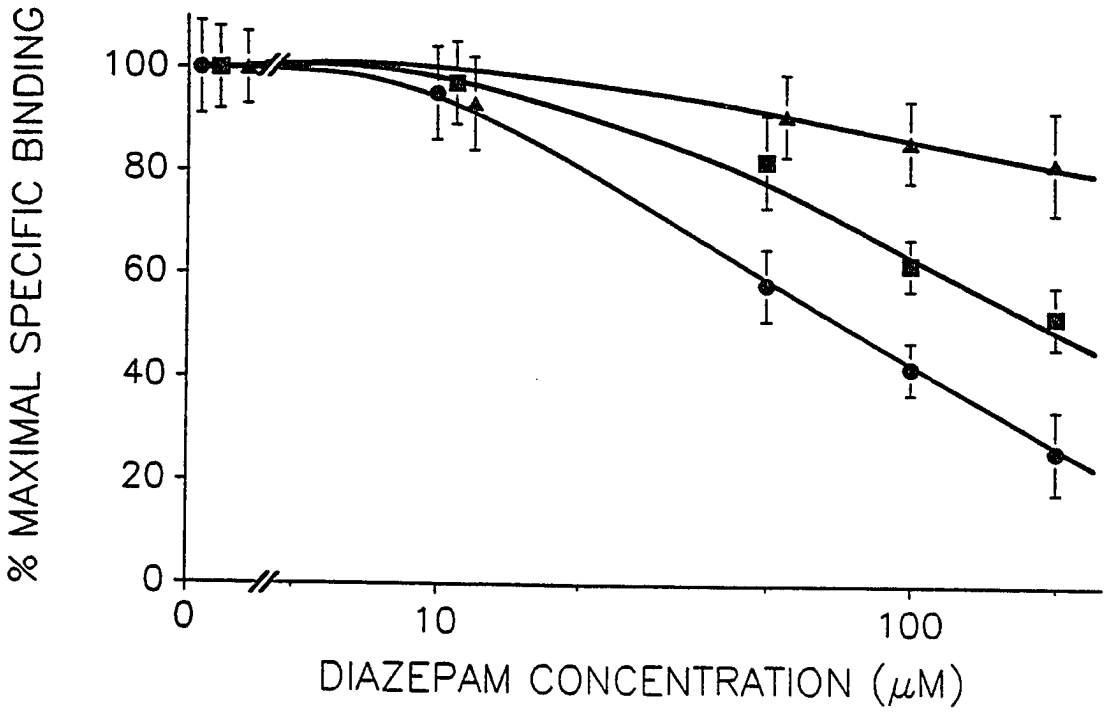


Fig. 19. Displacement of specific [^{3}H]-CNZ photolabeling by diazepam at different illumination times. Cytosolic fractions were incubated with 600 nM [^{3}H]-CNZ and the indicated concentrations of diazepam for 30 min and then irradiated for 15 min (solid circles), 30 min (solid squares), or 60 min (solid triangles).

compounds. Specific photolabeling was altered less than 10% by these agents (Table 6).

F. Purification of the Photolabeled Protein. For all column chromatography purification procedures, eluates were screened by separating proteins on 25 cm long SDS-PAGE and noting proteins that comigrated with the 65,000 dalton photolabeled protein. Employing this procedure, chromatography on Affi-Gel Blue was found to be an extremely effective initial purification procedure. Cytosol was loaded on the column with no NaCl present, and the column was washed extensively with the loading buffer. The eluate did not contain any proteins that appeared to comigrate with the photolabeled protein (Fig. 20). Sequential elutions were then performed with 20 mM NaCl, 50 mM NaCl, and 500 mM NaCl. The majority of a protein that comigrated with the photolabeled protein was found to elute at 50 mM NaCl from the Affi-Gel Blue column (Fig. 20). These fractions, referred to as the "Blue Fraction," contained a 65,000 dalton protein as a major constituent, along with 4-6 other proteins (Fig. 21). Subsequent photolabeling experiments demonstrated that these fractions contained a 65,000 dalton protein that exhibited specific [^{3}H]-FNZ photolabeling (Fig. 21). Displacement studies were conducted on the Blue Fraction with five different NO_2-containing benzodiazepines. The absolute and relative potencies exhibited by the Blue Fraction for these

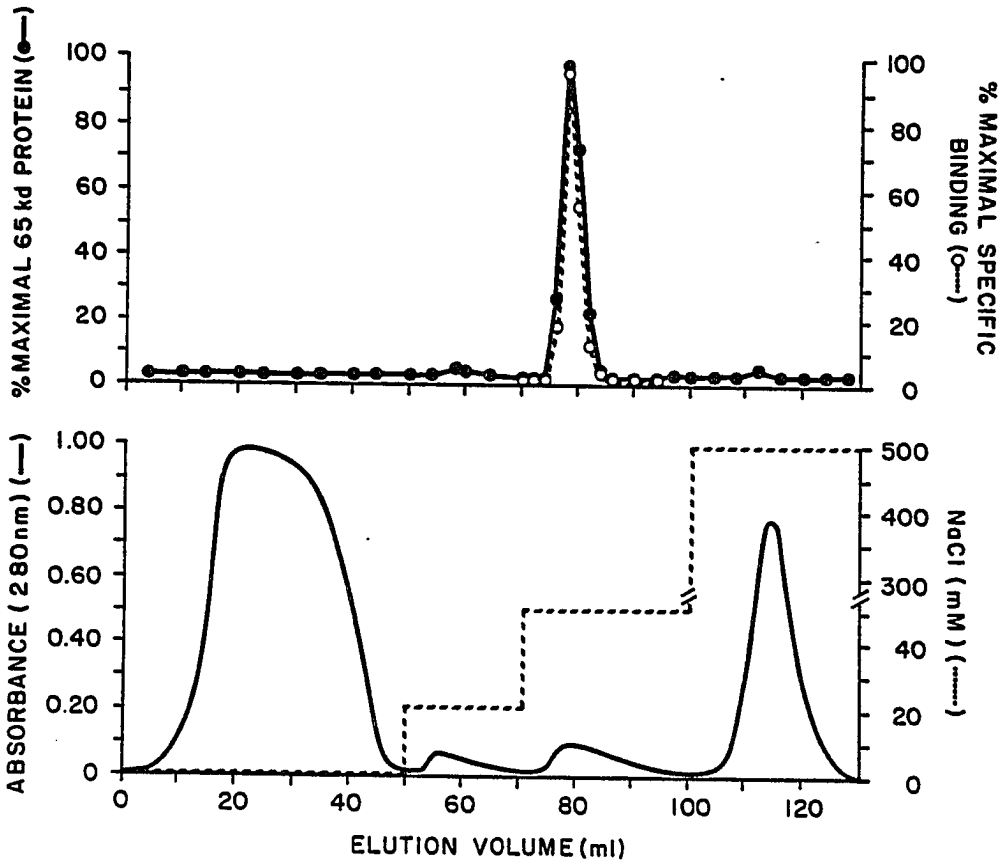


Fig. 20. Elution profile for Affi-Gel Blue column chromatography of brain cytosol. Profile depicts 65,000 dalton protein staining (upper panel, solid line), specific photolabeling of the 65,000 dalton protein (upper panel, dashed line), and total protein as detected by absorbance at 280 nm (lower panel, solid line). Cytosol was applied to the column, and sequential elutions were performed at 20 mM NaCl, 50 mM NaCl, and 500 mM NaCl (lower panel, dashed line). Protein staining at 65,000 daltons was determined by densitometric scanning of silver-stained protein bands

that appeared to comigrate with the 65,000 dalton photolabeled protein on 25 cm long SDS-PAGE. Specific photolabeling of the 65,000 dalton protein was determined by photolabeling column fractions with 600 nM [^{3}H]-FNZ in the presence (total photolabeling) and absence (nonspecific photolabeling) of 50 uM CNZ. Maximal specific photolabeling represents 197 pmol/mg protein. Total protein was monitored continuously at 280 nm. Typically, fractions with a total pooled volume of approximately 10 ml were combined to form the "Blue Fraction." In this experiment, the fractions from an elution volume of 75 ml to 85 ml were pooled. The depicted experiment is representative of 15 different column elutions.

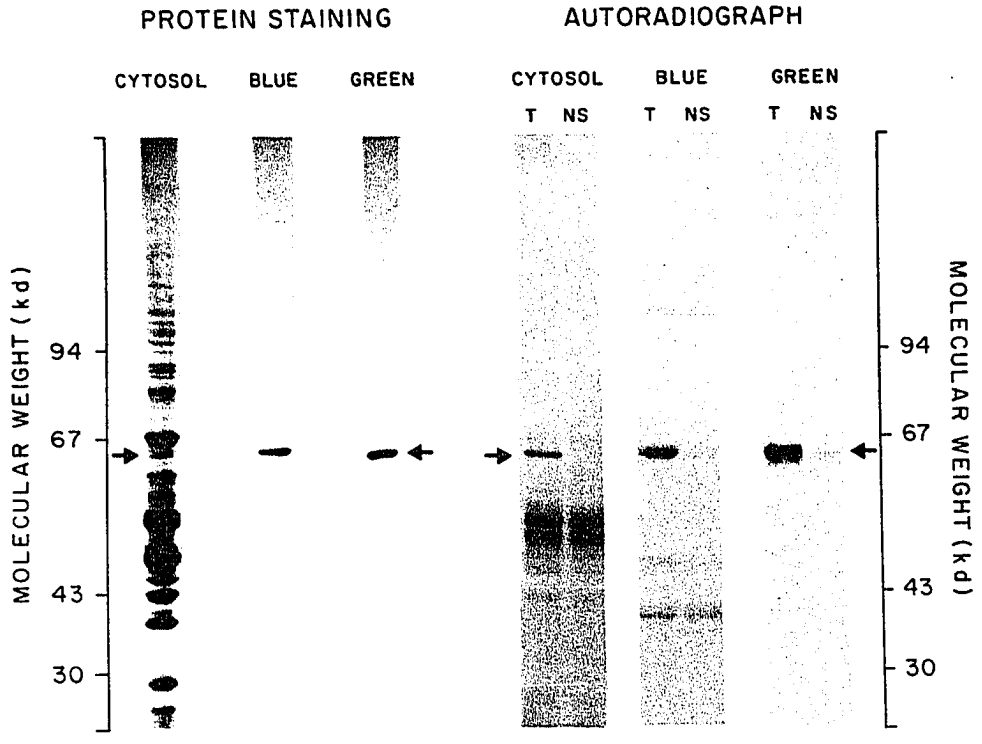


Fig. 21. Protein pattern and ^{3}H autoradiography for cytosol and fractions from the purification procedure. Samples included cytosol, the Blue Fraction (Blue), and the Green Fraction (Green). For protein staining, proteins were separated by SDS-PAGE and silver stained. For autoradiography, samples were incubated for 30 min and photolabeled for 60 min with 600 nM [^{3}H]-FNZ in the presence (T, total photolabeling) or absence (NS, nonspecific photolabeling) of 50 uM CNZ to detect photolabeling of the 65,000 dalton protein (arrow).

benzodiazepines were similar to the potencies obtained with whole cytosol (Table 7, Fig. 22). The displacement studies with B9(+) and B9(-), benzodiazepine enantiomers with markedly different pharmacologic potencies, demonstrated that photolabeling of the protein in the Blue Fraction, like that in cytosol, was stereoselective (Table 7). The Affi-Gel Blue column resulted in an 855-fold enrichment of the photolabeled protein (Table 8).

The Blue Fraction was further purified by Agarose Green column chromatography (Fig. 23). Following loading of the column with the Blue Fraction and washing with the loading buffer, a NaCl gradient from 50 mM to 200 mM was applied to the column. This gradient generally produced two peaks of absorbance at 280 nm, one at approximately 125 mM NaCl and the other at approximately 200 mM NaCl. SDS-PAGE of the column fractions revealed that elution of a 65,000 dalton protein corresponded to the peak at 125 mM NaCl (Fig. 23). Furthermore, this 65,000 dalton protein was the only protein detectable in these fractions by protein staining (Fig. 21). Fractions from the 125 mM NaCl peak were found to exhibit specific [^{3}H]-FNZ photolabeling (Fig. 21). Pooled fractions from the peak, termed the "Green Fraction," exhibited a potency profile for five different benzodiazepines that was similar to that determined for cytosol, including stereoselectivity for B9(+) and B9(-) (Table 7, Fig. 24). These findings

Table 7. IC_{50} values of five different benzodiazepines for cytosol, Blue Fraction, and Green Fraction.

Compound	Cytosol	Blue Fraction	Green Fraction
Flunitrazepam	0.60±0.12	0.49±0.08	1.19±0.13
B9(+)	1.17±0.13	0.58±0.07	1.34±0.16
Clonazepam	1.47±0.13	2.02±0.31	2.07±0.31
Ro5-6219	6.73±1.60	25.11±2.13	12.22±1.05
B9(-)	13.58±1.23	39.79±1.51	16.60±1.12

Displacement experiments were performed with 600 nM [^{3}H]-CNZ. IC_{50} values were determined from Hill plots.

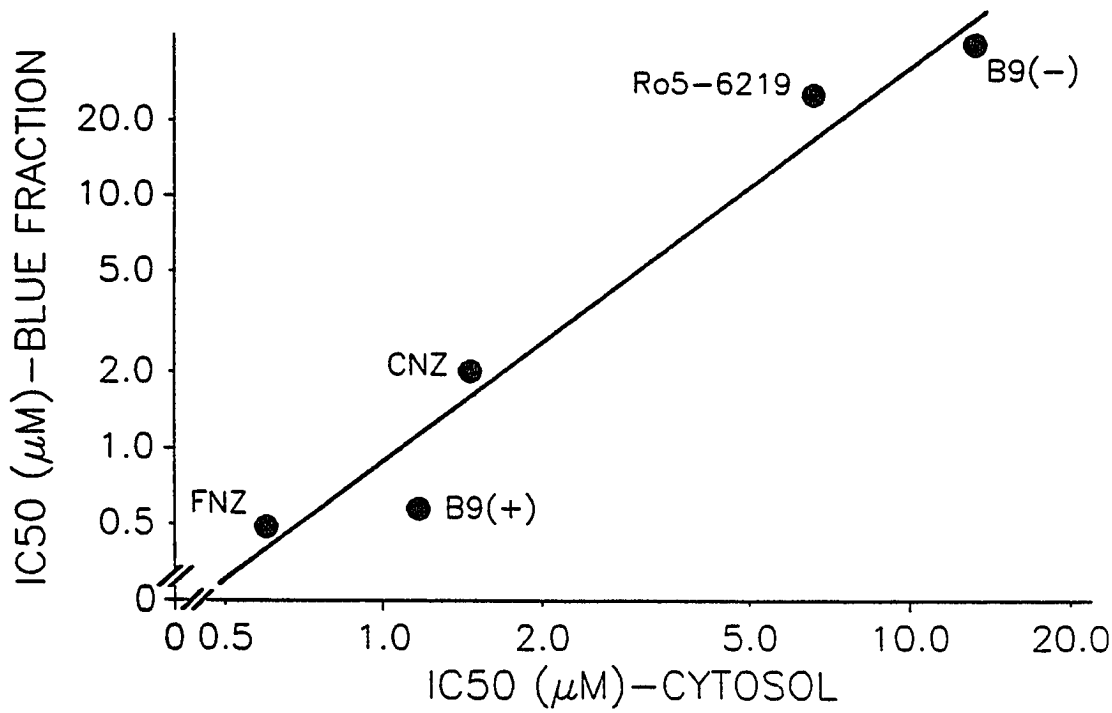


Fig. 22. Correlation of IC_{50} values determined for cytosol and Blue Fraction. IC_{50} values were calculated from displacement studies with 600 nM [^{3}H]-CNZ and represent the means of at least three separate determinations. The value of r for the regression was 0.987.

Table 8. Recovery and enrichment values for purification of the 65,000 dalton protein.

Fraction	Total Vol. (ml)	Prot. Conc. (mg/ml)	Spec. Labeling (pmol/mg)	Labeling (total pmol)	Purif. (-fold)	Yield (%)
Cytosol	7.5	19.1	0.113	16.2	1	100
Blue	10.4	0.009	96.6	9.08	855	56
Green	5.2	0.004	273	5.72	2416	35

Specific photolabeling was determined by photolabeling cytosol, the Blue Fraction ("Blue"), or the Green Fraction ("Green") with 600 nM [^{3}H]-FNZ in the presence and absence of 50 uM CNZ. The values shown are representative of five separate determinations.

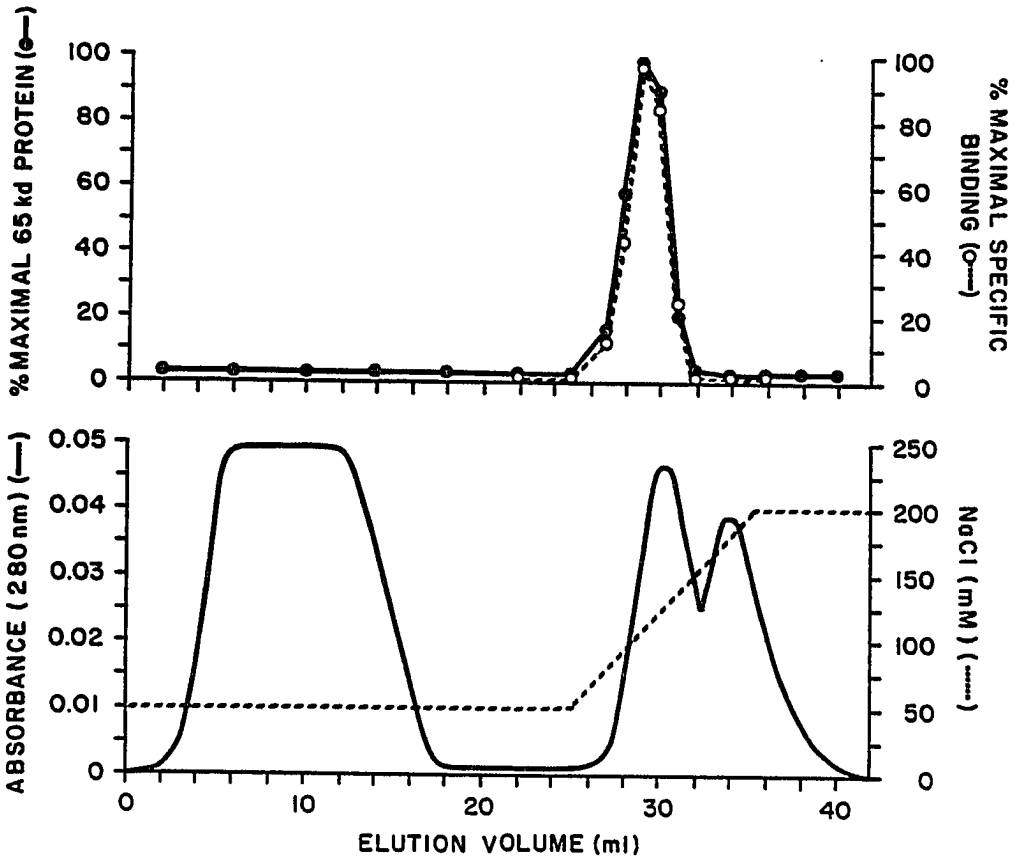


Fig. 23. Elution profile for Green 19-Agarose column chromatography of Blue Fraction. Profile is shown for protein staining at 65,000 daltons (upper panel, solid line), specific photolabeling of the 65,000 dalton protein (upper panel, dashed line), and absorbance at 280 nm (lower panel, solid line). Blue Fraction was applied to the column and eluted with a continuous NaCl gradient from 50 mM to 200 mM (lower panel, dashed line). Protein staining at 65,000 daltons was quantitated by densitometric scanning of protein bands that appeared to comigrate with the 65,000

dalton photolabeled protein on 25 cm long SDS-PAGE. Specific photolabeling was determined by [^{3}H]-FNZ photolabeling of column fractions. Maximal specific photolabeling represents 508 pmol/mg protein. Routinely, the "Green Fraction" constituted pooled fractions with a total volume of approximately 5 ml. In the depicted experiment, fractions from an elution volume of 27 ml to 32 ml were pooled. This profile is representative of 15 different column elutions.

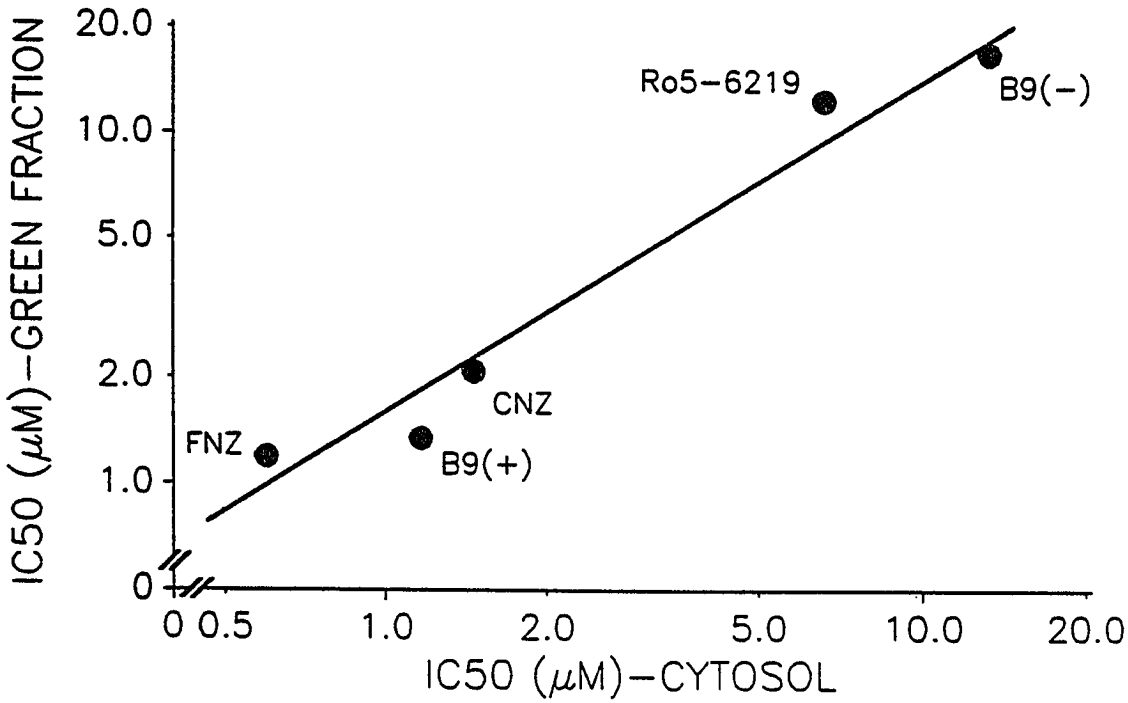


Fig. 24. Correlation of IC_{50} values determined for cytosol and Green Fraction. IC_{50} values were determined from displacement experiments with 600 nM [^{3}H]-CNZ and represent the means of at least three separate determinations. The value of r for the regression analysis was 0.972.

demonstrate that the single protein detected in the Green Fraction is the 65,000 dalton photolabeled protein initially identified in cytosol. Relative to cytosol, the Green Fraction represented a more than 2000-fold purification of the photolabeled protein (Table 8). The overall yield was 35% (Table 8). Employing this purification scheme, it was generally possible to obtain the Green Fraction within 8 hr of decapitation.

G. Biochemical Characterization of The Purified Protein. The native molecular weight of the binding protein was examined by employing gel filtration with Sephadex G-100. Five ug of protein from the Green Fraction was applied to the column after calibration with known protein standards. The 65,000 dalton was found to elute at a molecular weight of 62,500 daltons (Fig. 25), indicating that the purified protein exists as a monomer.

The amino acid composition of the purified protein was determined by analysis of approximately 5 ug of protein from a dialyzed preparation of the Green Fraction (Table 9). These studies revealed an unusually large relative abundance of glycine of 17.2%. The percentage of hydrophobic residues was calculated to be 42.6%. N-terminal amino acid sequencing was performed on a dialyzed preparation of the Green Fraction. The sequence that was obtained was compared with other known amino acid sequences. The sequence was not identical to any of the

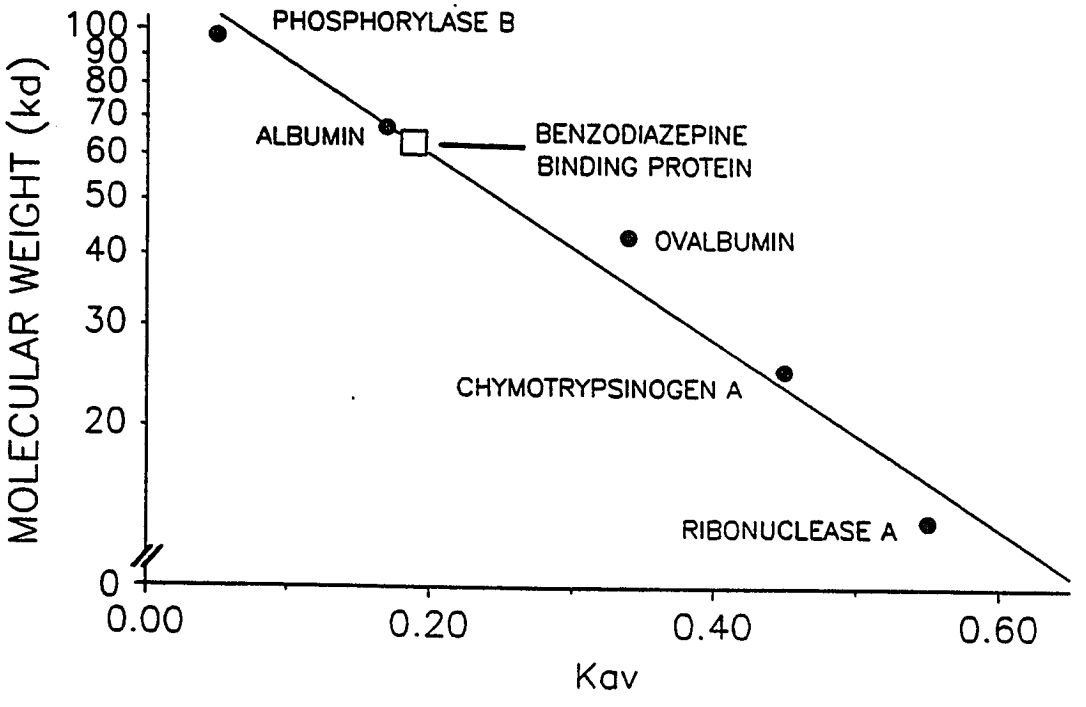


Fig. 25. Sephadex G-100 column chromatography of the purified protein. Approximately 5 ug of purified 65,000 dalton protein was applied to the column. The protein chromatographed with an apparent molecular weight of 62,517 daltons as determined by linear regression analysis. The value of r for the regression was 0.985.

Table 9. Amino acid composition of the purified protein.

Amino Acid	Mol %
Asx	10.1±0.3
Thr	3.6±0.2
Ser	2.8±0.1
Glx	9.4±0.3
Gly	17.2±1.7
Ala	11.6±0.3
Val	8.1±0.2
Met	1.8±0.1
Ile	4.7±0.1
Leu	9.4±0.1
Tyr	2.4±0.1
Phe	4.6±0.1
His	4.1±0.1
Lys	6.4±0.2
Arg	3.8±0.1

Amino acid composition studies were performed with approximately 5 ug of purified protein. Proline, cysteine, and tryptophan were not determined. The values represent the means±sem for three separate determinations.

sequences collected by the NBRF-PIR protein sequence data bank. However, homology of 66.7% was found between the N-terminus of the binding protein and the N-terminus of the beta chain of hemoglobin, including a five-residue sequence of exact homology in a region corresponding to the first alpha-helix of beta-hemoglobin (Table 10). Sequence homologies of 50% or greater were also obtained for the N-terminus of the beta chain of hemoglobin from mole rat (61.9%), mouse (57.1%), musk rat (57.1%), rock hyrax (57.1%), golden hamster (57.1%), llama (52.4%), pig (52.4%), and yellow-cheeked mole (52.4%). Homologies of 50% or greater were also obtained for 50S ribosomal protein L3 from E. coli (53.3%), E_2 protein from bovine papilloma-virus (50.0%), and DNA-directed RNA polymerase (50.0%). Unlike the homology with hemoglobin, these homologies did not correspond to the N-termini of the proteins. Homologies of 50% or greater were not obtained for any other protein in the data base, including rat serum albumin and pig 68,000 dalton neurofilament protein. Only a single peak was present on HPLC analysis during the sequencing, indicating that at least 95% of the Green Fraction was the 65,000 dalton protein. Isoelectric focusing of the protein was not possible due to inadequate resolution and inadequate entry of sample into the gel matrix.

I. Reversible Binding. Reversible binding to the purified protein was investigated by employing the Green Fraction in

Table 10. Amino acid sequence of the N-terminus of the 65,000 dalton protein and the N-terminus of the beta chain of rat hemoglobin.

65 kd protein:	V	L	L	A	D	A	K	K	N	A	V	N	G	L	G	K	K	V	N	H	D
Beta-hemoglobin:	V	H	L	T	D	A	E	K	A	A	V	N	G	L	W	G	K	V	N	P	D

Sequencing was performed in duplicate with approximately 1 nmol (65 ug) of purified protein. For each residue, only a single peak was detected on HPLC. The resulting sequence was tested against the NBRF-PIR protein sequence data base. A sequence homology of 66.7% was obtained with the N-terminus of the beta chain of rat hemoglobin.

several different assay systems. Due to the relatively high concentrations of radioligand necessary to study the site, radioactivity in control samples (no protein) was excessively high for the PEI-treated filter method (at 600 nM [^{3}H]-CNZ, approximately 300,000 DPM/filter), equilibrium dialysis (at 600 nM [^{3}H]-FNZ, approximately 300,000 DPM/5ul reaction mixture), and gel filtration (at 600 nM [^{3}H]-CNZ, approximately 150,000 DPM/100 ul eluate). The PEG-gamma-globulin precipitation method yielded lower levels of radioactivity (approximately 8,000 DPM/filter). However, significant, reproducible binding was not detected at 4° C or 20° C by this method, presumably due to dissociation during the washing process.

IV. DISCUSSION

A. A Protein That is Photolabeled by NO_2-Benzodiazepines. This study describes the characterization and purification of a protein in rat brain that is photolabeled by NO_2-containing benzodiazepines. The protein has a molecular weight of 65,000 daltons and exhibits selective, saturable, stereoselective photolabeling by NO_2-containing benzodiazepines in the high nanomolar-low micromolar range. The protein appears to be distinct from any previously-described benzodiazepine-binding proteins. Based on relative mobility on SDS-PAGE, it is clearly distinct from serum albumin (67,000 daltons), which has been shown to bind benzodiazepines in the high micromolar range (Muller and Wollert, 1976; Brodersen et al., 1977; Maruyama, 1985). Although the 65,000 dalton protein and serum albumin possess similar molecular weights, they are separated by 4-5 mm on the high resolution SDS-polyacrylamide gels employed in this study. The protein identified in this investigation is distinguishable from the central-type receptor and the peripheral site on the basis of several criteria. First, the relative mobilities on SDS-PAGE are markedly different for the newly-characterized binding protein at 65,000 daltons, the central receptor at approximately 50,000 daltons (Mohler et al., 1980; Thomas

and Tallman, 1981), and the peripheral site at approximately 20,000 daltons (Burgevin et al., 1986). Second, the potencies of various NO_2-containing benzodiazepines for the 65,000 dalton protein are in the high nanomolar-low micromolar concentration range, while those for the central (Mohler and Okada, 1977, Squires and Braestrup, 1977) and peripheral (Marangos et al., 1982; Schoemaker et al., 1983) sites are generally in the low nanomolar range. Third, the cytosolic enrichment of the protein distinguishes it from the central receptor, which is enriched in the crude synaptosomal fraction (Mohler and Okada, 1977; Marangos et al., 1982), and the peripheral site, which is enriched in the mitochondrial fraction (Basile and Skolnick, 1986; Anholt et al., 1986). Fourth, the 65,000 dalton protein is present in brain, liver, spleen, and kidney, while the central receptor is only present in brain and the peripheral site is present in brain and many peripheral tissues, such as heart and lung (Anholt et al, 1985; DeSouza et al, 1985), that do not exhibit specific photolabeling of the 65,000 dalton protein.

The domains associated with the 65,000 dalton protein appear to be homogeneous sites that are selective for benzodiazepines. The Scatchard plot for the binding site was linear, and all of the Hill coefficients were close to unity. These findings indicate that it is a single

homogeneous population of non-cooperative sites. The displacement of [^{3}H]-CNZ photolabeling by nine different NO_2-containing benzodiazepines indicates that the 65,000 dalton protein is able to bind many different benzodiazepine structures, while the lack of an effect on photolabeling by more than 70 different non-benzodiazepine, centrally-active compounds demonstrates the selectivity of the site for benzodiazepines. Since most of the non-benzodiazepine compounds included in this study were not photoactivated, it is possible that some of these compounds produce an effect that is overcome by the photoactivated, irreversibly-bound benzodiazepines and thus is not detected. As a result, definitive selectivity studies are not possible until a method is developed for measuring reversible binding to the 65,000 dalton protein.

Several structure-affinity relationships are apparent from the displacement studies with the different benzodiazepines. Electronegative halogens in the 2'-C position increase potency, and bulky substituents in the 1-N and 2-C positions decrease potency. The 3-C position also is important for photolabeling as demonstrated by the difference in potencies for B9(+) and B9(-), compounds that differ only in the orientation of the $-CH_3$ group on the 3-C. This stereoselectivity for 3-C enantiomers indicates that the 3-C is directly involved in photolabeling the site or that different orientations of the 3-C substituent

impart different conformations on some other portion of the molecule that is integral for photolabeling.

The K_i values for the benzodiazepines tested were in the 0.40-12 uM concentration range, and the K_D for [^{3}H]-CNZ determined by Scatchard analysis was 1.1 uM. This range of affinities is less potent than those reported for the central (Mohler and Okada, 1977; Squires and Braestrup, 1977) and peripheral (Marangos et al., 1982; Schoemaker et al., 1983) sites. However, therapeutic plasma concentrations of benzodiazepines are 0.1-10 uM (Booker and Celesia, 1973; Bond et al, 1977; Lister et al., 1983a), and corresponding brain concentrations are 2-3 times larger (Lister et al., 1983a,b). Thus, therapeutic doses of benzodiazepines produce brain concentrations that would be adequate to bind significantly to the 65,000 dalton protein. In addition, all of the photolabeling studies in this investigation were performed at 4° C and it is possible that the protein exhibits a higher affinity at 37° C. This possibility was not examined because excessive heating of the reaction mixture during the irradiation time period made it extremely difficult to maintain a constant, controlled temperature at temperatures greater than 4° C.

While performing pH studies, it was noted that photolabeling at pH 4.0 resulted in photolabeling of a 59,000 dalton protein. Remarkably, this 59,000 dalton protein retained its ability to exhibit irreversible

binding by NO_2-containing benzodiazepines at pH 4.0 and exhibited an IC_{50} value for CNZ that was similar to that obtained for the 65,000 dalton protein at pH 7.4. These findings suggest that the 65,000 dalton protein is converted into the 59,000 dalton protein at pH 4.0. It is possible that this molecular weight shift is due to removal of post-translational modifications (such as glycosylated sites), removal of prosthetic groups, acid-induced activation of a protease that acts on the 65,000 dalton protein, or acid-induced "local denaturation" of the 65,000 dalton protein such that it becomes susceptible to proteolytic digestion.

The tissue distribution for the protein indicates that, in addition to its existence in brain, it is also present in other tissues, including liver, spleen, and kidney. The protein may or may not perform essential functions in these other organs. It is possible that the 65,000 dalton protein in peripheral tissues is not functionally significant or that the protein in the brain, due to its function or accessibility, is more sensitive to the effects of the benzodiazepines than is the protein in peripheral tissues.

Photolabeling of the 65,000 dalton protein was only detected in the cytosolic fraction. Significant photolabeling of a 65,000 dalton protein was not observed when crude membrane fractions (100,000xg pellet) and lysed

P_2 fractions were prepared in the presence and absence of Ca^{2+}, Mg^{2+}, and NaCl, or when these fractions were photolabeled under a wide range of protein concentrations, irradiation times, and [^{3}H]-CNZ concentrations. These findings suggest that the protein is either localized in the cytosol or associated so loosely with the membrane that it becomes dissociated from the membrane during the fractionation procedure.

Benzodiazepines are extremely hydrophobic molecules (Leo et al., 1971; Borea and Bonora, 1983), and, as a result, are able to diffuse through membranes readily. Thus it is likely that benzodiazepines are present in the cytoplasm of neurons. In fact, in vivo labeling studies with radiolabeled benzodiazepines have demonstrated labeling of the nuclear membrane (Bosmann et al., 1980). It is possible that benzodiazepines produce some of their effects by binding to cytosolic receptors. Alternatively, the 65,000 dalton protein may be a cytosolic protein that associates transiently with the membrane. Due to their high hydrophobicity, the greatest concentration of benzodiazepines is likely to be in the membrane, and, in a manner analogous to that of phorbol esters and protein kinase C (Wolf et al., 1985), the benzodiazepines may exert their effects on the protein by promoting or prolonging its association with the membrane.

B. Purification of the Protein. The 65,000 dalton protein was purified to apparent homogeneity from rat brain cytosol through a two-step column chromatography procedure employing Affi-Gel Blue and Agarose Green. This purification procedure resulted in a more than 2000-fold enrichment of the protein with a 35% yield. The final preparation of the protein, the Green Fraction, was shown to be essentially homogeneous both by protein staining on SDS-PAGE and by HPLC analysis of sequential amino acid degradations obtained during the N-terminal amino acid sequencing.

After each of the column chromatography steps, the protein was shown to possess a pharmacologic profile very similar to that exhibited by the photolabeled protein in whole cytosol. The purified preparation of the protein retained stereoselectivity, a property exhibited by the protein in cytosol, and also possessed absolute and relative potencies for five different benzodiazepines that were similar to those obtained in cytosol.

It is interesting to note that the 65,000 dalton protein bound to Affi-Gel Blue resin. This resin, as well as similar resins which also contain the dye Cibacron Blue F3GA, have been employed to purify proteins containing a "dinucleotide fold." This form of supersecondary structure is composed of a beta-sheet core of five or six parallel strands that are stabilized by alpha-helical intrastrand

loops above and below the beta sheet (Rossmann et al., 1974; Schulz and Schirmer, 1974; Thompson et al., 1975). The dinucleotide fold forms the ATP-binding site in phosphoglycerate kinase and the NAD-binding site in lactate, malate, and glyceraldehyde-phosphate dehydrogenase (Rossmann et al., 1974; Schulz and Schirmer, 1974; Thompson et al., 1975). Consequently, Affi-Gel Blue has been an extremely effective resin for the purification of many dehydrogenases and kinases (Rossman et al., 1974; Schulz and Schirmer, 1974; Thompson et al., 1975). The affinity of the 65,000 dalton protein for the resin is lower than is generally observed. The protein eluted with a NaCl concentration of 50 mM, while most dinucleotide fold-containing proteins require a NaCl concentration of at least 100 mM for effective elution (Thompson et al., 1975). Nevertheless, the possibility of a dinucleotide fold is notable since purines displace benzodiazepines from the central site with a low affinity (Tallman et al., 1980) and the binding site for the central receptor exhibits significant amino acid sequence homology with the adenine nucleotide-binding portion of several ATPases (Tallman, 1986). The benzodiazepine-binding protein identified in this investigation did not exhibit any significant displacement of specific [^{3}H]-CNZ photolabeling by several purines, nucleotides, and dinucleotides, but these displacement studies may not have been sensitive enough to

detect the effect of a reversibly-bound ligand. It is interesting to note that the dinucleotide fold of equine liver alcohol dehydrogenase contains approximately 17% glycine residues (Rossman et al., 1974), a value similar to that determined for the binding protein. However, the dinucleotide folds of several other proteins contain only 7-11% glycine residues (Rossman et al., 1974).

C. Characterization of the Purified Protein. Gel filtration studies performed on the purified protein demonstrated that it exhibits a native molecular weight of 62,500 daltons. These findings indicate that the purified protein exists as a monomer. In cytosol, it is possible that the binding protein exists with other proteins or with itself as a loosely-associated complex that then becomes dissociated during the fractionation or purification procedures. The binding protein may also transiently associate with other cytosolic proteins.

Amino acid composition studies were performed on the purified protein. These studies demonstrated that the protein possesses a characteristically high glycine content of 17.2%. Rat collagen contains 33.8% glycine residues (Bornstein and Traub, 1979), one of the highest relative abundance values for glycine for any known mammalian protein. The large number of glycine residues in the binding protein may be required to satisfy specific steric requirements and may allow for high-density structures,

such as tightly-packed helices or beta-pleated sheets. The protein was found to contain 42.6% hydrophobic residues, greater than the average of approximately 30% for a soluble protein and less than the average of approximately 50% for a membrane protein (Rosenberg and Guidotti, 1969; Tanford, 1980). This is especially notable due to the high relative abundance of glycine, which frequently acts a hydrophobic residue (Cantor and Schimmel, 1980). It is possible that the 65,000 dalton protein, like protein kinase C (Nishizuka, 1984), has a hydrophobic domain that contains a high percentage of hydrophobic residues.

The N-terminus of the binding protein was sequenced. These studies demonstrated that the N-terminal amino acid sequence of the protein possesses significant homology with the beta chain of hemoglobin. This homology corresponds exactly to the N-terminus of the beta chain. It is interesting to note that the molecular weight of 65,000 daltons for the binding protein is approximately four times the molecular weight of approximately 16,000 daltons reported for the beta chain of hemoglobin (Braunitzer et al., 1964). In addition, porphyrins, the organic portion of the heme moiety, have been shown to inhibit binding to the peripheral benzodiazepine-binding site (Verma et al., 1987). Inhibition studies with photolabeling to the 65,000 dalton protein in cytosol did not demonstrate any effect with four different porphyrins, including protoporphyrin

IX, mesoporphyrin IX, deuteroporphyrin IX, and hemin. As noted, however, these studies measuring competition of irreversible radioligand binding by non-photoactivated compounds may not be sensitive enough to detect the effect of a reversibly-bound ligand. Finally, with regard to the sequence homology with hemoglobin, it is interesting to note that benzodiazepines are capable of inducing the synthesis of hemoglobin in Friend erythroleukemia cells by mechanisms that are independent of the central or peripheral binding sites (Clarke and Ryan, 1980; Wang et al., 1984). The 65,000 dalton protein appears to be distinct from hemoglobin based on the lack of exact homology between the N-termini of the two proteins, the absence of any detectable specific photolabeling of hemoglobin in rat serum, and the difference in glycine content of 9.6% for the rat beta chain of hemoglobin (Braunitzer et al., 1964) and 17.2% for the 65,000 dalton protein.

D. Possible Identity and Function of the Protein. It is possible that the 65,000 dalton protein is an enzyme. The presence of the protein in the liver suggests that it could be a catabolic enzyme, but this is not consistent with its existence in spleen and kidney. In addition, no specific [^{3}H]-CNZ photolabeling was detected in the crude microsomal fraction and no major proteins of the rat liver cytochrome P-450 system exhibit molecular weights in the 60,000-70,000

dalton range (Guengerich et al., 1982; Ryan et al., 1982; Vlasuk et al., 1982). The 65,000 dalton protein may represent an anabolic enzyme. Recent studies have indicated that benzodiazepine-like compounds exist in the brains (Sangameswaran and DeBlas, 1985; Sangameswaran et al., 1986; DeBlas et al., 1987) and serum (Wildmann et al., 1986) of animals with no known exposure to the benzodiazepines. These compounds may be obtained from the diet, but it is also possible that they are synthesized in the brain or other organs. The possibility of endogenous synthesis of benzodiazepines is raised by the finding that benzodiazepine-like immunoreactivity is detectable in NG105-15 cells after being grown for three months in serum-free medium (DeBlas et al., 1987). The 65,000 dalton protein identified in this investigation may be part of a benzodiazepine-synthesizing system. Actually, benzodiazepine synthesis has been demonstrated in Actinomycetes, a bacterium, and in Penicillium cyclopium, a fungus (Luckner, 1984). In Actinomycetes, it is interesting to note that a centrally-active compound, L-dihydroxyphenylalanine (L-DOPA), is the precursor for benzodiazepine synthesis (Luckner, 1984).

The binding protein may be a precursor of the central or peripheral sites. However, a precursor would probably be vesicle-associated and would not be enriched in the cytosolic fraction (Appel and Day, 1976). In addition, the

potencies for benzodiazepines and the tissue distribution further distinguish the 65,000 dalton protein from the central and peripheral sites.

Several previously-characterized proteins that are present in the cytosolic fraction exhibit a molecular weight similar to that of the protein identified in this study. These proteins include 68,000 dalton neurofilament protein (Scott et al., 1985), phosphatidylinositol-specific phospholipase C at 65,000 daltons (Hofmann and Majerus, 1982), calelectrin at 67,000 daltons (Sudhof et al., 1984), corticosteroid binder II at 65,000 daltons (Litwack et al., 1973), choline acetyltransferase at 68,000 daltons (Bruce et al., 1985), calpastatin at 68,000 daltons (Takano et al., 1986), cyclic GMP-stimulated cyclic AMP phosphodiesterase at 67,000 daltons (Pyne et al., 1986), and heat-stable inhibitor of the Ca^{2+}-activated cyclic nucleotide phosphodiesterase at 68,000 daltons (Sharma et al., 1978). The glycine content of 17.2% for the 65,000 dalton, photolabeled protein distinguishes it from the 68,000 dalton neurofilament protein with 4.3% glycine residues (Hogue-Angletti et al., 1982), calelectrin with 9.0% (Sudhoff et al., 1984), choline acetyltransferase with 8.4% (Braun et al., 1987), serum albumin with 4.7% (Glenney et al., 1981), and calpastatin with 5.3% (Takano et al., 1986). Furthermore, the N-terminal sequence for pig choline acetyltransferase (PILEKTPPKMA; Braun et al., 1987)

and the sequences for rat albumin and pig 68,000 dalton neurofilament protein (from the NBRF-PIR protein sequence data base) do not exhibit significant homologies with the 65,000 dalton protein.

It is difficult to clearly associate the 65,000 dalton protein with any of the actions of the benzodiazepines that do not appear to be mediated by the central or peripheral sites, such as effects on neuronal excitability, neurotransmitter release, calcium uptake, and growth and differentiation. The finding that CNZ exhibited a relatively high potency appears to distinguish photolabeling of the 65,000 dalton protein from benzodiazepine effects in which CNZ displays a relatively low potency or is completely inactive. These effects include facilitation of neurotransmitter release (Mitchell and Martin, 1978; Martin and Mitchell, 1979; Mitchell and Martin, 1980), inhibition of adenosine uptake (Phillis et al., 1981), inhibition of nerve growth factor-induced neurite outgrowth in PC 12 cells (Morgan et al., 1985), and induction of differentiation in Friend erythroleukemia cells (Wang et al., 1984). For the other benzodiazepine effects, the potency data is too limited to make a strong positive or negative correlation with the binding studies.

E. Conclusion. This investigation describes a 65,000 dalton protein in rat brain that exhibits selective, saturable, and stereoselective photolabeling by

therapeutically-relevant concentrations of NO_2-containing benzodiazepines. This protein is distinct from the central- and peripheral-type sites based on molecular weight, absolute potency of NO_2-benzodiazepines, subcellular distribution, and tissue distribution. The protein was purified to apparent homogeneity by column chromatography. The purified protein exists as a monomer, is glycine-rich, and possesses sequence homology with the beta chain of hemoglobin.

Additional studies must be performed to examine the function of the 65,000 dalton protein. Two important areas for investigation are enzyme activity and physiologic effects of the protein. To examine the possibility that the protein is a benzodiazepine-synthesizing enzyme, purified protein would be incubated with benzodiazepines and then low molecular weight components of the reaction mixture would be separated by HPLC. These studies would be designed to reverse the reaction by adding an excess of benzodiazepine such that a detectable level of substrate would be present after incubation. In addition to purified protein, these reactions would also be conducted with cytosol or low molecular weight cytosolic components added back to the purified protein to replace any essential cofactors or prosthetic groups that were removed during the purification process. Similar incubations of the purified protein with several different benzodiazepines would be

performed to examine for benzodiazepine degradation by the protein. In these studies, the reaction mixtures would also be supplemented by liver cytosol or low molecular weight components of liver cytosol. Physiologic effects of the 65,000 dalton protein would be investigated by injecting the purified protein into cells and measuring the effects of injection on sustained repetitive firing, threshold for action potential activity, and membrane conductance. Appropriate control injections in these studies would include buffer, boiled protein, and photolabeled protein. In this system, it would also be valuable to examine the effect of injecting a monoclonal antibody against the 65,000 dalton protein. Thus, further studies must be performed to investigate the possible functional significance of the 65,000 dalton protein and to determine if the protein mediates any of the effects of the benzodiazepines that cannot be accounted for by the central or peripheral sites.

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