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Comparative Biochemistry of Drug-Binding Proteins in Calmodulin/S100 Family

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COMPARATIVE BIOCHEMISTRY OF DRUG-BINDING PROTEINS IN THE CALMODULIN/S100 FAMILY

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INTRODUCTION

Cells respond to hormonal, neurochemical and nutritional signals by adjusting their metabolic and biosynthetic machinery. Some of these responses are mediated by effector proteins which regulate various enzymes. Several calcium modulated proteins are effector proteins whose regulatory activities are modulated by fluctuations in the intracellular free calcium ion concentration. These calcium modulated effector proteins are involved in fundamental cellular processes such as glucose metabolism, muscle contraction, cell motility, secretion, and mitosis. The experiments presented in this thesis are part of an effort to compare the biochemical, immunochemical and pharmacological properties of several calcium modulated effector proteins. An understanding of the molecular physiology of calcium modulated proteins provides the foundation for elucidating the mechanisms of calcium action in cellular physiology.

Effector Proteins

A variety of enzymes employ regulatory effector proteins which modulate the enzymatic response to external stimuli. These enzymes include protein kinases (1-2), adenosine triphosphatases (3), acetyl coenzyme A carboxylase (4), fatty acid synthetase (4-6), and lactose synthetase (7-9). Although these enzymes have diverse functions, their effector proteins form structurally and functionally related classes of proteins.

The regulatory subunit of the cyclic adenosine monophosphate dependent protein kinase is an effector protein which binds cAMP, leading to its dissociation from the catalytic subunit (1). The free catalytic subunit carries out the transfer of phosphate from ATP to specific hydroxyl groups of protein

substrates. In this manner the regulatory subunit mediates the responses of cells to hormones which cause changes in the intracellular cAMP levels. For example, when β-adrenergic agonists stimulate cAMP production in hepatocytes, the regulatory subunit transduces this signal into activation of the protein kinase cascade involved in glycogen breakdown (2).

Another effector protein, α-lactalbumin, regulates the synthesis of lactose by mammary tissue (7). The enzyme galactosyl transferase (UDP galactose-N-acetylglucosamine-β -4-galactosyl transferase) catalyzes the condensation of galactose and glucose to form the disaccharide, lactose (7). The transferase requires α-lactalbumin to specify glucose as the substrate(8-9), and the level of α-lactalbumin in the tissue helps to control the rate of lactose synthesis (7-9). Prolactin acts in conjunction with insulin and steroids to induce α-lactalbumin synthesis in the mammary tissue of adult females post partum (7).

The acyl carrier protein (ACP) of fatty acid synthetase is an effector protein (5-6) which forms a covalent intermediate with the growing acyl chain. Although other parts of the synthetase complex catalyze the addition of two carbon units to the growing acyl chain, the acyl carrier protein is essential for proper enzymatic function (4). The prosthetic group, 4'-phosphopantetheine, is linked through a phosphodiester bond to the hydroxyl of a serine residue in the ACP (6). The levels of synthetase activity are linked to the presence of the water soluble vitamin pantothenate in the diet (4). Similarly, the biotin carboxyl carrier protein (BCCP) forms a covalent complex with biotin and acts as an accessory to the acetyl coenzyme A carboxylase enzyme complex (4,10-11). The activity of the enzyme is dependent on biotin, and citrate is an allosteric regulator of carboxylase activity (4).

Several calcium modulated proteins are examples of effector proteins,

each conferring calcium ion sensitivity on enzymes. Troponin C is a calcium modulated effector protein which confers calcium sensitivity on the actomyosin ATPase of skeletal and cardiac muscle (3). Troponin C regulates muscle contraction by mediating the flux of calcium ions stimulated by acetyl choline release at the neuromuscular junction.

In smooth muscle, two calcium modulated effector proteins are involved in regulating muscle contraction. The regulatory light chain of myosin is a calcium modulated protein (12-13) which regulates the myosin ATPase (14). Smooth muscle contraction may be regulated by phosphorylation of the light chain by the enzyme myosin light chain kinase (15). This kinase is stimulated by calcium and calmodulin (13,16-17), another calcium modulated effector protein. In liver and striated muscle, calmodulin is an integral subunit of phosphorylase kinase (18), and calmodulin also activates glycogen synthase kinase in liver (19), thus regulating both the breakdown and synthesis of glycogen.

Hormones, neurotransmitters, vitamins and drugs may affect the level of messengers such as Ca^{2+} or cAMP, which signal effector proteins such as calmodulin and the regulatory subunit of cAMP dependent protein kinase. Alternatively, the endocrine stimulation of a cell may induce the synthesis of an effector protein as in α-lactalbumin. A third category of effector proteins is exemplified by ACP and BCCP which covalently link the dietary vitamins and cofactors necessary for metabolic functions. Of these categories of effector proteins, the calcium modulated effector proteins illustrate how physiological systems use calcium ions as transducers of endocrine and neuronal signals.

Calcium Modulated Proteins

Calcium is required for optimal growth and functioning of most living organisms, and studies of the molecular basis of calcium action indicate that proteins act as calcium ion receptors (12-13). Calcium binding proteins may be divided into four classes reflecting different ways in which proteins bind and use calcium. First, proteins containing γ-carboxyglutamic acid bind calcium through bidentate chelation. The proteins involved in blood clotting such as prothrombin (20) and Factor X (21) are the best characterized proteins containing γ-carboxyglutamic acid. These proteins bind calcium with dissociation constants in the millimolar range, approximately the serum levels of calcium (13,20-21). Second, certain proteins use calcium ions to stabilize a particular conformation. Calcium binding lectins such as concanavalin A (22) and the calcium binding enzyme, thermolysin (23), use calcium in paired divalent cation binding sites which appear to enhance the stability of the protein. These proteins use mainly oxygen-containing side chains of amino acids to form polyhedral calcium binding sites with relatively high affinities for calcium. Third, calcium binding enzymes may use calcium ions in substrate binding and enzymic catalysis. For example, pancreatic phospholipase A_2 contains a calcium binding site near the active site of the enzyme (24). The bound calcium acts to stabilize the bound phospholipid substrate and to help catalyze the hydrolysis of the fatty acid glycerol ester bond (25). The calcium ion in phospholipase A_2 is mainly coordinated by peptide carbonyl oxygens and water molecules (24). Other hydrolytic enzymes such as trypsin, chymotrypsin and staphyloccocol nuclease also contain calcium binding sites (13). Fourth, calcium modulated proteins bind calcium reversibly at physiological ionic strength and pH with dissocia-

tion constants in the micromolar concentration range (12-13). The calcium binding structures of calcium modulated proteins are octahedral, employing oxygen-containing side chains as well as peptide carbonyl oxygens to coordinate the calcium ion. Examples of calcium modulated proteins are troponin C, parvalbumin, calmodulin, S100, myosin regulatory light chains, and the vitamin D dependent calcium binding protein.

Kretsinger (12) has proposed that proteins which contain EF hand structures are the targets of calcium acting as a second messenger intracellularly. All calcium moulated proteins for which an amino acid sequence is known contain potential calcium binding structures known as EF hand structures. The term EF hand is derived from the calcium binding structure in carp parvalbumin for which the crystal structure has been determined. The EF hand is formed by the E-α helix and the F-α helix connected by a peptide loop. The loop contains the amino acid residues which have the ligating oxygens of the octahedral calcium site. In the EF hand region of carp parvalbumin, four of the calcium ligands are formed by carboxylate moieties of aspartic acid and glutamic acid side chains. One ligand, the -Y vertex of the octahedron, employs the oxygen of the peptide carbonyl. In this case, there is no restriction on the nature of the side chain of the -Y amino acid (12). The sixth ligand is a water molecule which intercalates between the peptide carbonyl of a glycine residue and the calcium ion. All potential EF hand structures contain amino acid sequence homology with the parvalbumin EF hand region. In addition to the ligating amino acids, the presence of a glycine between the Y and Z vertices may be required to allow the peptide loop to make a sharp bend while wrapping itself around the calcium ion.

Flanking the calcium binding loop are the two α helices, the E helix and the F helix. These helices are amphipathic in nature because the side chains

of residues on the inner faces of the helices are hydrophobic, while those on the outer faces are generally hydrophilic. In the EF hand model of Kretsinger (12), the hydrophobic surfaces of the helices face the hydrophobic core of the protein. Pairs of EF hand structures may be stabilized by hydrophobic interactions between helices as well as interactions between side chains of the loops. However, it is not clear from the crystal structure of parvalbumin (26) what generally determines how multiple pairs of EF hand structures interact in proteins. Until the three dimensional structure of a protein with four or more EF hand structures is known, the model is restricted to units of two EF hands.

During the past decade the primary structures of several calcium modulated proteins have been determined (12-13,27-34). From muscle research, the pioneering experiments of Ebashi (3) led to the isolation of troponin C and its identification as the calcium sensitizing subunit of actomyosin ATPase. Collins et al. (27) determined the complete amino acid sequence of the protein from rabbit skeletal muscle, and Van Eerd et al. (28) determined the primary structure of bovine cardiac troponin C. Both of these molecules have four domains containing potential EF hand structures and have internal amino acid sequence homology. The skeletal muscle troponin C binds four moles of calcium per mole of protein while the cardiac troponin C binds only three (12). The lack of one functional calcium binding domain in cardiac troponin C may be due to several amino acid substitutions which modify the EF hand structure of the second domain (28).

Through another channel of research on muscle proteins, the regulatory light chains of myosin were shown to be calcium modulated proteins (12-14). Among vertebrates, light chains range from 16,000-21,000 molecular weight and are defined by their ability to resensitize myosin ATPase to Ca^{2+} (14).

The regulatory light chains bind only one mole of Ca^{2+} per mole of protein when associated with the myosin heavy chain (14), although regulatory light chains contain four domains with amino acid sequence homology to the EF hand structure (12). Several amino acid changes and deletions in the non-binding domains may account for the lack of calcium binding function, as in the second domain of cardiac troponin C (12,28). In addition, regulatory light chains are phosphorylated at serine residues by the enzyme myosin light chain kinase (1,15-17). Hartshorne and Persechini (15) have suggested that phosphorylation of the light chain regulates myosin ATPase activity in smooth muscle. However, it is not clear how phosphorylation of light chains affect the calcium binding properties of the potential EF hand structures.

Calcium modulated proteins are also found in non-muscle tissue (12-13). The vitamin D-dependent, calcium binding protein from mammalian intestine is an acidic protein with $M_r \approx 10,000$ (13,29). Based on crystallographic studies of the bovine protein (30) there is one region which contains an EF hand structure and binds one mole of calcium. In addition, a second calcium binding site occurs in the amino terminal half of the protein. This site is similar to EF hand structure, but the exact nature of the ligating species is still under investigation. A second group of vitamin D-dependent calcium binding proteins are found in the avian intestine and mammalian kidney (13,31). These proteins have $M_r \simeq 28,000$ (13,31). The structures of these larger vitamin D-dependent proteins have not been determined, but these proteins may prove to be an interesting type of calcium modulated proteins.

Brain tissue has been a rich source of three calcium modulated proteins: calmodulin, S100α and S100β (32-34). Because these proteins are the subject of most of the studies presented in this thesis, it is instructive to examine calmodulin, S100α and S100β in detail. The isolation and characterization of

the unique chemical structures, calmodulin, S100α and S100β, has enabled researchers to begin study of structure-function relationships in these proteins.

Calmodulin

Calmodulin is the name proposed (35) for a calcium binding protein which was first purified to chemical homogeneity by Watterson *et al.* (36) as a troponin C-like protein of bovine brain. Most eukaryotic cells contain calmodulin, and the amino acid sequence of calmodulin is highly conserved (13,37). Vertebrate calmodulin is an acidic, calcium-binding protein consisting of 148 amino acid residues. Bovine brain calmodulin contains no tryptophan, cysteine, phosphate or carbohydrate (32,36) and contains one residue of N^{ϵ}-trimethyllysine (32). Calmodulin has four structural domains that are similar to each other and similar to the four domains of skeletal muscle troponin C (27,32). Each of the four calmodulin domains contains the requirements for a model EF hand structure (12-13), and the protein binds four moles of calcium per mole of protein. The direct demonstration of the calcium ligating species in the protein will depend on data from the crystallographic studies of Cook *et al.* (38) and Kretsinger *et al.* (39).

A comparison of the amino acid sequences of calmodulin from four species is shown in Figure 1. The amino acid sequence studies of calmodulins from various species indicate that vertebrate calmodulins may be identical (32,40-43). These species include bovine (32), human (40), chicken (41), rat (42) and rabbit (43). Nearly complete amino acid sequences have been determined for calmodulin from the invertebrates *Renilla reniformis* (44) and *Metridium senile* (45). These proteins have several amino acid substitutions from the bovine brain sequence occurring in the third and fourth domains. Two

of these substitutions are changes in the amidation state of Asn-97 and Gln-135. In addition, the Renilla protein has a deletion of the glutamine at position 3 (44).

The amino acid sequence of calmodulin from the protozoan Tetrahymena pyriformis (46) has thirteen differences from the vertebrate protein. The majority of these are in the third and fourth domains, as in the invertebrate calmodulin, and the threonine at position 146 has apparently been deleted. In addition, three amino acid substitutions occur in the second domain: the amidation state of asparagine-60, a threonine to serine substitution at position 70, and a methionine to leucine substitution at position 71. Eleven of the thirteen amino acid substitutions can be accounted for by single nucleotide changes in the codon structure.

Calmodulin from spinach leaves has twelve amino acid substitutions from the vertebrate sequence (47-49). Again, most of these are in the third and fourth domains of the molecule, with several notable exceptions. The glycine at position 96 of vertebrate calmodulin is replaced by a glutamine in the spinach sequence. This substitution is only compatible with a minimum two nucleotide change in the DNA sequence of the gene for calmodulin. More importantly, glutamine at position 96 occurs between the Y and Z vertices of the model EF hand calcium binding loop of domain two (12-13,47-48). It is not clear how this substitution affects the binding of calcium. Spinach calmodulin also contains a single cysteine residue at position 26 (45), which corresponds to the -Y vertex of the model EF hand of domain one. The functional significance of this substitution is not known, since detailed calcium binding studies of the spinach protein have not been reported. The cysteine as well as the other amino acid substitutions suggest that plant calmodulins may have functionally significant amino acid sequence differences from ver-

tebrate calmodulin.

Recently, the partial amino acid sequences of two novel calmodulins have been determined (50). These sequences are shown in Figure 2. Calmodulins from the green alga Chlamydomonas reinhardtii (50) and the slime mold Dictyostelium discoideum (51) do not contain N $^{\varepsilon}$-trimethyllysine and have lysine at position 115. This difference could be due to the absence of the methylation enzymes. Alternatively, other amino acid substitutions in this region of Chlamydomonas calmodulin may affect the susceptibility of lysine 115 to methylation. Notably, the threonine at position 117 and the aspartic acid at position 118 are replaced by serine and glutamic acid. These substitutions make the region at positions 117-120 of Chlamydomonas calmodulin homologous to residues 81-84 in the third domain of vertebrate calmodulin. This region has been implicated in the interaction of vertebrate calmodulin with troponin I (37,52). The appearance of an identical sequence in the fourth domain of Chlamydomonas calmodulin raises the possibility that the protein may have significant functional properties that are different from vertebrate calmodulin. Amino acid sequence studies of calmodulin from Dictyostelium discoidium indicate that the slime mold protein is similar to other calmodulins, but does not contain N $^{\varepsilon}$-trimethyllysine. In addition, Dictyostelium calmodulin contains serine at position 111, asparagine at position 118, and leucine at position 130. All three of these differences from the bovine brain protein are compatible with single nucleotide changes in the codon structures. The amino acid sequence determination of the Dictyostelium protein is shown in the Appendix.

A number of investigators have identified calmodulin-like proteins in Chlamydomonas flagellae (53), barley (54), and mushrooms (55). However, since calmodulin is a member of a family of structurally and functionally

related calcium binding proteins, it is reasonable that calmodulin-like proteins exist in these tissues. Troponin C could be considered a tissue specific, calmodulin-like protein, but troponin C has a well defined function in striated muscle (3). The biochemical characterization of calmodulin-like proteins by amino acid sequence analysis and functional properties should clarify the relationships among four-domain calcium modulated proteins.

Calmodulin has no known enzymatic activity but has been shown to stimulate numerous enzymes in vitro in a calcium dependent manner (37). These include: 1) enzymes of cyclic nucleotide metabolism (56-62), 2) various AT-Pases (63-67), 3) NAD kinases in plants (68) and invertebrates (69), 4) protein kinases (18-19,70-72), and a protein phosphatase (73). In some cases, these in vitro effector activities of calmodulin may reflect true physiological functions. The holoenzyme of muscle phosphorylase kinase has been purified to chemical and enzymatic homogeneity and has been found to consist of four subunits, α, β, γ, and δ (18,43). The δ subunit was shown to be the calcium sensitizing component and to be identical to vertebrate calmodulin (18). According to similar criteria (16-17), myosin light chain kinase holoenzyme also contains calmodulin as an integral subunit.

The ability to demonstrate a calcium and calmodulin stimulation in vitro does not necessarily reflect a physiological function of calmodulin. In reconstituted actomyosin ATPase assays, calmodulin quantitatively substitutes for troponin C, the physiological calcium sensitizing factor of skeletal muscle actomyosin ATPase (74-75). It is possible that calmodulin could substitute for endogenous calcium dependent regulators in some of the other in vitro activities attributed to calmodulin.

Calmodulin stimulation of the enzymes of cyclic nucleotide metabolism has been an intriguing and controversial aspect of calmodulin function.

Several forms of cyclic nucleotide phosphodiesterase activity can be separated by ion exchange chromatography (76). One of these has a relatively low K_M for cAMP, can also utilize cGMP as substrate, and shows ten fold activation with calcium and calmodulin (76-77). Cheung (56) detected a protein activator of cyclic nucleotide phosphodiesterase,and Kakiuchi and Yamazaki (57) observed a calcium dependent stimulation of phosphodiesterase. Teo and Wang (78) later showed that the activator was a calcium binding protein. Watterson et al. (36) demonstrated that chemically homogeneous troponin C-like protein (calmodulin) from bovine brain could activate the enzyme in vitro. Klee (77) purified the porcine brain activator protein and showed that it was identical to calmodulin. However, the calmodulin stimulated phosphodiesterase accounts for only a fraction of the total cyclic nucleotide phosphodiesterase activity in brain extracts (76-77). Thus, the physiological role of calmodulin activation of phosphodiesterase remains unclear for brain and other tissues.

A similar situation exists for calmodulin stimulation of brain adenylate cyclase. The adenylate cyclase activity in brain membrane fractions can be stimulated about four fold by calmodulin and calcium (58-59). Storm and his colleagues (60) initially reported that the guanyl nucleotide regulatory subunit was required for calmodulin activation of adenylate cyclase. Recently, Salter et al. (61) have demonstrated that calmodulin activates the isolated catalytic subunit of the enzyme after gel filtration in the presence of ammonium sulfate. Recent work with forskolin and manganese ion activation of adenylate cyclase indicates that the state of the catalytic subunit may indeed be modulated by other factors in addition to the guanyl nucleotide regulatory subunit (79).

Adenylate cyclase from Bordetella pertussis can be activated by cal-

modulin in a calcium dependent manner (80). This observation is particularly intriguing because the Bordetella enzyme is extracellular (80), unlike most mammalian cyclases which are intracellular, plasma membrane-associated proteins (61,79). In addition, Bordetella synthesizes a protein toxin which catalyzes the ADP-ribosylation of the inhibitory guanyl nucleotide regulatory protein in pancreatic islet (81) and neuroblastoma-glioma hybrid cell (82) adenylate cyclase systems.

The guanylate cyclase from the protozoan Tetrahymena pyriformis is stimulated only by Tetrahymena calmodulin, and not by vertebrate or invertebrate calmodulins (83). However, Tetrahymena calmodulin activates vertebrate phosphodiesterases (83-84), adenylate cyclase, myosin light chain kinase, and (Ca^{2+}-Mg^{2+}) ATPase (85) as well as plant NAD kinase (85). Several of the amino acid sequence differences between Tetrahymena and vertebrate calmodulin occur in the fourth domain (46). These changes include a histidine residue substituted for the glutamine at position 135 of the vertebrate protein, an arginine at position 143, and a deletion of the threonine at position 146. These substitutions in the fourth domain alter the reactivity of the protein to the site specific antiserum developed by Van Eldik and Watterson (86). However, it is not known how these amino acid substitutions in Tetrahymena calmodulin alter the specificity for activation of the guanylate cyclase.

In addition to calcium dependent activation of enzymes, calmodulin displays calcium dependent binding to troponin I (74), spectrin (87), caldesmon (88), fodrin (89), and a microvillus protein (90), and calcium independent binding to gap junction (13), histones (91-92), and myelin basic protein (91,93). In order to evaluate the physiological relevance of these interactions, it is important to examine the limitations of the methods used to iden-

tify calmodulin binding proteins. Three methods have generally been used to demonstrate calmodulin binding to proteins (13,48,94-95). First, proteins are shown to bind to calmodulin-Sepharose conjugates in the presence of calcium and to elute with buffers containing a calcium chelator (48). Studies of this type are limited by the amount of active calmodulin which has been covalently coupled to the Sepharose. Experiments using calmodulin-Sepharose conjugates must also use buffers which are relatively isotonic. Some proteins, such as the tau protein of microtubules, bind to calmodulin-Sepharose when applied in hypotonic buffers (96). Such an interaction is not sufficient to establish that tau is the target for calmodulin in the regulation of microtubule depolymerization in vitro (48,96). Second, calmodulin binding proteins are identified by the binding of radioiodinated calmodulin to proteins which have been separated by electrophoresis in polyacrylamide gels (90,97-98). An important consideration in this method is the procedure for the renaturation of the proteins in the gel matrix following electrophoresis. Third, crosslinking of calmodulin to a bound protein or peptide has been used to demonstrate an interaction (99). For example, Giedroc et al. (99) found stoichiometrically cross-linked species of calmodulin and endorphin peptides using bis(sulfosuccinimidyl) suberate.

Calmodulin appears to interact with various supramolecular structures and cellular organelles (89,97-98,100-102). Calmodulin binding proteins have been examined in subcellular fractions of chicken embryo fibroblasts (100). While 77-93% of the calmodulin was present in the soluble fraction, a small but reproducible amount of calmodulin was found in the nuclear, mitochondrial/lysosomal, and microsomal fractions (100). These particulate fractions also contained a number of calmodulin binding proteins as determined by a gel overlay method (98,100). These results suggest that some of the

targets of calmodulin action may reside on various subcellular organelles or structures, and that calmodulin binding proteins are not limited to soluble enzymes.

Siekevitz and colleagues (89,101) have demonstrated that calmodulin is present in a preparation of postsynaptic densities from canine cerebral cortex. These supramolecular complexes also contain fodrin, an actin-binding protein which also binds iodinated calmodulin in a gel overlay procedure (89,97). Immunocytochemical localization of calmodulin at the level of the electron microscope also indicates that postsynaptic densities contain calmodulin (102).

S100

S100 refers to a heterogeneous fraction of low molecular weight, acidic proteins which bind calcium. In 1965, Moore and McGregor (103) identified S100 as prominent in soluble extracts of rat brain, but not apparent in extracts of rat liver. Moore (104) developed a procedure to isolate this fraction, called S100 to signify its partial solubility in saturated ammonium sulfate at neutral pH. Since that first report, a number of methods for the isolation of S100 from bovine brain have been described (105-111). These involve routine techniques of protein chemistry such as salt fractionation, column chromatography, and, in some cases, preparative gel electrophoresis. Isobe and co-workers (33-34,112) have purified two polypeptides, S100α and S100β, by more extensive use of these procedures. During the course of the studies presented in this thesis, we have demonstrated (113) the purification of polypeptides of the S100 fraction by the combined use of salt fractionation, ion exchange chromatography, affinity-based adsorption chromatography on phenothiazine-Sepharose conjugates, and reverse phase high performance

liquid chromatography. Hidaka and colleagues (114) have recently described the purification of one of the S100 components using affinity-based chromatography on immobilized naphthalene sulfonamide derivatives.

During the last several years, purified S100α and S100β have become available. Most of the earlier studies of S100 have utilized or monitored a heterogeneous mixture of proteins (105-110). As a result, a large body of confusing literature has evolved. Three guidelines are useful in the evaluation of this literature. First, S100 is a protein fraction and the relative amounts of S100α and S100β have not been determined. Variable amounts of calmodulin and calmodulin fragments are also present in some S100 preparations (36,112,115-116). Second, since there is no known function for calmodulin-depleted S100, its protein components should not be considered subunits of an enzymatic, structural or regulatory complex. Isobe et al. (117) have presented evidence that in solution S100α and S100β exist as dimers of the form $\alpha_1 \beta_1$ (S100a) and β_2 (S100b), but the significance of this dimerization is not clear. Third, S100 is usually monitored by immunoreactivity with heterologous antisera prepared against a heterogeneous protein mixture. Thus, most antisera directed against S100 do not define a single structure. In this thesis we have addressed this problem through immunochemical studies of purified polypeptides.

S100α (33) and S100β (34) are polypeptides of 93 and 91 amino acids, respectively, which share 54 identical amino acids. The amino acid sequences of S100α and S100β are shown in Figure 3. Of the 39 sequence differences between S100α and S100β, 23 are compatible with single nucleotide substitutions, and S100α contains a tryptophan residue which is lacking in S100β. Both S100α and S100β contain the sequence requirements for the formation of a EF hand calcium binding structure. In addition, residues 20-32 in both S100 proteins share

amino acid sequence homology with the amino terminal calcium binding structure in the bovine vitamin D dependent calcium binding protein (30). Szebenyi and Moffat (30) have suggested that the S100 proteins contain calcium binding sites in this region, although crystallographic data are necessary to establish these sites. Crystals suitable for diffraction studies have been reported for S100β (39), but electron density maps are not yet available.

The physicochemical properties of S100 are also subject to reevaluation. Calissano et al. (118) examined the calcium binding properties of S100 using changes in tryptophan fluorescence and equilibrium dialysis. Only S100α contains tryptophan (33), however, although S100β may display anomalous tyrosine fluorescence (119). In addition, the affinity of S100 for calcium may vary with the state of the sulfhydryls (120). Thus, three properties of S100 are important to our understanding of the proteins: the native oxidation states of the sulfhydryls, the affinities and stoichiometries of calcium binding, and the nature of the ultraviolet absorption spectra.

The function of the S100 proteins is not known, but several in vitro activities have been described (113-114,121-124). Apparent calcium dependent activities include interaction with synaptosomal (121) and artificial (122) membranes, binding to immobilized phenothiazines (113) and naphthalenes (114), stimulation of nucleolar RNA polymerase activity (123) and depolymerization of tubulin (124). The only activities which have been examined using purified polypeptides are the interactions with drugs (113-114) and the depolymerization of tubulin (124).

Until recently, S100 has been considered a nervous system-specific protein antigen (125-126), which is primarily localized to glial cells in the central nervous system and to Schwann cells in the periphery. Hidaka et al. (127) have isolated S100β from bovine abdominal adipose tissue, and Kanamori

et al. (128) have found immunoreactive S100 in human T lymphocytes. These findings indicate that S100 is not specific to the nervous system and suggest that the tissue distribution of S100α and S100β should be examined with specific antisera.

Molecular Pharmacology

The pharmacology of calcium modulated effector proteins is still in its infancy. Honda et al. (129) reported that the activation of cyclic nucleotide phosphodiesterase by calcium and an unidentified protein could be inhibited by chlorpromazine. Levin and Weiss (130) found that a number of phenothiazine derivatives bind to calmodulin in a calcium dependent manner and block the activation of phosphodiesterase (131). Since these initial reports, phenothiazine derivatives have been shown to block calmodulin stimulated enzymes in vitro when the calmodulin- drug complex is formed prior to incubation with the enzyme (37). However, phenothiazine drugs cannot dissociate the phosphorylase kinase δ subunit from the holoenzyme (132). The exact nature of the drug interaction with calmodulin in the absence and presence of various enzymes has not been determined.

Phenothiazines are heterocyclic compounds that are used pharmacologically as antipsychotics, antiemetics and tranquilizers (133). The structures of three phenothiazine derivatives which have been used in our studies are shown in Figure 4. Levin and Weiss (134) reported that the affinity of calmodulin for various phenothiazines and other neuroleptic drugs correlated with their potency as neuroleptic agents. However, the affinity of all classes of neuroleptic drugs for calmodulin does not correlate with pharmacological potency (135-136). For example, the optical isomers of butaclamol have similar affinities for calmodulin, but the + isomer is much

more potent clinically than the - isomer (137). Thus, the involvement of calmodulin in the mechanism of neuroleptic drug action has not been established by pharmacological correlations.

Phenothiazine drugs and dyes have been used for many years to study the active sites of enzymes (138), structural domains of albumins (139), and structures on neurotransmitter receptors (140). Phenothiazines also interact with membranes (141), induce shape changes in erythrocytes (142), and intercalate into DNA (153-144). Phenothiazine ligands have been useful in demonstrating structurally homologous nucleotide binding domains in dehydrogenases (145). Phenothiazine dyes, such as methylene blue, have been used to probe the active sites of enzymes through dye-sensitized photooxidation (138). Serum albumins have separate sites for various anion and cationic drugs and dyes, including phenothiazines (139,146). Phenothiazines block dopamine receptor agonist binding in membrane preparations from neural tissue (140,147-148), and dopamine receptor blockade has been invoked (147) to explain the antipsychotic actions of phenothiazines. The phenothiazines can also produce tardive dyskinesias as the major side effect of chronic or, in some cases, acute doses of drugs (149). Thus, the physiological and pharmacological role of phenothiazine drugs is an important question in clinical pharmacology and psychiatry (147). The answer, however, includes many potential targets of action.

Regardless of the pharmacological relevance of the interaction between calmodulin and phenothiazines, these drugs have been used as inhibitors of calmodulin stimulated enzymes (37) and as affinity ligands for calmodulin purification (150-151). Phenothiazines have also been used in cell systems where calmodulin and calcium were thought to mediate a physiological event. In several studies (152-162), inhibition of a process by a phenothiazine drug

has been used as evidence for a calmodulin-specific process. Affinity based chromatography on immobilized phenothiazines has been used as the final step in calmodulin purification (150-151). However, other purified calcium-modulated proteins were shown to interact with immobilized phenothiazines in a calcium dependent manner (113). In addition, Moore and Dedman (163) have observed that various unidentified proteins from crude tissue extracts bind to phenothiazine-Sepharose conjugates. Phenothiazines also block the lipid activation of a protein kinase which does not require calmodulin (164). These data indicate that phenothiazine drugs are not calmodulin-specific inhibitors. When our studies were initiated, the binding of trifluoperazine and other phenothiazines to calmodulin (130) was assumed to be responsible for the inhibition of calmodulin stimulated enzymes. However, the relationship between binding activity and inhibitory activity of the drugs was not established.

The calcium dependent binding of phenothiazines to calmodulin remains a useful tool to probe the structural and functional domains of the protein. Storm and colleagues (165) have observed that the hydrophobic probe 1-anilinonaphthalene-8-sulfonate binds to calmodulin in a calcium dependent manner. Steiner and Sternberg (166) found that the binding of the fluorescent probe changes when calmodulin is part of the phosphorylase kinase complex. A variety of local anesthetics including mepacrine (167) and adrenergic drugs including propranolol (167) and phenoxybenzamine (168) interact with calmodulin. Hidaka and colleagues (169-172) have demonstrated that naphthalene sulfonamide derivatives block calmodulin activation of several enzymes. These drugs are smooth muscle relaxants, and can be used as affinity ligands for calmodulin and S100β purification (114). The method of coupling of the drug to the solid support may affect the specificity of the interaction with

calmodulin and S100β (114).

Summary

Calmodulin is a member of a class of structurally and functionally related proteins. Calmodulin is widely distributed among eukaryotes, and the amino acid sequence of calmodulin is highly conserved. The protein contains multiple structural domains and has multiple activities *in vitro*, including calcium dependent drug binding. In the studies presented in this thesis, we have used phenothiazines as tools to probe the relationships between structural domains and functional domains in calmodulin.

The approach to the problem of structure-function relationships can be framed in three questions:

Do other calcium modulated proteins show calcium dependent drug binding activity?

What are the structural requirements for drug binding in calmodulin?

How is the drug binding activity of calmodulin related to its enzyme activator and protein binding activities?

In these questions lies the essence of our approach to the molecular aspects of the relationship between the inhibitory drug binding acitivities and the functional activities of calmodulin. While other investigators (134-137) have studied the inhibitory actions of many classes of drugs on calmodulin, we have examined the properties of the protein and structurally related proteins in order to relate structure and function.

MATERIALS AND METHODS

Preparation of Calcium Modulated Proteins from Bovine Brain

Calmodulin, S100α, and S100β were isolated from bovine brains using a modification (113) of a procedure for the isolation of spinach calmodulin (176). Mature bovine brains (PelFreez) were thawed at 4 °C and stripped of meninges and accompanying blood vessels. All of the following procedures were done at 4 °C unless otherwise indicated. The brains (1-2 kg) were homogenized in a Waring blender with three bursts of 30 seconds each in three volumes (1 l/kg) of buffer H (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM 2-mercaptoethanol). A homogenate supernatant (S_1) was collected after centrifugation at 10,000 x g for one hour and filtering the supernatant through two layers of cheesecloth. The homogenate supernatant was brought to 60% saturation by the addition of solid ammonium sulfate while stirring. The mixture was allowed to stir for one hour and was centrifuged at 10,000 x g for one hour. The supernatant (S_2) was collected by filtering through two layers of cheesecloth and brought to pH 4.0 by the addition of 50% H_2SO_4. The mixture was stirred for 1.5 hours and centrifuged at 10,000 x g for one hour. The supernatant (S_3) was decanted, and the pellet (P_3) was redissolved in water with a few drops of 1 M Trizma (Sigma) to pH ~7.5. This solution was dialyzed against two changes of 20 liters of water over 4 hours and then overnight against 4 liters buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.2 M NaCl). Following dialysis, the redissolved P_3 was centrifuged at 50,000 xg for 1 hour, and the supernatant was applied to a column

(4.0 x 15.0 cm) of diethylaminoethyl-Sephadex A-50 which was equilibrated in buffer B. The non-adsorbed material was eluted with 1 liter buffer B at 1-1.5 ml/min. The remaining material was eluted with a linear salt gradient using 1 liter buffer B (0.2 M NaCl) and 1 liter buffer B with 0.5 M added NaCl (0.7 M NaCl, final) as the limiting solutions. Fractions of 10 ml were collected and analyzed by polyacrylamide gel electrophoresis. Fractions containing material that comigrated in electrophoresis with calmodulin or S100 standard were pooled separately and dialyzed against two changes of 4 liters water overnight and for 4 hours against 4 liters buffer F (10 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 mM $CaCl_2$). The dialyzed material was applied to a column (2.0 x 14 cm) of phenothiazine- Sepharose 4B equilibrated in buffer F at room temperature. Non-adsorbed material was eluted with 100 ml buffer F and 240 ml of buffer F containing 0.4 M NaCl. Calmodulin or S100 protein was eluted with 80 ml of buffer E (10 mM Tris-HCl, pH 8.0, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.4 M NaCl). The column effluent was collected in fractions of 6 ml, and these fractions were analyzed by electrophoresis on polyacrylamide gels and by measuring the optical absorbance at 235 nm. The fractions containing absorbing material were pooled, dialyzed overnight against at least three changes of 100 volumes of water, and then lyophilized. The lyophilized material which had been eluted with buffer E was redissolved in water with a few microliters of 1 N NaOH to adjust the pH to 7. Proteins were purified to apparent homogeneity by high performance liquid chromatography at 30 $^{\circ}C$ on a Waters μBondapak Phenyl column (0.78 x 30 cm) using a modification of the procedure reported by Klee et al. (177). Solvent A consisted of 10 mM potassium phosphate, pH 6.1, 0.5 mM EGTA and solvent B was acetonitrile. Two ml of material were applied to the column which had been equilibrated in 20% B. Calmodulin eluted by changing the solvent composition

to 28% B, S100α eluted with 32%B, and S100β eluted with 36% B. In experiments with different columns, the solvent composition for elution varied slightly, but the relative elution of proteins was identical on all columns tested. Pools of material eluted from the column were collected, rotary evaporated to remove the acetonitrile, dialyzed exhaustively against 10 mM ammonium bicarbonate and water, and lyophilized. The lyophilized protein was stored at -20 °C. The overall yield was 30-50 mg of calmodulin and 10-20 mg each of S100α and S100β from each kg (wet weight) of stripped bovine brain.

Absorption Spectra

The absorption spectra of protein samples were measured using a Hewlett-Packard 8450 ultraviolet-visible spectrophotometer. Samples were dissolved in 0.1 M ammonium bicarbonate and measured in 1 cm quartz cuvettes.

Amino Acid Analysis

Samples for amino acid analysis were rotary evaporated to dryness in 18 x 150 mm Pyrex ignition tubes. To each tube was added 0.5-1.0 ml of 6 N HCl (J.T. Baker) and a small crystal of phenol to protect tyrosine. The tubes were sealed under vacuum and incubated 20-24 hours at 115 °C. Following incubation, the samples were rotary evaporated and redissolved in sample dilution buffer (Pierce) consisting of 0.2 N sodium citrate, pH 2.20 containing 2% thiodiglycol and 0.1% phenol. Samples were analyzed on a Durrum D500 or LKB 4400 instrument. Analyses of samples for N^{ε}-trimethyllysine were performed using an isothermal program as described by Van Eldik et al. (173).

Samples to be analyzed for tryptophan were dried in 12 x 75 mm Pyrex tubes, and 0.050 ml of 4 N methane sulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Pierce) was added to each tube. The tubes were sealed

under vacuum and incubated for 22 hours at 115 °C. Following incubation, each sample was neutralized with 0.045 ml of 4 N sodium hydroxide and diluted with 0.105 ml of sample dilution buffer.

Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate was performed using the discontinuous buffer system described by Laemmli (174). Gels were either large slabs of 20 x 20 x 0.2 cm or smaller slabs of 10 x 12.5 x 0.03 cm.

Polyacylamide gel electrophoresis in the absence of detergent was performed using the method described by Watterson et al. (36), in large slabs or tubes (7.5 x 0.5 cm).

Following electrophoresis, gels were stained with 0.1 % (w/v) Coomassie brilliant blue in 50% methanol, 9% acetic acid, and destained in 5% methanol, 9% acetic acid.

Alkylation of Sulfhydryls

Bovine S100α and S100β were alkylated using ^{14}C-iodoacetate (Amersham, 54 mCi/mmol). Lyophilized S100α (62.5 nmol) and S100β (53 nmol) were dissolved in 1.0 ml of 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.12 mM DTT. Samples were incubated for 30 minutes at room temperature. Iodoacetate (^{14}C-labeled) was added to a final concentration of 51 μM and the samples were incubated for 2 hours at 35 °C. The reactions were stopped by the addition of 50 μl of 20 mM DTT to each. The reaction solutions were dialyzed for 15 hours at 4 ° against 4 liters of 100 mM ammonium bicarbonate and for 12 hours at 4 °C against 4 liters of 10 mM ammonium bicarbonate in cellulose dialysis tubing (Spectrum Laboratories) with 6000-8000 nominal molecular weight cutoff.

Samples were lyophilized and redissolved in 1.0 ml of 5 mM ammonium bicarbonate.

Performic Acid Oxidation

Protein was oxidized with performic acid according to procedures described by Hirs (175). Formic acid (Eastman or Aldrich, reagent grade) was mixed with 30% hydrogen peroxide at a ratio of 19:1 (v/v). This performic acid reagent was incubated at room temperature for two hours and then chilled to 0 °C in an ice bath. Protein samples were dissolved in formic acid to 1-2 mg/ml and chilled to 0 °C for 30 minutes. A 2-3 fold excess by volume of ice cold performic acid reagent was added to the protein solution and incubated on ice for 2.5 to 3 hours. The sample was rotary evaporated to dryness, redissolved in water and lyophilized.

Digestion with Proteases

Protein samples were dissolved at 1-3 mg/ml in 0.1 M ammonium bicarbonate containing 1 mM EGTA. Trypsin (Worthington, TPCK treated) was dissolved in 1 mM HCl at 1 mg/ml. Trypsin solution was added to the protein samples at 1:100 (w/w) ratio. After incubation for two hours at 37 °C, a second aliquot of trypsin solution was added. The final ratio of trypsin to sample was 1:50 (w/w). Samples were covered tightly and incubated at 37 °C overnight, usually 14-18 hours total. Following the incubation, samples were lyophilized and redissolved at ~2 mg/ml in water.

Tryptic peptides of calmodulin, S100α and S100β were separated by high performance liquid chromatography on a Whatman ODS-3 column (0.94 x 25 cm) using a variation of the methods described by Watterson et al. (176). Solvent A was 0.01 N HCl (Ultrex, J.T. Baker) and solvent B was acetonitrile (Fisher

HPLC grade). A 1-2 ml aliquot of the tryptic digest (~ 2 mg/ml) was applied to the column which was equilibrated in 95% A, 5% B. Non-absorbed material including salts and amino acids were eluted by pumping at 5% B at 2 ml/min, 30 °C. Peptides were eluted with increasing amounts of acetonitrile in the solvent mixture. The effluent was monitored for optical absorbance at 215 nm. Fractions were collected either as 0.5 min/tube or as pools of absorbance peaks, rotary evaporated to dryness, redissolved in water, and lyophilized. The peptides were redissolved in water and stored at -20 °C or dried and stored at -20 °C.

Digestion of samples with mouse submaxillary gland protease (Boehringer) was performed exactly as described for trypsin.

Cyanogen Bromide Digestion

Samples for cyanogen bromide digestion were dissolved in 70% formic acid at 5-10 mg/ml. Cyanogen bromide was added as a 0.64 g/ml solution in acetonitrile to a 100-400 fold molar excess of cyanogen bromide over methionyl residues. The samples were mixed, sealed, and incubated for 22 hours at room temperature. Following incubation, the samples were rotary evaporated to dryness, redissolved in water, and lyophilized. The lyophilized material was dissolved in water and stored at -20 °C.

Citraconylation

Protein samples were dissolved at 1 mg/ml in water and adjusted to pH 8.5 with 10 N NaOH. Five aliquots of 100 µl citraconic anhydride (Pierce) were added at 10 minute intervals while stirring the sample. The pH was maintained at 7.5 - 8.5 by the addition of 10N NaOH. The total reaction time was 5 minutes. The sample was desalted on a Sephadex G-25 column (2.0 x 30 cm) in 0.1

M $NH_4 HCO_3$. The eluant was collected in 3.5 ml fractions. The material which eluted at the void volume of the column was clear to light yellow in color and contained most of the citraconylated protein. This material was lyophilized and redissolved in 1.0 ml 0.1M $NH_4 HCO_3$, 1 mM EGTA, and then digested with tryspin for four hours at 37 °C. The reaction was inhibited by the addition of a soybean trypsin inhibitor (Worthington) to a concentration equal to that of the trypsin. This mixture was incubated for 30 minutes at 37 °C, and lyophilized. The digested sample was redissolved in 1 ml of 10% formic acid, incubated four hours at 37 °C, and rotary evaporated. This material was dissolved in 1 ml of 50% acetic acid (J.T. Baker Photrex) and incubated overnight at 4 °C. The solution was rotary evaporated and redissolved in 600 µl of water. The peptides were separated exactly as described for tryptic peptides except an extra 10 minutes was allowed for the elution of non-adsorbed material at 5%B.

Iodination of Proteins

Bovine brain calmodulin, S100α and S100β were iodinated for radioimmunoassays using the procedure of Dorval et al. (182) as modified by Van Eldik and Watterson (86). Briefly, 5-10 µg of protein in 0.15 M sodium phosphate, pH 7.5 was iodinated at 23 °C for 25 seconds using 4 µg of Chloramine-T and 1 mCi of Na ^{125}I (Amersham, 100mCi/ml, carrier free). For S100β the reaction time was extended to 5 minutes. The reaction mixture was immediately transferred to a column (1x15 cm) of Sephadex G-10 (Pharmacia) equilibrated in 0.15 M sodium phosphate pH 7.5. Aliquots of iodinated protein were stored at -20 °C. A sample of the iodinated protein was routinely subjected to electrophoresis on polyacylamide gels in the presence of sodium dodecyl sulfate. Autoradiograms of these gels showed that the iodinated protein comigrated

with unlabeled protein. Bovine brain and chicken gizzard calmodulin were iodinated according to the method of Richman and Klee (180) or as modified by Marshak et al. (113) for use in phenothiazine-Sepharose binding assays and gel overlay proedures (98). Calmodulin (5 μg) was incubated at room temperature in a mixture containing 0.3 μg lactoperoxidase (Calbiochem), hydrogen peroxide (final dilution, 1:5000 of 30%, v/v), 1 mCi Na ^{125}I (Amersham, 100 mCi/ml, carrier free), 0.4 mM $CaCl_2$, and 0.15 M sodium phosphate, pH 7.5. Following incubation for 14 minutes, the free Na ^{125}I was removed by gel filtration on a column (1 x 15 cm) of Sephadex G-10 equilibrated in 50 mM Tris-HCl, pH 7.4. The iodinated protein was collected, stored, and monitored as described above.

Production of Antisera

Antisera were produced in New Zealand white female rabbits using procedures described by Van Eldik and Watterson (86). Performic acid-oxidized S100α and S100β were injected according to the following protocol: 0.4 mg in complete Freund's adjuvant (Difco) on day 1 followed by 0.1 mg in incomplete Freund's adjuvant (Difco) on days 7, 9, 13, 15, and 17. The first serum was obtained from blood collected on day 27. Thereafter, rabbits were given booster injections of 0.1 mg antigen in incomplete Freund's adjuvant every two weeks. After several weeks, booster injections were given every 4-6 weeks as recommended by Van Eldik and Watterson (86). Unoxidized S100β was injected according to a similar protocol, but the initial injection contained 1 mg of protein antigen, and the booster injections contained 0.5 mg of S100β per rabbit.

Peptide ME13B was synthesized as described below, removed from the support using hydrogen fluoride (183), and purified by reverse phase high per-

formance liquid chromatography. The composition of the peptide was verified by amino acid analysis. The peptide was crosslinked to chicken ovalbumin using glutaraldehyde according to the procedure of Audibert et al. (184). The peptide (1430 nmol) was dissolved in 2.71 ml of 0.1 M sodium bicarbonate, and 2.44 mg of chicken ovalbumin in 0.5 ml of 0.1 M sodium bicarbonate was added. This mixture contained a 1.2 fold molar excess of free amino groups on the peptide over the carrier protein. The solution was stirred for 60 minutes at room temperature, and 0.017 ml of 2.5% glutaraldehyde (v/v) was added. Following incubation at room temperature for 45 minutes in the dark, another aliquot of 0.017 ml 2.5% glutaraldehyde was added to give a final glutaraldehyde concentration of 2.63 mM, and the solution was allowed to stir in the dark for an additional five hours. The mixture was dialyzed at 4 °C against three changes of 100 ml of 0.1 M ammonium bicarbonate for one hour each. The dialysate was collected, lyophilyzed, and hydrolyzed in vacuo in 6 N HCl for 24 hours. Amino acid analysis showed that less than 2% of the ME13B peptide was found in the pooled dialysate. The peptide-ovalbumin solution was further dialyzed for 6.5 hours at 4 °C against two liters of 0.01 M sodium phosphate, pH 7.2, 0.15 M sodium chloride. The conjugate was stored in aliquots at -80 °C.

For injection, an aliquot of the ME13B-ovalbumin conjugate (100 nmol peptide) was emulsified in 1.0 ml of complete or incomplete Freunds adjuvant. The injection protocol was the same as decribed above.

Radioimmunoassays

Radioimmunoassays were performed as described by Van Eldik and Watterson (86). Direct radioimmunoassays were performed in RIA buffer (0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris-HCl, pH 7.6) containing 0.2% (w/v) bovine serum albumin. Reaction mixtures of 0.10 ml contained a fixed amount of ^{125}I- labeled

protein (50,000 cpm; 1 ng), various concentrations of antisera, and enough normal rabbit serum (Cappel Laboratories) to give a final serum volume of 5 to 10 μl in each assay tube. Following overnight incubation at 4 °C, 50 μl of goat anti-rabbit serum (Cappel Laboratories) were added, and the incubation was continued at 4 °C for 6-10 hours. RIA buffer (0.5 ml) was added, and the mixture was centrifuged at 900 x g for 10 minutes. The resulting supernatant was removed, and the radioactivity of the pellet was measured in a γ radiation spectrometer (Packard or Beckman).

Competition radioimmunoassays were performed as above using an amount of antiserum determined to give precipitation of 50% of the radioactivity, as determined by direct radioimmunoassay. Various concentrations of competing antigens were incubated with the antisera overnight at 4 °C. The iodinated antigen was then added (50,000 cpm; 1 ng) and the mixture was incubated overnight at 4 °C. Goat anti-rabbit serum was then added, and the assay mixtures were incubated and processed as described above.

Peptide Synthesis

All the methods used for solid phase peptide synthesis are reviewed by Barany and Merrifield (182). Peptides were synthesized on p-methylbenzhydrylamine-resin (U.S. Biochemicals). The resin consisted of 1% divinylbenzene cross-linked polystyrene beads (100-200 mesh) derivatized to 0.96 mM nitrogen per gram. Boc-amino acids (Peninsula Laboratories) were coupled either directly using dicyclohexylcarbodiimmide (Pierce) or as the symmetric anhydride. Dichloromethane (Fisher) was distilled from sodium carbonate prior to use.

Peptides AE21, FF25, and AF20 were provided by K. Fok and B. Erickson, The Rockefeller University, New York.

Equilibrium Binding of Chlorpromazine

The equilibrium for the binding of chlorpromazine by calmodulin was measured using gel exclusion chromatography as described by Hummel and Dreyer (178). Binding experiments were performed in buffer HD1 [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and various concentrations of 2-chloro-10-(N,N-dimethyl)3-aminopropyl phenothiazine (chlorpromazine), a gift of Dr. Harry Green, Smith Kline and French Laboratories]. Bovine brain calmodulin was dissolved in buffer HD1 at a concentration of 62.5 μM (1.04 mg/ml) as determined by amino acid analysis. The concentration of chlorpromazine was determined by absorbance at 254 nm using ε^{M}=30,000 (179). The calmodulin solution was allowed to equilibrate for at least 30 minutes at room temperature. An aliquot (20-80 μl) of the calmodulin solution was applied to a column (0.5 x 30 cm) of Bio-Gel P6 (100-200 mesh, BioRad) which had been equilibrated previously in buffer HD1 containing the same concentration of chlorpromazine as in the calmodulin solution. The column was eluted with buffer HD1 at a flow rate of 0.3 ml/min using a Waters 6000A pump, and the absorbance of the effluent was monitored at 254 nm using either a Waters 480 or 441 detector. Absorbance measurements were transmitted to a Hewlett Packard 1000 computer using a Hewlett Packard analog/digital converter. The areas of peaks and troughs in the chromatogram were integrated using Hewlett Packard 3356 Laboratory Automation System software.

Standard solutions of chlorpromazine in buffer HD1 for calibration contained 0.01 and 0.1 mM chlorpromazine above the concentration of chlorpromazine in the buffer. Concentrations of drug were determined spectrophotmetrically as described above.

Internal calibration of the binding of chlorpromazine to calmodulin was

performed as described by Hummel and Dreyer (178). An aliquot of the calmodulin solution was diluted with various amounts of standard chlorpromazine solutions to give the desired excess amount of drug. This solution was equilibrated for at least 30 minutes at room temperature and filtered on the BioGel P6 column as described above.

Binding experiments were performed exactly as described above using buffer HD2 containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA and various concentrations of chlorpromazine.

Phenothiazine-Sepharose Preparation

Phenothiazine-Sepharose conjugates were prepared using 2-chloro-10-(3 amino propyl) phenothiazine (a gift of Dr. Albert Manian, National Institutes of Health) according to procedures described by Van Eldik et al. (53). Cyanogen bromide activated Sepharose 4B (Pharmacia) was swelled overnight in 1 mM HCl at 4 °C and washed at room temperature with 1 mM HCl, water, and 0.2 M $NaHCO_3$ to pH ~8. The phenothiazine derivative was dissolved at 50 mg/ml in 50% ethanol, pH 6. The drug solution was added dropwise to a slurry of activated Sepharose 4B (1:1, v/v in 0.2 M $NaHCO_3$) while stirring slowly at a ratio of 100 mg drug/10 g dry Sepharose 4B. The reaction proceeded with shaking for 2 hours at room temperature in a covered vessel. The resin was washed as before, and left as a 1:1 (v/v) slurry in 0.2 M $NaHCO_3$. To the slurry was added an equal volume of 0.5 M ethanolamine, and the mixture was incubated with agitation for 35 minutes at room temperature. The derivatized Sepharose was washed as above and stored as a 1:1 (v/v) slurry in buffer E (10 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 1 mM EGTA, 1 mM $MgCl_2$). The amount of 2-chloro-10-(3-aminopropyl) phenothiazine coupled to the resin was estimated spectrophotometrically by measuring the difference in optical absorbance at

308 nm between settled resin and settled, unmodified Sepharose 4B. Assuming an extinction coefficient of 4500 (179), the concentration of coupled phenothiazine was 3 mM.

Phenothiazine-Sepharose Binding Assays

Binding experiments using phenothiazine-Sepharose conjugates were performed according to the procedure described by Marshak et al. (113). In direct binding experiments, 4-18 ng of ^{125}I-labeled chicken gizzard or bovine brain calmodulin (180) were incubated for 60 minutes at room temperature with various amounts of phenothiazine-Sepharose conjugates and unmodified Sepharose 4B. Each mixture contained 50 µl of packed resin, 10µl of iodinated calmodulin and 50µl of buffer 1 (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 2 mM $CaCl_2$, 5 mg/ml bovine serum albumin). Following incubation, the resin was washed once with 1.0 ml of buffer 1, three times with 1.0 ml each of buffer 2 (buffer 1 containing 0.2 M NaCl) and three times each with buffer 3 (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 2 mM EGTA, 0.2 M NaCl, 5 mg/ml bovine serum albumin). The washes with buffers 1 and 2 (unbound material) and buffer 3 (bound material) were pooled separately and the amount of isotope was measured in a γ radiation spectrometer (Packard model 578 or Beckman Gamma 7000).

The design of competitive inhibition assays was similar to that used in competition radioimmunoassays. Each assay mixture contained 2.5 µl of packed phenothiazine resin, 47.5 µl of Sepharose 4B, 50 µl of buffer 1, and 10µl of unlabeled protein diluted in buffer 1. The reaction mixtures were incubated at room temperature for 60 minutes. Subsequent incubation with iodinated calmodulin and buffer washes were done exactly as in binding experiments.

Cyclic Nucleotide Phosphodiesterase Assays

Assays for cyclic nucleotide phosphodiesterase were performed as described by Watterson *et al.* (181). Some assays were done using buffer HD1 (see above) with 0.4 mM $MnCl_2$, at room temperature (22-24 °C) in order to duplicate the conditions used for equilibrium drug binding experiments. For measurements of drug inhibition of phosphodiesterase activity, the appropriate amount of drug was incubated for ten minutes at room temperature with calmodulin. The cAMP substrate was added, and the reaction was initialized by the addition of the enzyme preparation. In all cases, the amount of AMP produced was measured within the linear range of incubation time and enzyme concentration (181).

Gel Overlay Experiments

Gel overlay experiments were performed using the procedure described by Carlin *et al.* (97) as modified by Burgess *et al.* (98). Following electrophoresis, polyacrylamide gels were washed in 25% 2-propanol, 10% acetic acid to remove detergent. The proteins were renatured in the gel matrix by subsequent washes in overlay buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl) and overlay buffer containing 0.2% bovine serum albumin (Sigma). The gels were then incubated overnight in overlay buffer containing 4×10^6 cpm of radioiodinated calmodulin in the presence of 1 mM calcium chloride, 4 mM EDTA, or 1 mM calcium chloride and chlorpromazine. The unbound radioactivity was removed with subsequent washes, and the gels were stained and destained as described above. The dried gels were exposed to Cronex film (DuPont) at -80 °C using an intensifying screen.

Other Materials

All chemicals were ACS reagent grade from Fisher. Water was from a Darco water sytem (Durham, North Carolina). Vitamin D-dependent calcium binding proteins were gifts of S. Christakos, carp parvalbumin was a gift of R. Kretsinger, and phosphorylase kinase was a gift of T. Chrisman. Myosin light chain kinase, rabbit skeletal muscle troponin C, and subcellular fractions of chicken embryo fibroblasts were provided by L. Van Eldik and W. Burgess.

RESULTS

Isolation and Characterization of S100α and S100β

Calmodulin, S100α and S100β were isolated from bovine brains using a combination of ammonium sulfate fractionation, anion exchange chromatography, affinity-based adsorption chromatography, and reverse phase high performance liquid chromatography. The initial homogenization and ammonium sulfate fractionation steps were based on procedures previously described (36,176). Anion exchange chromatography was performed on diethylaminoethyl-Sephadex A-50 at pH 7.5 to obtain partial separation of S100 and calmodulin as originally described by Watterson et al. (36). Figure 5A shows the Coomassie blue staining pattern of fractions from the ion exchange chromatographic step which have been separated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. The separation of S100 and calmodulin by ion exchange chromatography is highly dependent on pH. At pH 8.0 the S100 and calmodulin elute together from the column, while at pH 7.1, neither protein is retained in the presence of 0.2 M sodium chloride. Subsequent chromatography of the S100 and calmodulin pools on phenothiazine-Sepharose 4B removed high molecular weight contaminants as shown in Figure 5B. Separation of S100α and S100β was achieved by chromatogrphy on μBondapak phenyl resin using step elutions with increasing amounts of acetonitrile in the mobile phase. This procedure also separated calmodulin from S100α and S100β. Figure 6 shows the chromatogram obtained when the absorbance of the effluent from a preparation of S100α and S100β is monitored at 235 nm.

The ultraviolet absorption spectra of S100α and S100β are shown in

Figure 7. Based on these spectra, S100α has a single residue of tryptophan which produces the large absorbance maximum at 278 nm. S100β contains no tryptophan, and shows a distinct phenylalanine fine spectrum with four relative absorbance maxima between 250 and 270 nm. The amino acid compositions of S100α and S100β are shown in Table 1. The phenylalanine:tyrosine ratio of 7:1 is consistent with the spectrum of S100β. The amino acid compositions of S100α and S100β shown in Table 1 are indistinguishable from those calculated from the amino acid sequences reported for S100α and S100β by Isobe and Okuyama (33-34). It is not clear why slightly low valine values were observed for both S100α and S100β (Table 1). Isobe and Okuyama (33-34) also obtained low valine values for hydrolysates of S100α and S100β. Since S100α contains no arginine and S100β contains no tryptophan, these amino acids could be used to evaluate the separation of S100α and S100β. In each case these values were less than 0.1 mole of amino acid per mole of protein. S100α and S100β were alkylated using ^{14}C-labeled iodoacetic acid in denaturing conditions. This procedure led to the incorporation of 0.37 moles of ^{14}C per mole of S100α and 0.54 moles of ^{14}C per mole of S100β. These values are consistent with the relative amounts of cysteine in each protein.

In order to further characterize the S100β isolated from bovine brain, the protein was subjected to digestions with cyanogen bromide or trypsin, and the resulting peptides were isolated and characterized. Following alkylation, S100β was digested with cyanogen bromide, and the peptide mixture was fractionated by reverse phase high performance liquid chromatography. The separation of cyanogen bromide peptides of alkylated S100β is shown in Figure 8A. Four fractions of the effluent were collected corresponding to peaks of absorbance at 215 nm and were found to contain peptides. The amino acid compositions and recoveries of these peptides, shown in Table 2, account for the

entire S100β molecule. Equal amounts (5.5 nmols) of radioactivity were recoverd in two of the peptides, corresponding to peptides 58-74 and 80-91, each of which contains a single residue of cysteine (34).

Tryptic digests of S100β and performic acid oxidized S100β were fractionated by reverse phase high performance liquid chromatography. Digests of the unmodified S100β contained large amounts of undigested protein, while those of oxidized S100β contained no detectable undigested protein. The aborbance of the effluent at 215 nm is shown in Figure 8B. Seven pools containing peptides were recovered from this separation, and the amino acid compositions and recoveries of these peptides are shown in Table 2. These peptides account for the entire S100β molecule. An assortment of short peptides (fraction numbers 1 and 2) were derived from residues 24-34 which include five lysine residues. A large peptide containing residues 34-91 was recovered, indicating that lysine 48 and lysine 54 were relatively insensitive to digestion with trypsin. These results are similar to those reported by Isobe and Okuyama (34).

Limited amino acid sequence analysis of peptides from cyanogen bromide and tryptic digests of S100β were performed using automated Edman degradation. The amino terminal sequences of cyanogen bromide peptides 80-91 and 58-74 and of tryptic peptide 6-20 were consistent with the sequences reported by Isobe and Okuyama (34). A complete summary of the amino acid sequence data for S100β peptides appears in the Appendix.

S100 proteins were isolated from human and rat brains using affinity-based adsorption chromatography on phenothiazine-Sepharose. Human brain S100 proteins were isolated from crude S100 fraction prepared according to Watterson et al. (36). Following chromatography on phenothiazine-Sepharose, the S100 pool was fractionated on μBondapak phenyl as described for bovine

S100. Two peaks of ultraviolet absorbing material eluted from the column at 36% acetonitrile, and one peak eluted at 40% acetonitrile in the mobile phase. The amino acid compositions of each of these pools is shown in Table 3. Each of these species of human S100 was tested for immunoreactivity with antisera raised against performic acid oxidized bovine S100α. As shown in Figure 9, all three species of S100 reacted to varying degrees with this antiserum. S100 protein was also isolated from rat brains exactly as described for bovine brains. Chromatography on μBondapak phenyl revealed two peaks of absorbance which eluted at 34% acetonitrile. Two fractions of the effluent were collected and the amino acid compositions of these fractions are shown in Table 3. These amino acid compositions are similar to but distinct from those of the bovine or human proteins.

Immunological Studies of S100α and S100β

In order to develop immunological tools for purified S100α and S100β, various immunization procedures were used to prepare antisera in rabbits. Performic acid oxidized S100α and S100β were injected subcutaneously in the backs of rabbits using the protocol described in Materials and Methods. This protocol has been shown to generate antisera against calmodulin in a reproducible fashion (86). The responses of rabbits to immunization with performic acid oxidized S100β are shown in Figure 10. In direct radioimmunoassays, consecutive sera from these rabbits bound increasing amounts of ^{125}I-S100β. The responses of rabbits to immunizations with performic acid oxidized S100α are shown in Figure 11. Consecutive sera collected during the first 70 days following the initial injections of these rabbits bound increasing amounts of ^{125}I-S100α antigen in direct radioimmunoassays. The amount of antigen bound to sera collected after day 70 was lower than that for

earlier sera. Sera collected after day 90 again showed an increasing ability to bind antigen, as shown in Figure 11.

In competitive inhibition assays, antisera from rabbits injected with performic acid oxidized S100β did not react with bovine brain calmodulin or rabbit skeletal muscle troponin C, as shown in Figure 12. The amount of S100β necessary to inhibit 50% of the binding of ^{125}I-S100β to the first serum from rabbits injected with performic acid oxidized S100β was fifty-fold lower than that amount of S100α. As shown in Figure 12, however, the fifth serum showed no discrimination between the two proteins. In contrast, antisera from rabbits injected with performic acid oxidized S100α showed an approximate 800-1000 fold specificity for S100α over S100β, as shown in Figure 13. This specificity was stable for sera collected over a period of several months. It is not clear whether the 0.1-0.2% reaction of these antisera with S100β is due to contaminating S100β or to a small population of antibody molecules which do not discriminate between S100α and S100β.

Two rabbits were injected with unoxidized S100β according to protocols of Watterson et al. (185). Antisera from these rabbits showed a high reactivity with ^{125}I-S100β in direct radioimmunoassays as shown in Figure 14. However, these antisera did not discriminate between S100α and S100β in competitive inhibition radioimmunoassays (Figure 15).

In order to test the feasibility of generating a site-directed antiserum for S100β, we synthesized the tridecapeptide ME13B: Met(O)-Ala-Phe-Val-Ala-Met(O)-Ile-Thr-Thr-Ala-Cys(ACM)-His-Glu-NH_2 . This peptide encompasses residues 74-86 of S100β, a region which contains eight amino acid sequence differences from S100α. The synthetic peptide ME13B was crosslinked to chicken ovalbumin using glutaraldehyde according to the method described by Audibert et al. (184). The ME13B-ovalbumin conjugate was injected sub-

cutaneously according to the schedule shown in Figure 16. Sera from both of these rabbits show increasing ability to bind ^{125}I-S100β, as illustrated in Figure 16. These results suggested that antisera reacting with S100β could be produced using a synthetic peptide immunogen. Further studies of synthetic immunogens for S100β have been initiated to develop a library of site-directed antisera.

Phenothiazine Binding to Calcium Modulated Proteins

Phenothiazine-Sepharose 4B resin was used to rapidly screen proteins for interactions with phenothiazines. Bovine brain calmodulin or chicken gizzard calmodulin which was iodinated according to the procedure of Richman and Klee (180), bound to phenothiazine-Sepharose in calcium-containing buffer and was eluted with buffer containing chelator. The amount of calmodulin bound increased linearly with the amount of phenothiazine-Sepharose resin, reaching saturation at approximately 60-70% of the total radioactivity, as shown in Figure 17. The binding of ^{125}I-calmodulin to phenothiazine-Sepharose increased over an incubation period of 0-30 minutes and was unchanged after 45 minutes for at least 4 hours. All incubations were performed for 60 minutes at room temperature. The binding of ^{125}I-calmodulin to phenothiazine-Sepharose was identical at pH 7.0, 7.4 and 8.0.

Because iodinated calmodulin was a useful tool in radioimmunoassays and immobilized phenothiazine binding assays, we investigated the site of iodination by lactoperoxidase and by chloramine T in the absence and presence of calcium. Bovine brain calmodulin contains two tyrosines, at position 99 in the third domain and at position 138 in the fourth domain. Bovine brain calmodulin was iodinated according to a modification of the method of Richman and Klee (180) using lactoperoxidase either in the presence of $CaCl_2$ or EGTA. In

addition, calmodulin was iodinated using a chloramine T procedure (182) according to the protocol of Van Eldik and Watterson (176). Following removal of the free Na ^{125}I by gel filtration, each of the iodinated proteins was digested with trypsin in a mixture with 100μg unlabeled bovine brain calmodulin. The tryptic peptides were separated by chromatography on Whatman ODS-3 resin according to methods described (176). The effluents were collected in 2 ml fractions, and the amount of radioactivity in each fraction was measured. Tryptic peptide standards were iodinated using the chloramine T procedure (182), and the retention times of the iodinated standard peptides were measured in separate experiments under chromatographic conditions identical to those for the tryptic digests. According to the peptide nomenclature of Watterson et al. (32), peptide T-5B contains tyrosine 99, and peptide T-1B contains tyrosine 138. Figure 18 shows the distribution of ^{125}I in calmodulin after lactoperoxidase iodination in the presence of calcium. In this case ~80% of the radioactivity recovered was found in a peak which comigrated with iodinated T-5B. The remaining 20% of the radioactivity recovered was found in T-1B. Similar results were obtained for iodination using chloramine T or using lactoperoxidase in the presence of EGTA. The overall recovery was 81-89% of the radioactivity applied to the column.

Various purified calcium modulated proteins were tested for the ability to block iodinated calmodulin binding to phenothiazine-Sepharose. Figure 19A shows the inhibition curves for calmodulin isolated from various animal and plant sources. All of these calmodulins blocked the binding of iodinated vertebrate calmodulin to immobilized phenothiazines. The amount of vertebrate calmodulin required to inhibit 50% of the ^{125}I-calmodulin binding was 33±6 (mean±SEM) pmol, determined in fourteen separate experiments, and that amount for plant calmodulins was 40±8 (mean±SEM) pmol, determined in four ex-

periments. Results for rabbit skeletal muscle troponin C, bovine brain S100β and carp parvalbumin are shown in Figure 19B. Troponin C and S100β blocked ^{125}I-calmodulin binding to the immobilized phenothiazines, but parvalbumin showed no inhibition. In other experiments, bovine brain S100α inhibited in a manner similar to S100β, while the chicken and rat vitamin D-dependent calcium binding proteins showed no inhibition. These results indicated that a subclass of calcium modulated proteins interacted with immobilized phenothiazines in the apparent relative order calmodulin>troponin C>S100α≈S100β.

Purified S100β and troponin C were also tested for the ability to interact directly with phenothiazine-Sepharose conjugates. The results of an experiment with bovine brain S100β are shown in Figure 20. S100β (25 μg) adsorbed to a column (0.65 x 3.0 cm) containing phenothiazine-Sepharose conjugates in the presence of calcium and eluted with buffers conaining chelator. The protein was not retained by the column when applied in the presence of buffers containing calcium chelators. Identical results were obtained for rabbit skeletal muscle troponin C. Although these results with immobilized phenothiazines were indicative of drug-protein interactions, the experiments were based on a calcium dependent adsorption to an immobilized matrix.

In order to determine whether the activity of proteins in immobilized phenothiazine binding assays reflected the binding of phenothiazine drugs in solution, binding experiments were performed using the method of Hummel and Dreyer (178). As shown in Figure 21, the protein sample with bound drug eluted as a peak of absorbance at the void volume of the gel filtration column. The trough in Figure 21 corresponded to the depletion of drug from the buffer, caused by drug binding to the protein. The area of this trough represented the

amount of drug bound to the protein sample. In order to calculate the amount of drug represented by the area of the trough, it was necessary to calibrate the response of the detector to known amounts of drug. An example of this external calibration is shown in Figure 22. At each chlorpromazine concentration, the response of the detector was linear with the amount of drug, and the amount of drug corresponding to the area of the trough was calculated using this relationship, as shown in Figure 22. The amount of drug represented by the trough was also calculated by internal calibration. By adding excess drug to the sample of protein, the area of the trough was reduced or converted to a peak, as illustrated in Figure 23. The relationship between the areas of these peaks or troughs and the amount of excess drug added to the sample was linear, as shown in Figure 24. The amount of drug bound to the protein is equal to the amount of excess drug required to bring the area of the trough to zero, shown by the dashed line in Figure 24. The values for the amount of chlorpromazine bound to bovine brain calmodulin calculated by external and internal calibrations differed by <5% below 20 μM drug and <20% at higher drug concentrations.

The binding of chlorpromazine by bovine brain calmodulin was measured over a range (0.5-100 μM) of chlorpromazine concentrations. Using the method of Hummel and Dreyer (178) allowed the measurement of the number of moles of drug bound per mole of protein at thermodynamic equilibrium for each drug concentration. The results of these measurements are shown in Figure 25. In the presence of calcium calmodulin bound 5-6 moles of drug per mole of protein with an apparent dissociation constant of 17-20 μM. In the presence of 4 mM $MgCl_2$ and 1 mM EGTA, calmodulin bound less than 0.5 moles of drug per mole of protein (Figure 25).

Spinach calmodulin and Dictyostelium calmodulin also bound chlor-

promazine under equilibrium conditions, as shown in Table 4. These calmodulins bind up to 4-5 moles of drug per mole of protein. In order to evaluate these data in terms of the comparative structure of the proteins, the amino acid sequence of Dictyostelium calmodulin was investigated. Dictyostelium calmodulin (provided by M. Clarke, Newark, N.J.) was purified as described (51) and subjected to chromatography on a μBondapak phenyl column as described in Materials and Methods. The purified protein was treated with protease from mouse submaxillary gland, and the resulting peptides were separated by reverse phase liquid chromatography as shown in Figure 26. Four peptides were isolated from fractions of the effluent, designated 16,17,18, and 21. The amino acid compositions of these peptides are shown in Table 5. The amino acid sequence of the first 36 residues of peptide 17 were elucidated, and the peptide was found to be homologous to residues 107-148 of other calmodulins, as shown in Table 2. The amino acid sequence determination data are shown in the Appendix.

Other proteins were tested for their ability to bind chlorpromazine under equilibrium conditions. These results are summarized in Figure 27. Rabbit skeletal muscle troponin C bound up to 2 moles of drug per mole of protein at 50 and 100 μM chlorpromazine, while S100α and S100β bound up to 1 mole of drug per mole of protein. Carp parvalbumin bound very little chlorpromazine, even at 100 μM chlorpromazine. This apparent order of binding activities was similar to that observed using the immobilized phenothiazine binding assay: calmodulin>troponin C> S100α≃S100β.

Phenothiazine Binding to Fragments of Calmodulin

Peptides and whole digests of bovine brain calmodulin were tested for their abilities to interact with immobilized phenothiazines. Digestion of

vertebrate calmodulin with trypsin or cyanogen bromide abolished the ability of the protein to block ^{125}I-calmodulin binding to phenothiazine-Sepharose as shown in Figure 28. Tryptic digestion of citraconylated calmodulin produced a mixture of polypeptides which inhibited ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates to the same extent as native protein, as shown in Figure 29. Digests of calmodulin using the protease from mouse submaxillary gland also inhibited ^{125}I-calmodulin binding to immobilized phenothiazines, as shown in Figure 30.

Bovine brain calmodulin was digested with mouse submaxillary gland protease, and individual peptides were isolated by reverse phase liquid chromatography. The amino acid compositions of these peptides are shown in Table 6. Samples of each of these peptides were tested for their activity in immobilized phenothiazine binding assays. Peptide 1-90 reduced the binding of iodinated calmodulin to phenothiazine- Sepharose by 22%. All other peptides showed no detectable activity in this assay. Synthetic peptides whose amino acid sequences covered two regions of calmodulin were tested for their activity in immobilized phenothiazine binding assays. The amino acid sequences of these peptides are listed in Table 7. Peptide FF25 comprises residues 68-93 of the bovine brain calmodulin sequence and peptide AF20 covers residues 73-93. Both are in the interdomain region between domains 2-3. Peptide AE21 encompasses residues 102-123, the interdomain region between domains 3 and 4. As shown in Figure 31, none of these peptides inhibited ^{125}I-calmodulin binding to phenothiazine-Sepharose at a 200 to 500 fold molar excess of peptide. Mixing AE21 and FF25 also did not produce an active species in this assay (Figure 31).

Peptides from the submaxillary gland protease digestion of calmodulin were tested for drug binding using Hummel-Dreyer analysis at 10 and 100 μM

chlorpromazine, and the results are summarized on Table 8. Peptide 1-90 was able to bind chlorpromazine to 5% of the level of the native protein. Smaller peptides displayed even less drug binding activity. Peptide 1-37 bound 2% of the amount of chlorpromazine bound by the intact protein, while peptide 107-148 bound drug at the level of 1% that of the intact protein at the higher drug concentration, 100 μM chlorpromazine.

Relationship between Drug Binding and Protein Binding Activities of Calmodulin

The correlation between phenothiazine binding and protein binding activity of calmodulin was tested in several ways. First, performic acid oxidized calmodulin retained activity in immobilized phenothiazine binding experiments as shown in Figure 32. However, performic acid oxidized calmodulin does not activate phosphodiesterase, although it is not clear if it can inhibit calmodulin activation of phosphodiesterase (185). In equilibrium binding studies, performic acid oxidized calmodulin bound 1.3 moles of chlorpromazine per mole of protein only at 100 μM drug, as shown in Figure 27. These data suggest that performic acid oxidation reduces the affinity of calmodulin for chlorpromazine but does not eliminate its ability to interact with the drug. After digestion with submaxillary gland protease, calmodulin retained drug binding activity (Figure 30), but did not activate phosphodiesterase, as shown in Figure 33.

Second, bovine brain calmodulin was digested with increasing amounts of trypsin for 14 hours, and the digests were measured for phosphodiesterase activator activity and for immobilized phenothiazine binding activity. As shown in Figure 34, immobilized phenothiazine binding is lost after digestion with a smaller amount of trypsin than is phosphodiesterase activator ac-

tivity.

Third, the inhibition of calmodulin stimulated phosphodiesterase by phenothiazines was tested under conditions of temperature, ionic strength, and pH identical to those for equilibrium binding experiments. The results are shown in Figure 35. The concentration of chlorpromazine needed to block 50% of the activator activity was approximately 27 μM.

Fourth, chlorpromazine was included in the incubation of ^{125}I-calmodulin in a gel overlay experiment. The results of this experiment are shown in Figure 36. Samples of chicken gizzard myosin light chain kinase, rabbit skeletal muscle phosphorylase kinase, and microsomal and supernatant subcellular fractions of chicken embryo fibroblasts were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Following electrophoresis, the gels were washed and stained as described in Materials and Methods. ^{125}I-calmodulin was preincubated for thirty minutes at room temperature in buffer containing 0, 10 μM or 100 μM chlorpromazine in the presence of calcium, and these solutions were subsequently incubated with identical polyacrylamide gels. As shown in Figure 36, results for 0 and 10 μM chlorpromazine were indistinguishable, while 100 μM chlorpromazine inhibited most of the calcium dependent binding. Some, but not all, of the proteins in the microsomal fraction which show binding in the presence of EDTA also show binding in 100 μM chlorpromazine.

DISCUSSION

As part of an effort to characterize structural and functional domains on calmodulin and other calcium modulated proteins, the experiments presented in this thesis were designed to investigate the interactions between phenothiazine drugs and calcium modulated proteins. The results of these studies support three general conclusions.

First, a subclass of calcium modulated proteins binds phenothiazines in a calcium dependent manner. Quantitative measurements in solution at thermodynamic equilibrium established the stoichiometry and affinity of drug binding for several structurally related calcium modulated proteins. In addition, immobilized phenothiazines were used to isolate S100α and S100β from bovine brain, and these proteins were characterized structurally and immunologically.

Second, the binding of phenothiazines by calmodulin appears to be a complex phenomenon that involves multiple sites and that requires an extended region of the calmodulin molecule. Chemically modified calmodulin, proteolytic and synthetic fragments of calmodulin, and structural analogs of bovine brain calmodulin had drug binding properties different from the native protein.

Third, within the limitations of the methods employed, phenothiazine binding to calmodulin correlated with inhibition of calmodulin's enzyme activation and protein binding functions. However, several results suggested that drug binding and protein binding activities of calmodulin could be dissociated under certain experimental conditions. The following discussion contains a section on the biochemical and immunological characterization of S100, followed by a section on the three conclusions concerning drug-protein

interactions.

S100

S100 proteins were isolated from cow, rat, and human brain extracts using affinity-based adsorption chromatography on phenothiazine-Sepharose conjugates. Two polypeptide components of bovine brain S100 were separated by preparative reverse phase high performance liquid chromatography. According to ultraviolet absorption spectra, amino acid analysis, peptide maps and partial amino acid sequence analysis, S100α and S100β isolated from bovine brain using immobilized phenothiazines were indistinguishable from the polypeptides isolated by Isobe et al. (33-34). There have been no reports of quantitative recovery of S100α and S100β from bovine brain because no specific functional parameter could be monitored throughout the preparation. Since no function of S100α and S100β has been established, immunochemical methods are the most useful methods for detection of the proteins.

In order to produce antisera against S100α and S100β, we used the procedure of Van Eldik and Watterson (86). This method established the precedent for reproducible production of antisera against calmodulin, and the immunoreactive site on calmodulin for one of these antisera was characterized in detail. Antisera from rabbits injected with performic acid oxidized S100α showed a specificity for S100α over S100β. This specificity was seen in sera collected during the first nine weeks following the initial injection. Rabbits injected with performic acid oxidized S100β initially produced antisera with relative specificity for S100β, but this specificity was reduced over a period of ten weeks. Injections of unoxidized S100β in rabbits produced antisera with a high titer to S100β but with no discrimination between S100α and S100β.

A more recent approach has been to raise antisera against synthetic peptides modeled from regions of the S100β molecule which are dissimilar to S100α. Such antisera might react with a unique region of the native protein. This method of engineering antisera has been used successfully by Watterson et al. (185) for the calmodulin molecule. In our studies, peptide-ovalbumin conjugates have elicited a limited response to S100β in two rabbits. These results suggest the feasibility of producing antisera which react with S100β by using synthetic peptides as immunogens.

Antisera against S100 proteins were originally produced by Levine and Moore (186) using crude bovine S100 coupled to methylated bovine serum albumin. S100 has been characterized as a very poor immunogen (125,186). In contrast, we found that purified S100β is an excellent antigen and produced a high titer antiserum. The difficulty in obtaining sera specific for S100β over S100α may be due to the high structural homology between the two proteins. Haan et al. (187) have reported the production of monoclonal antibodies against S100. However, even these monoclonal antibodies apparently react with both S100α and S100β. Thus, the use of synthetic peptides to produce site directed antisera seems to be a rational approach to generating highly specific antibodies.

Drug-Protein Interactions

The isolation of S100α and S100β using an immobilized phenothiazine led to the finding that certain calcium modulated proteins interact with phenothiazines. Calmodulin, troponin C, S100α and S100β interact with immobilized phenothiazines (113) and bind chlorpromazine in solution. Our studies of parvalbumin and vitamin D-dependent calcium binding proteins indicate that this drug-protein interaction is not a general feature of calcium

modulated proteins. The EF hand calcium binding structure alone cannot account for drug binding activity since parvalbumin (26) and vitamin D-dependent calcium binding protein (30) both contain these structures yet do not bind phenothiazines. Parvalbumin also contains an internal hydrophobic domain (12,26), but this domain is evidently not sufficient for the protein to bind phenothiazines under the conditions of our assays. In contrast, a cationic drug binding domain on bovine serum albumin is accessible to phenothiazine interaction (139,146).

The structural requirements of the drug binding domains on calmodulin were addressed by examining drug binding activities of calmodulin digests and purified peptides. Tryptic and cyanogen bromide digestion destroyed the ability of calmodulin to block ^{125}I-calmodulin binding to phenothiazine-Sepharose. However, digestion of calmodulin with mouse submaxillary gland protease or of citraconylated calmodulin with trypsin resulted in digests which retained the ability to inhibit calmodulin binding to immobilized phenothiazines. These digests showed activities similar to calmodulin on a molar basis. This indirect study implicated regions of calmodulin which are different between complete tryptic digestion and cleavage only at arginine residues. These encompass domain 1 and the interdomain region between domains 2-3. Synthetic peptides in this interdomain region showed no activity in the immobilized drug binding assay. However, these peptides were relatively short and did not encompass a complete structural domain of the calmodulin molecule.

Several large fragments of calmodulin retained the ability to interact with immobilized phenothiazines. One of these (116) contained the carboxy terminal region as judged by immunoreactivity with the site specific antisera of Van Eldik and Watterson (86). Head et al. (188) identified a fragment from

a cyanogen bromide digest of calmodulin which bound to immobilized phenothiazines in the presence of calcium and was eluted with EGTA. That fragment also encompassed part of the caboxy terminal half of the molecule (residues 77-124). However, the adsorption of a fragment of calmodulin to immobilized phenothiazines does not necessarily imply that the fragment has stoichiometric, high affinity drug binding properties in solution. An inherent limitation of both of these studies (116,188) is that the binding affinity was not quantitated by elution with free phenothiazine.

In order to quantitate phenothiazine binding, several large fragments of calmodulin were tested for their ability to bind chlorpromazine in solution under equilibrium conditions. The isolated peptide 1-90 showed some binding to chlorpromazine in solution. Although not stoichiometric, this was the first demonstration that an isolated peptide from calmodulin retained drug binding capacity in solution. However, even peptide 1-90, which included nearly two-thirds of the protein had only 5% of the drug binding activity of the intact protein. These data indicate that chlorpromazine binding is not a simple phenomenon and suggests that high affinity, calcium dependent drug binding resides in an extended region of the calmodulin molecule. For example, an interaction between two halves of the protein may be necessary for high affinity drug binding. The EF hand model (12) does not predict the nature of the interaction between the amino terminal half (domains one and two) and the carboxy terminal half (domains three and four). The x-ray crytallographic structure of calmodulin may reveal these relationships.

Results of several experiments suggested that the phenothiazine drug binding activity and enzyme activator activity of calmodulin were dissociable. Performic acid oxidized calmodulin retained the ability to in-

teract with immobilized phenothiazines but did not activate phosphodiesterase. In solution, performic acid oxidized calmodulin displayed the ability to bind more than 1 mole of chlorpromazine per mole of protein at 100 μM drug. This level of drug binding is less than that of the unmodified protein but appears to be sufficient to inhibit iodinated calmodulin binding to immobilized phenothiazines. Performic acid oxidation quantitatively converts the methionines in calmodulin to methionine sulfone (175). This modification may allow calmodulin to retain some of its secondary structure and to adsorb to phenothiazine-Sepharose conjugates, while abolishing calmodulin's ability to activate phosphodiesterase. In contrast, when calmodulin was digested with increasing amounts of trypsin, immobilized phenothiazine binding was lost with a lower concentration of trypsin than was phosphodiesterase activator activity. This experiment has limitations because it was a comparison of two assays which varied in their sensitivities and in their abilities to discriminate activities. Nevertheless, the data obtained from partial trypsin digestion and from performic acid oxidation suggested that different modifications of calmodulin led to the differential loss of its drug binding and enzyme activator functions.

Equilibrium binding studies of bovine brain calmodulin and chlorpromazine revealed a maximal binding of 5-6 moles of drug per mole of protein in the presence of calcium. In the presence of 1 mM EGTA and 4 mM magnesium, less than one mole of drug bound per mole of protein (Figure 25). Half maximal binding of chlorpromazine occured at 17-20 μM drug in the presence of calcium. Between 10 and 15 μM chlorpromazine, there was a slight inflection in the binding curve, suggesting biphasic drug binding. However, these data are not sufficient to establish two distinct populations of binding sites. It is significant that troponin C from rabbit skeletal muscle bound up to approx-

imately 2 moles of chlorpromazine per mole of protein (Figure 27). Troponin C and calmodulin have 60% sequence homology (32), but the two proteins apparently have different stoichiometries of chlorpromazine binding. Spinach calmodulin may have slightly different affinity for chlorpromazine than the bovine protein (Table 4, Figure 25), but more data are necessary to establish this difference. These results with bovine brain calmodulin and its structural analogs do indicate that chlorpromazine binding to calmodulin is a complex phenomenon involving multiple sites of interaction.

Several explanations that have been raised for other drug-protein interactions (189-191) may apply to the complex calmodulin-phenothiazine interactions. Molecules of drug could bind in a cooperative manner if higher drug concentrations cause an unfolding of the calmodulin structure. Alternatively, additional drug molecules may interact with drugs already bound to the protein. The stacking of drug molecules on calmodulin has been suggested by evidence for drug-drug interactions in nuclear magnetic resonance experiments (192-193), but these measurements were made at millimolar drug concentrations, near the critical micellar concentration of several phenothazines (194). The concentrations of chlorpromazine used in our experiments are at least an order of magnitude below the critical micellar concentration for chlorpromazine in 0.15 M sodium chloride (194). However, this does not exclude the possibility that small aggregates of drug molecules in solution bind to calmodulin. In fact, we observed lower solubility of chlorpromazine at concentrations above 200 μM in the buffers with a total ionic strength above 200 mM.

The complex nature of the chlorpromazine binding to calmodulin is reminiscent of the interactions between serum albumins and dyes. Horowitz and Klotz (189) observed that p-aminophenylmercuric acetate bound to bovine

serum albumin in a biphasic manner at pH 6.0-6.4. Up to 0.56 moles of dye per mole of protein bound in a linear fashion at 0.1 μM free dye. Above 15 μM dye, the moles of dye per mole of protein increased to values above 3 over a narrow range of dye concentrations. The interpretation of these data was similar to our analysis of the calmodulin-phenothiazine interaction. The dye may induce an unfolding of the albumin molecule, or interactions among adjacent dye molecules may thermodynamically favor the binding of additional dye molecules. These sorts of models have also been used to explain the binding of various organic ions to serum albumins (190-191).

The binding of phenothiazines by calmodulin was qualitatively dissimilar to the binding of phenothiazines by glutamate dehydrogenase (145). While the apparent dissociation constants for the binding of phenothiazines to glutamate dehydrogenase were in the micromolar range, the binding of the drugs was not dependent on calcium ions. These dissociation constants were correlated to kinetically-derived constants for enzyme inhibition. Phenothiazines inhibit the activity of glutamate dehydrogenase, and Veronese et al. (145) provided evidence that drug binding was related to the binding of the natural ligands, NADH and GTP, to the enzyme. The possibility remains that phenothiazines mimic the binding of some naturally occurring ligand to calmodulin.

The binding of chlorpromazine to calmodulin correlated with the inhibition of calmodulin function, within the limits of the experimental design. As shown in Figure 35, chlorpromazine blocked the activation of cyclic nucleotide phosphodiesterse by calmodulin. The concentration of chlorpromazine necessary for 50% inhibition of activator activity was 27 μM. The temperature, ionic strength and pH of these measurements were identical to those used for equilibrium drug binding mesurements. Under these conditions,

the value 27 μM is not significantly different from the apparent dissociation constant of 17-20 μM for chlorpromazine binding to calmodulin. However, this correlation does not exclude the possibility that only a population of drug binding sites are linked to functional inhibition.

In the gel overlay experiments shown in Figure 36, 10 μM chlorpromazine did not block the interaction of ^{125}I-calmodulin with binding proteins. In contrast, 100 μM chlorpromazine was sufficient to inhibit almost all of the binding of calmodulin to various proteins. These data indicate that 10 and 100 μM are the limits of the range of effective chlorpromazine concentrations under these conditions. This finding is consistent with an apparent dissociation constant for chlorpromazine binding of 17-20 μM and with an apparent IC_{50} for phosphodiesterase activator inhibition of 27 μM. In overlay inhibition experiments the concentration of drug required for 50% inhibition was not titrated precisely because of the quantal nature of the procedure. In addition, the exact affinity and stoichiometry of chlorpromazine binding to iodinated calmodulin are not known.

The results presented in this thesis have several implications for biological studies. The finding that chlorpromazine binding to calmodulin is a complex phenomenon with multiple sites of interaction suggests that the biological effects of low, non-cytotoxic doses of phenothiazines may not be due to the antagonism of calmodulin action. Levels of phenothiazine drugs sufficient to bind to calmodulin in substoichiometric amounts do not necessarily imply that the function of calmodulin has been altered. In the presence of multiple classes of phenothiazine binding proteins and subcellular structures, the extracellular concentration of drug may not reflect the true intracellular concentration of drug available to calmodulin. The availability of calmodulin in vivo to the drug is also unknown.

The studies presented here provide the necessary foundation for our current attempts to elucidate the molecular basis of specific drug-protein interactions. Our recent approach combines measurements of the equilibrium binding of drugs with the identification of covalent drug-protein adducts (168).

By studying the drug binding properties of calmodulin and related calcium modulated proteins, these investigations may provide a framework for the rational design of new drugs. Of particular interest are compounds that antagonize specific biological effects of calcium. Recently, the family of drugs which act as calcium channel blockers has gained prominence in clinical cardiology (195). However, the selective action of these drugs requires the adjustment of dosage to block only a portion of the calcium ion channels. An alternate approach to the antagonism of calcium action would involve the selective inhibition of specific receptors for calcium ions intracellularly. For example, inhibitors of tissue specific calcium modulated proteins, such as cardiac troponin C, may have fewer systemic effects than calcium channel blockers. Compounds which block the calmodulin activation of some enzymes but not others might be useful in treating pathophysiological states which involve calcium mediated responses.

The design of specific antagonists of calcium modulated proteins will rely on a detailed knowledge of the molecular physiology of these proteins. The studies presented in this thesis add to our knowledge of the molecular basis of the interactions between phenothiazine drugs and calcium modulated proteins.

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TABLE 1: Amino Acid Compositions of S100α and S100β Isolated from Bovine Brain

	S100α		S100β	
	moles per 10,400 g	residues, no. per molecule	moles per 10,507 g	residues, no. per molecule
Aspartic acid	13.2	13	9.9	9
Threonine	3.7	4	3.0	3
Serine	4.1	5	4.5	5
Glutamic acid	14.8	15	19.4	19
Proline	0	0	0	0
Glycine	5.8	6	4.2	4
Alanine	7.4	8	5.2	5
Cysteine[1]	1.3	1	2.4	2
Valine	7.2	8	6.3	7
Methionine	2.0	2	2.8	3
Isoleucine	1.3	1	3.9	4
Leucine	11.2	11	8.3	8
Tyrosine	2.0	2	1.1	1
Phenylalanine	5.1	5	6.9	7
Histidine	2.3	2	4.3	5
Lysine	9.4	9	8.2	8
Tryptophan[2]	0.7	1	<0.01	0
Arginine	<0.06	0	1.3	1

The number of moles was calculated from composition analysis using the molecular weight calculated from the amino acid sequences (33-34). The number of residues per molecule were calculated from the amino acid sequences (33-34).

[1] Determined as cysteic acid after performic acid oxidation
[2] Determined after hydrolysis in methane sulfonic acid

TABLE 2: Amino Acid Compositions[2] of S100β Peptides

	Cyanogen Bromide Digestion of Carboxymethylated S100β				Trypsin Digestion of Performic Acid Oxidized S100β						
	2	3	4	7	1	2	3	4	5	9	14
Aspartic acid			3.6	5.5	1.0	2.0				1.2	7.6
Threonine		1.5	1.0								3.0
Serine			1.3	3.2			1.0	1.0	1.2	1.3	2.2
Glutamic acid		3.1	3.9	12.0	1.0	2.0	1.0	1.1	2.0	1.2	12.1
Proline											
Glycine		0.3	2.6	2.9	0.9	2.9				1.3	2.2
Alanine	2.0	1.1		2.4						2.0	3.0
Cysteine[1]		0.5	0.6								2.3
Valine	1.2			5.2						2.5	3.6
Methionine											N.D.
Isoleucine		1.1		2.9						1.1	2.7
Leucine			1.1	6.8		1.8	1.1	0.9	0.9	1.1	3.8
Tyrosine				1.0						0.7	
Phenylalanine	0.9	2.3	1.9	2.4						0.8	5.4
Histidine		2.0		2.9	1.2					1.0	2.8
Lysine				7.9	1.9	4.6	1.8	1.0	0.9		1.9
Arginine				1.1						0.8	
Recovery (%)	50	32	21	54	48	49	64	45	79	34	46
Peptide Assignment[3]	75-79	80-91	58-74	1-57	21-24 25-26	21-24 27-28	29-33	30-33	1-5	6-20	34-91

[1] Determined as carboxymethyl cysteine in cyanogen bromide fragments and as cysteic acid in trypsin fragments.

[2] Compositions were calculated as molar ratios

[3] Peptide assignments are based on the amino acid sequence of bovine brain S100β (33-34).

N.D. Not Determined

TABLE 3: Amino Acid Compositions of Human and Rat S100 Proteins

	Human			Rat	
	H1	H2	H3	R1	R2
	(moles per 10,300g)			(moles per 10,300g)	
Aspartic acid	12.3	12.6	10.6	10.4	10.7
Threonine	3.6	4.0	3.0	3.9	3.4
Serine	6.1	6.9	4.1	10.8	5.2
Glutamic acid	17.3	19.2	18.8	16.9	17.9
Proline	0	0	0	0	0
Glycine	7.4	5.3	5.1	16.3	5.3
Alanine	6.4	7.2	5.2	5.2	5.0
Cysteine	N.D.	N.D.	N.D.	N.D.	N.D.
Valine	6.5	3.3	6.0	5.4	6.6
Methionine	2.4	2.9	4.1	2.4	2.3
Isoleucine	1.3	1.5	3.4	3.6	3.4
Leucine	7.5	5.9	8.8	6.9	8.4
Tyrosine	1.0	0	1.0	2.3	1.2
Phenylalanine	6.4	6.9	6.2	3.8	7.0
Histidine	3.6	4.4	4.8	2.9	4.8
Lysine	9.1	9.4	7.7	5.8	7.4
Tryptophan[1]	0	0	0	N.D	N.D.
Arginine	1.0	1.1	1.0	2.9	1.3

Calculated from composition analysis using a molecular weight of 10,300.

[1] Determined after hydrolysis in methane sulfonic acid

N.D. Not Determined

TABLE 4: Chlorpromazine Binding to Calmodulins

	Binding Ratio (Moles drug bound/mole protein)		
Chlorpromazine Concentration	9.3 μM	49 μM	100 μM
Spinach Calmodulin	0.96	4.84	4.20
Dictyostelium Calmodulin	2.56	4.01	2.55

TABLE 5: Amino Acid Compositions of *Dictyostelium* Calmodulin Peptides

	16	17	18	21
Aspartic acid	6.4	8.2	4.0	12.4
Threonine	2.1	2.0	4.0	3.6
Serine	1.9	1.5	4.0	2.2
Glutamic acid	6.9	7.6	9.1	11.2
Proline	0	0	0	2.0
Glycine	2.9	3.6	3.7	4.9
Alanine	3.1	1.4	3.1	4.9
Cysteine	N.D.	N.D.	N.D.	N.D.
Valine	2.9	4.3	1.3	2.4
Methionine	2.2	2.9	0	3.0
Isoleucine	1.8	1.6	2.0	3.2
Leucine	1.5	2.8	3.1	4.1
Tyrosine	1.0	1.0	0	0.7
Phenylalanine	1.7	1.0	3.0	3.2
Histidine	0	1.0	0	0
Lysine	0	2.0	3.2	2.1
Arginine	2.6	1.4	1.0	1.9
Recovery (%)	44	61	85	94
Peptide Assignment	75-106	107-148	1-37	38-74 38-106

Compositions were calculated by molar ratios.

Peptide assignments are based on the amino acid sequence of bovine brain calmodulin (32).

N.D. Not Determined

TABLE 6: Amino Acid Compositions of Calmodulin Peptides from Submaxillary Gland Protease Digest

	28	(91-106)*	48	(107-148)*	50	(1-37)*	63	(1-90)*
Aspartic Acid	2.7	3	6.9	7	5.5	4	12.8	13
Threonine	0.1	0	3.6	3	4.8	5	9.0	9
Serine	1.2	1	0	0	2.6	1	3.0	3
Glutamic acid	1.5	1	10.9	9	9.0	7	19.0	17
Proline	0	0	0	0	0	0	N.D.	2
Glycine	2.3	2	3.7	3	5.0	3	6.8	6
Alanine	2.1	2	2.2	2	3.5	3	7.5	7
Valine	1.1	1	4.3	4	2.0	1	2.5	2
Methionine	0.1	0	3.8	4	1.3	1	4.3	5
Isoleucine	1.0	1	2.3	2	2.3	2	5.5	5
Leucine	1.1	1	2.4	2	3.3	3	6.5	6
Tyrosine	1.0	1	1.2	1	0.7	0	0	0
Phenylalanine	1.0	1	1.2	1	2.8	3	6.2	6
Histidine	0.3	0	1.0	1	0.4	0	0	0
Trimethyllysine	0	0	0.9	1	0.4	0	0	0
Lysine	1.0	1	1.0	1	3.0	3	5.0	5
Arginine	0.4	1	0.5	2	1.5	1	1.6	4
Recovery (%)	45		55		15		66	

Compositions of peptides were calculated by molar ratios

* Number of residues per molecule for regions of calmodulin (shown in parentheses) from the amino acid sequence of bovine brain calmodulin.

N.D. Not Determined

TABLE 7: Synthetic Peptides of Calmodulin

```
                    75                  80
AF20  H-Ala-Arg-Lys-Met-Lys-Asp-Thr-Asp-Ser-Glu-

                    85                  90
        Glu-Glu-Ile-Arg-Glu-Ala-Phe-Arg-Val-Phe-NH2


                    70                  75                  80
FF25  H-Phe-Leu-Thr-Met-Met-Ala-Arg-Lys-Met-Lys-Asp-Thr-Asp-

                        85                  90
        Ser-Glu-Glu-Glu-Ile-Arg-Glu-Ala-Phe-Arg-Val-Phe-NH2


                   105                 110
AE21  H-Ala-Glu-Leu-Arg-His-Val-Met-Thr-Asn-Leu-Gly-

                115                 120
        Glu-Lys-Leu-Thr-Asp-Glu-Glu-Val-Asp-Glu-NH2
```

TABLE 8: Chlorpromazine Binding to Fragments of Bovine Brain Calmodulin

Chlorpromazine Concentration	Binding Ratio (Moles drug bound/mole peptide)	
	10 μM	100 μM
Calmodulin (1-148)	2.02	4.10
1-90	0.014	0.201
1-37	0.009	0.067
107-148	0.000	0.049

FIGURE 1: Comparison of the amino acid sequences of calmodulin from four species: bovine brain (32,95), Renilla reniformis (44), spinach (47-49), and Tetrahymena pyriformis (46). The amino acid residues of bovine brain calmodulin are numbered according to Watterson et al. (32). Blank spaces indicate amino acid residues identical to those found in bovine brain calmodulin, and # represents a reported deletion. Positions which have been postulated to be calcium ligands (12) are marked with an asterisk (*), and positions postulated (12) to be on the internal aspect of α-helices are marked with a bar (—). K' represents N $^{\varepsilon}$-trimethyllysine.

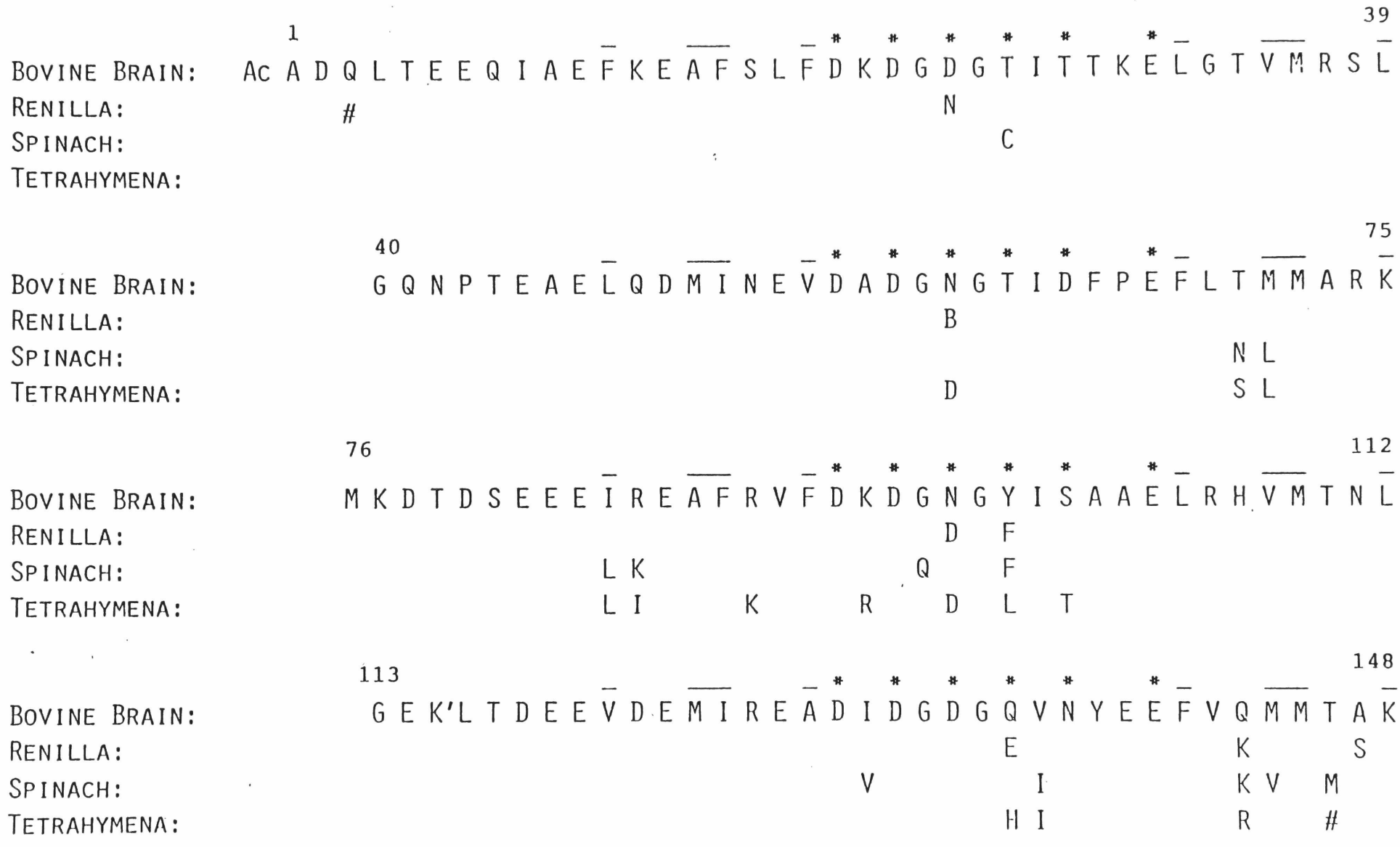

1
39
BOVINE BRAIN: Ac A D Q L T E E Q I A E F K E A F S L F D K D G D G T I T T K E L G T V M R S L
RENILLA: # N
SPINACH: C
TETRAHYMENA:
40
75
BOVINE BRAIN: G Q N P T E A E L Q D M I N E V D A D G N G T I D F P E F L T M M A R K
RENILLA: B
SPINACH: N L
TETRAHYMENA: D S L
76
112
BOVINE BRAIN: M K D T D S E E E I R E A F R V F D K D G N G Y I S A A E L R H V M T N L
RENILLA: D F
SPINACH: L K Q F
TETRAHYMENA: L I K R D L T
113
148
BOVINE BRAIN: G E K'L T D E E V D E M I R E A D I D G D G Q V N Y E E F V Q M M T A K
RENILLA: E K S
SPINACH: V I K V M
TETRAHYMENA: H I R #

FIGURE 2: Comparison of the known amino acid sequence differences in the carboxy terminal domain of calmodulin from Chlamydomonas reinhardtii (50) and Dictyostelium discoideum. The sequence determination for Dictyostelium calmodulin is documented in the Appendix. The symbols and the sequences of other calmodulins are the same as in Figure 1.


107
148
VINE BRAIN: H V M T N L G E K' L T D E E V D E M I R E A D I D G D G Q V N Y E E F V Q M M T A K
NILLA: E K S
TRAHYMENA: H I R #
INACH: V I K V M
LAMYDOMONAS: K S E V
CTYOSTELIUM: S K N L

FIGURE 3: Amino acid sequences of S100α and S100β from bovine brain (33-34). The amino acid residues of S100α are numbered and the sequence of S100β is aligned according to Isobe and Okuyama (33). Residues 1-88 of S100β are aligned with residues 2-89 of S100α, and residues 89-91 of S100β are aligned with residues 91-93 of S100α. The symbol # corresponds to a gap inserted in the sequence of S100β for the purpose of alignment. The asterisks (*) represent amino acid residues postulated (12,30,33-34) to be calcium ligands, and bars (—) indicate amino acid residues postulated (12,30,33-34) to be on the internal aspect of the α-helices. The letter B denotes an unidentified blocking group on the amino terminus of S100α.

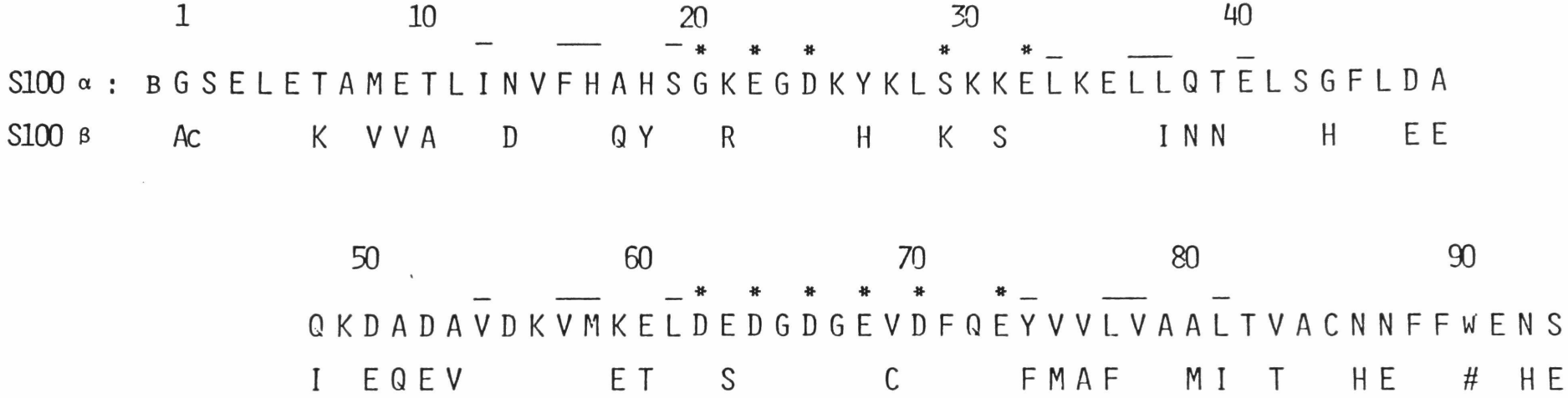

1 10 20 30 40
S100 α : B G S E L E T A M E T L I N V F H A H S G K E G D K Y K L S K K E L K E L L Q T E L S G F L D A
S100 β Ac K V V A D Q Y R H K S I N N H E E
50 60 70 80 90
Q K D A D A V D K V M K E L D E D G D G E V D F Q E Y V V L V A A L T V A C N N F F W E N S
I E Q E V E T S C F M A F M I T H E # H E

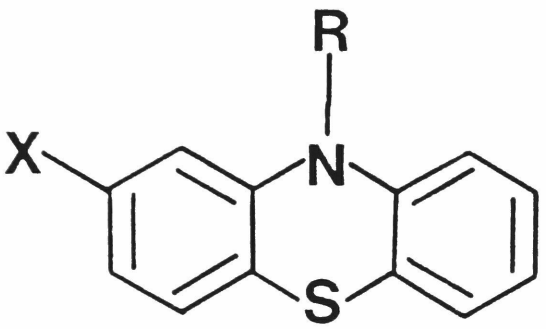
FIGURE 4: Structure of phenothiazine drugs.

CAPP: 2-chloro-10-(3-aminopropyl)phenothiazine

CPZ: chlorpromazine

TFP: trifluoperazine

Phenothiazines



	X	R
CAPP	Cl	$(CH_2)_3NH_2$
CPZ	Cl	$(CH_2)_3N(CH_3)_2$
TFP	CF_3	$(CH_2)_3$ 

FIGURE 5: Polyacrylamide gel electrophoresis of fractions from anion exchange (A) and affinity-based adsorption (B) chromatography of a preparation of bovine brain.

A. Aliquots (3.5 μl) of fractions from chromatography of bovine brain extract on diethylaminoethyl-Sephadex A-50 were analyzed by electrophoresis on a 15% (w/v) polyacrylamide gel in the presence of 0.1% (w/v) sodium dodecyl sulfate and 1 mM EGTA. The protein retained by the resin were eluted with a gradient (2 liters) of 0.2 M to 0.7 M NaCl as described in the text, and 200 fractions of 10 ml were collected. The Coomassie blue staining pattern is shown for several fractions, with the fraction number indicated. The lane marked S contains a mixture of bovine brain calmodulin and S100β for standards. The arrows mark the positions of the standards: calmodulin (CaM) and S100β (S100). Two pools of fractions were collected containing S100 (fractions 45-75) and calmodulin (fractions 76-100).

B. The pool of fractions containing S100 from the anion exchange chromatography described above was subjected to chromatography on phenothiazine-Sepharose conjugates as described in the text. Aliquots of material eluted by various buffers from the phenothiazine-Sepharose conjugates were analyzed by electrophoresis on a 15% (w/v) polyacrylamide gel in the presence of 0.1% (w/v) sodium dodecyl sulfate and 1 mM EGTA. The Coomassie blue staining pattern is shown: lanes 1,8, chicken gizzard calmodulin; lanes 2,7 molecular weight markers (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400); lane 3, elution with calcium buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM 2-mercaptoethanol); lane 4, elution with calcium buffer containing 0.4 M NaCl; lane 5, elution with chelator buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 2 mM EGTA) containing 0.4 M NaCl; lane 6, elution with chelator buffer containing 8 M urea.

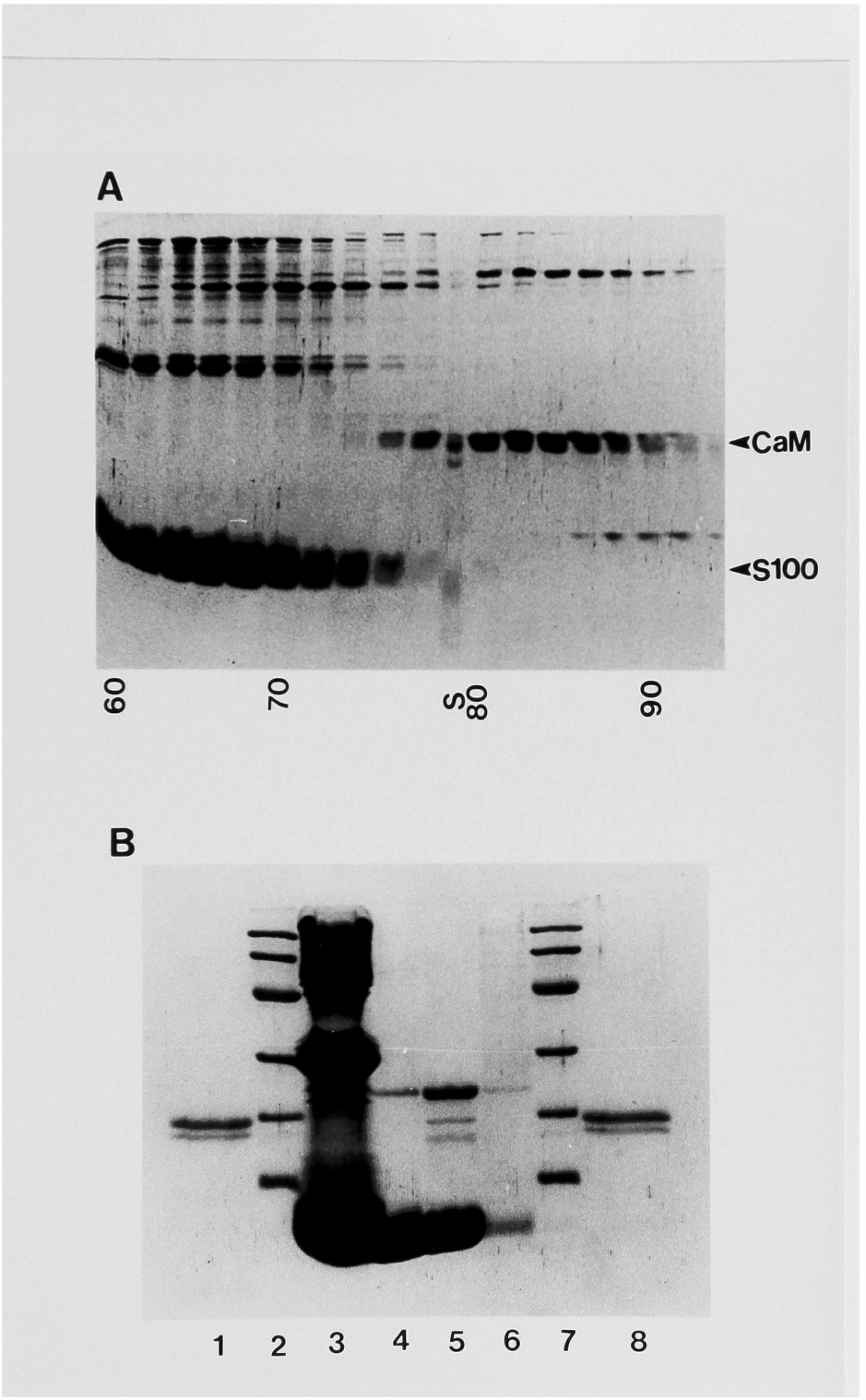

A
CaM
S100
60
70
S
80
90
B
1
2
3
4
5
6
7
8

FIGURE 6: Separation of bovine brain S100α and S100β by reverse phase chromatography on μBondapak phenyl resin. The material eluted from phenothiazine-Sepharose conjugates with EGTA and NaCl (lane 5, Figure 5B), was subjected to chromatography on a column (0.78 x 30 cm) of μBondapak phenyl resin equilibrated in 80% (v/v) 0.01 M potassium phosphate, pH 6.1, 0.5 mM EGTA, and 20% (v/v) acetonitrile. Calmodulin was eluted by changing the solvent compostion to 28% (v/v), S100α eluted with 32% (v/v) acetonitrile, and S100β eluted with 36% (v/v) acetonitrile. The ordinate shows the absorbance of the effluent at 235 nm as the amplitude of the detector response in millivolts. The abscissa shows the retention time (RT) in minutes. The retention times of the peaks are marked. Peaks corresponding to proteins are indicated: CaM, calmodulin; S100α; and S100β. The peaks occuring at 9.90 and 10.91 contained residual tris(hydroxymethyl) aminomethane, excess EGTA, and ammonia but no detectable amino acids by amino acid analysis.

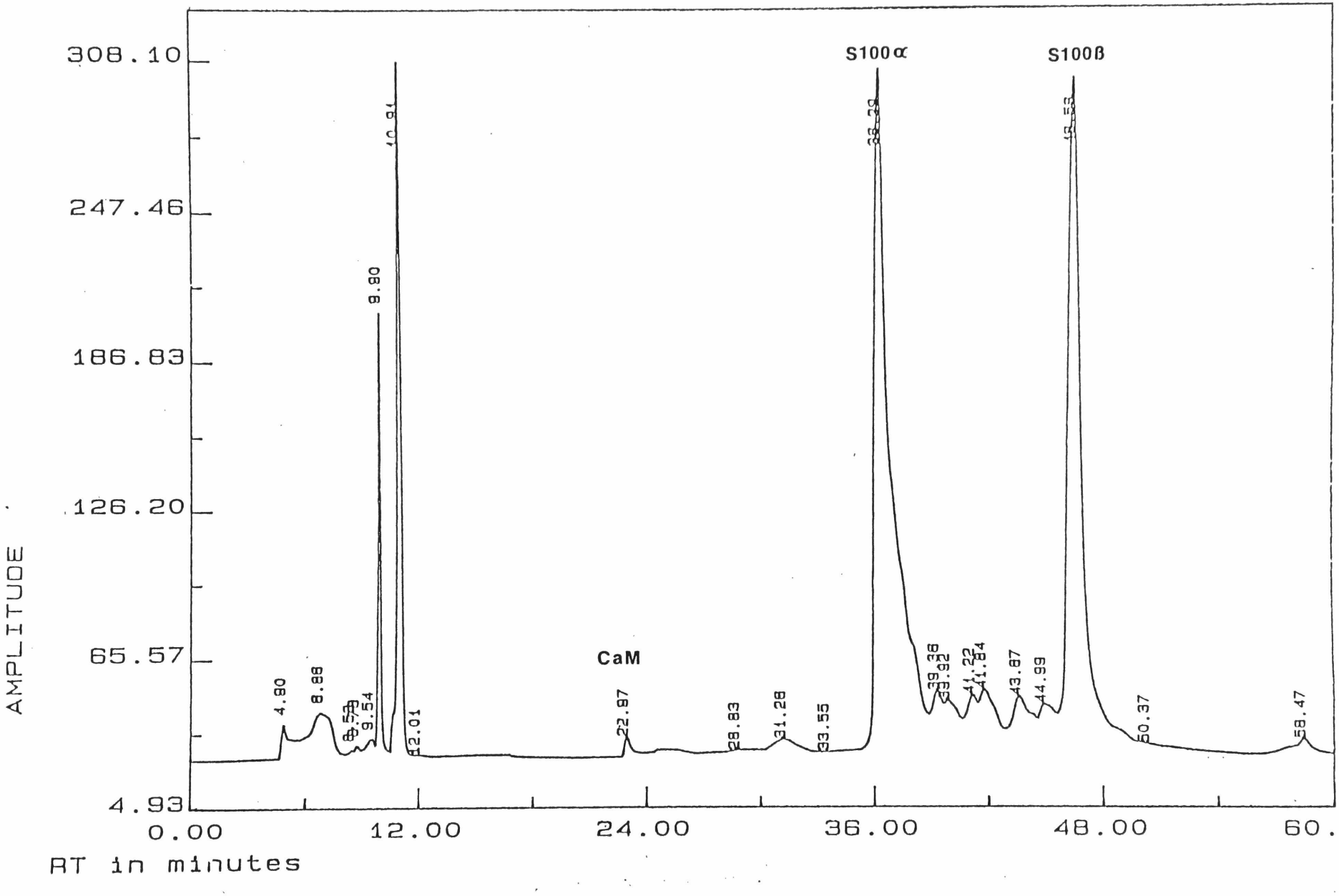
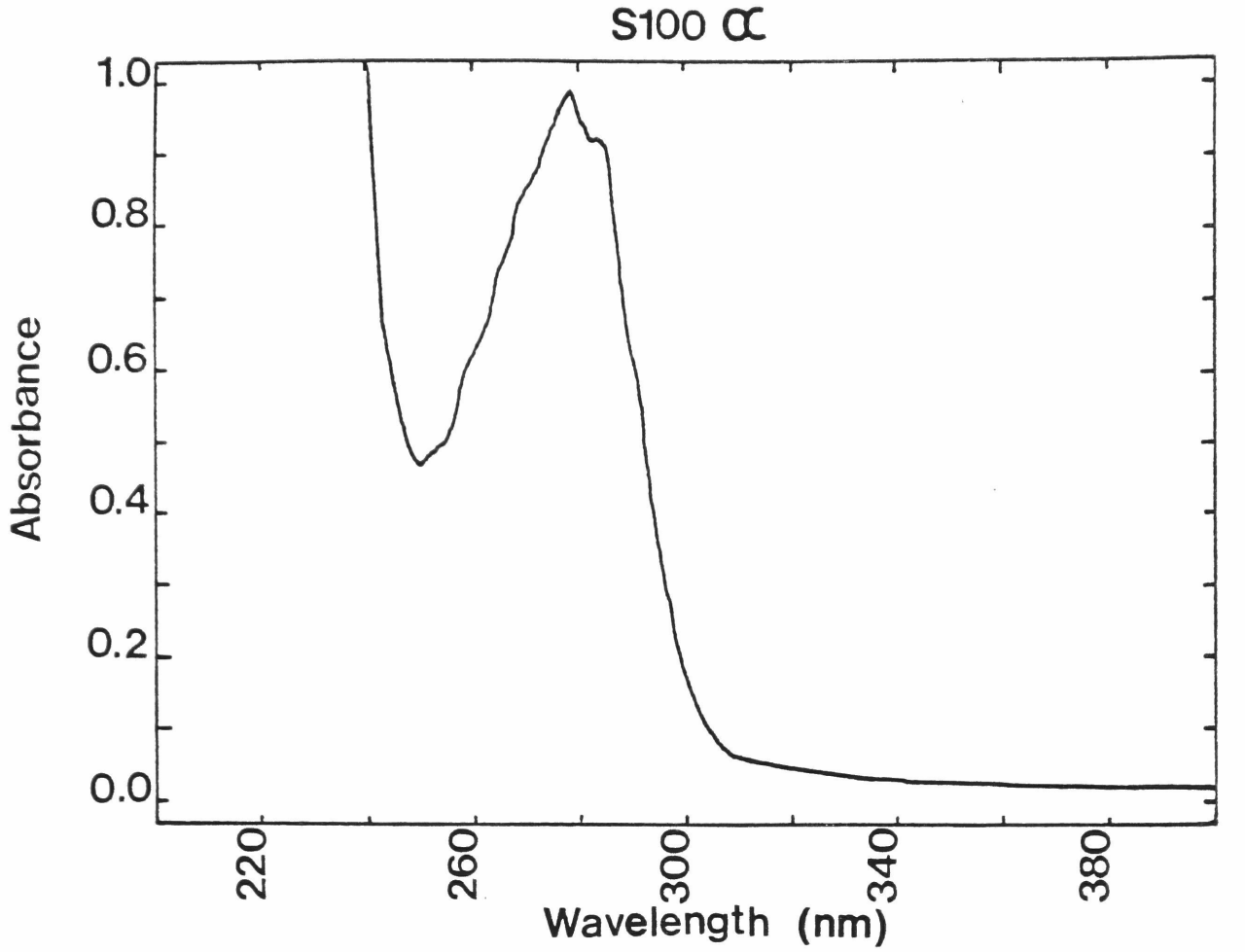

308.10
247.46
186.83
126.20
65.57
4.93
AMPLITUDE
0.00
12.00
24.00
36.00
48.00
60.
RT in minutes
S100α
S100β
CaM
4.90
8.88
9.54
9.80
10.91
12.01
22.97
28.83
31.28
33.55
38.38
39.92
41.22
41.84
43.87
44.99
50.37
58.47

FIGURE 7: Ultraviolet absorption spectra of S100α (top) and S100β (bottom). Proteins were dissolved in 0.1 M ammonium bicarbonate at 1.5 mg/ml.


S100 α
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
220
260
300
340
380
Wavelength (nm)

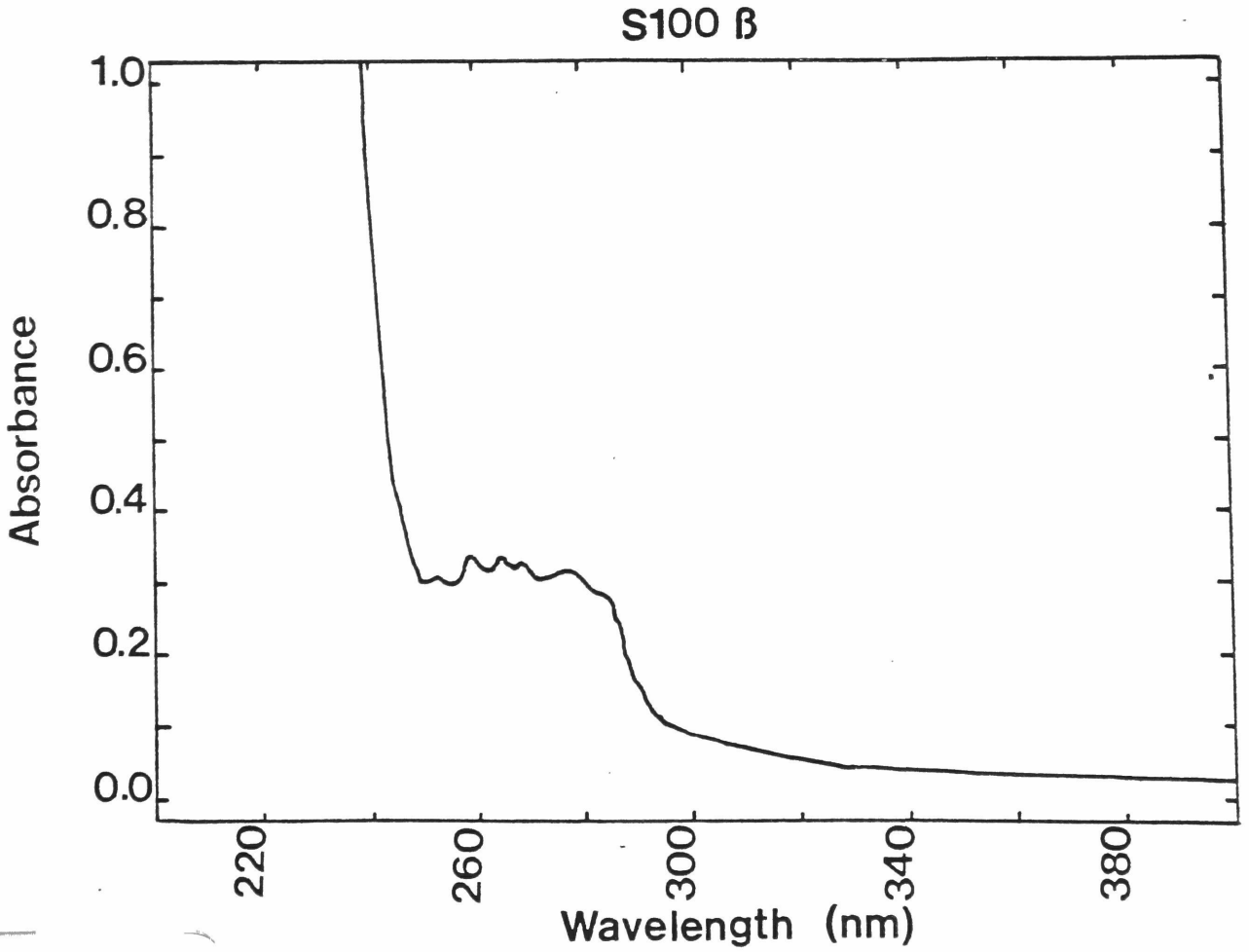
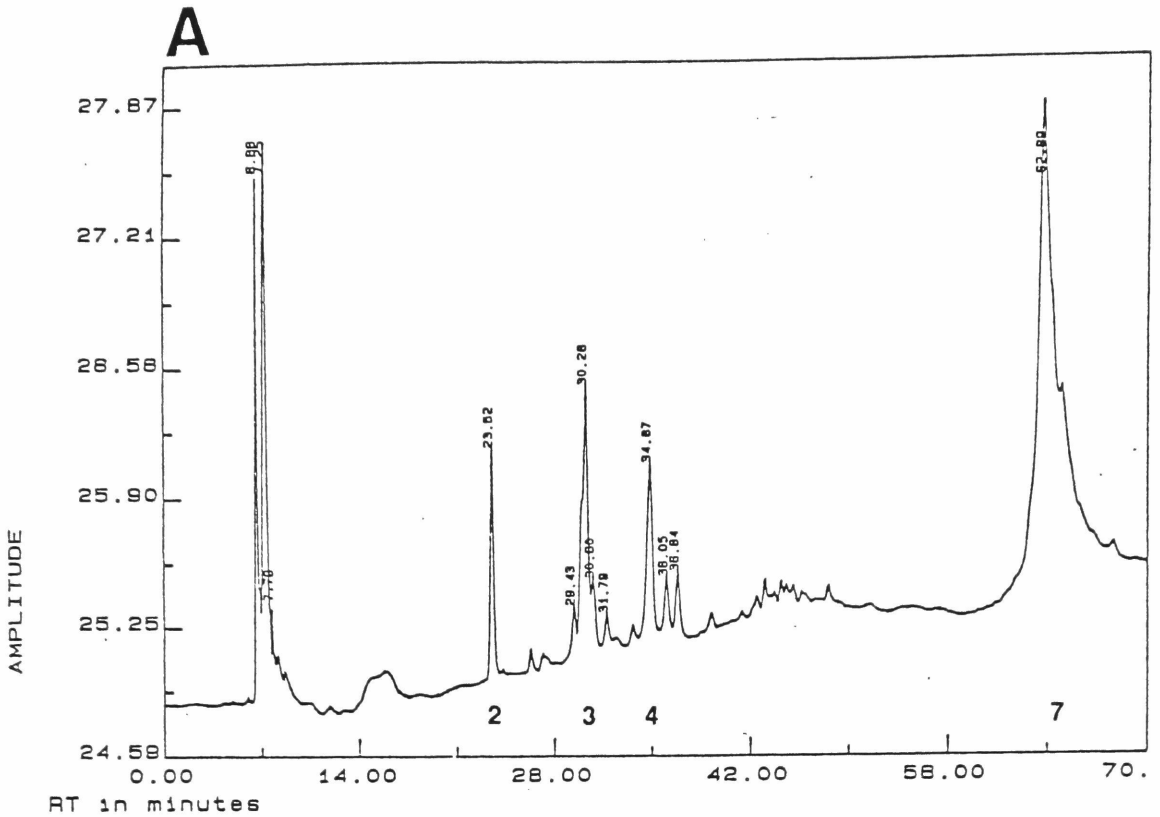

S100 β
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
220
260
300
340
380
Wavelength (nm)

FIGURE 8: Separation of S100β peptides by reverse phase chromatography. Carboxymethylated S100β was digested with cyanogen bromide and performic acid oxidized S100β was digested with trypsin as described in the text. The digests were applied to a column (0.94 x 25 cm) of octadecylsilanyl resin (ODS) equilibrated in 95% (v/v) 0.01 N HCl and 5% (v/v) acetonitrile. Peptides were eluted by increasing the amount of acetonitrile in the mobile phase at a flow rate of 2 ml/min. The ordinates show the absorbance of the effluent at 215 nm as the amplitude of the detector response in millivolts, and the abscissas show the retention time (RT) in minutes. The retention times of the peaks are marked, and the fractions which were collected for analysis are numbered.
A, carboxymethylated S100β digested with cyanogen bromide;
B, performic acid oxidized S100β digested with trypsin.


A
27.87
27.21
26.58
25.90
25.25
24.58
AMPLITUDE
0.00
14.00
28.00
42.00
58.00
70.
RT in minutes
23.62
30.26
34.87
52.89
2
3
4
7

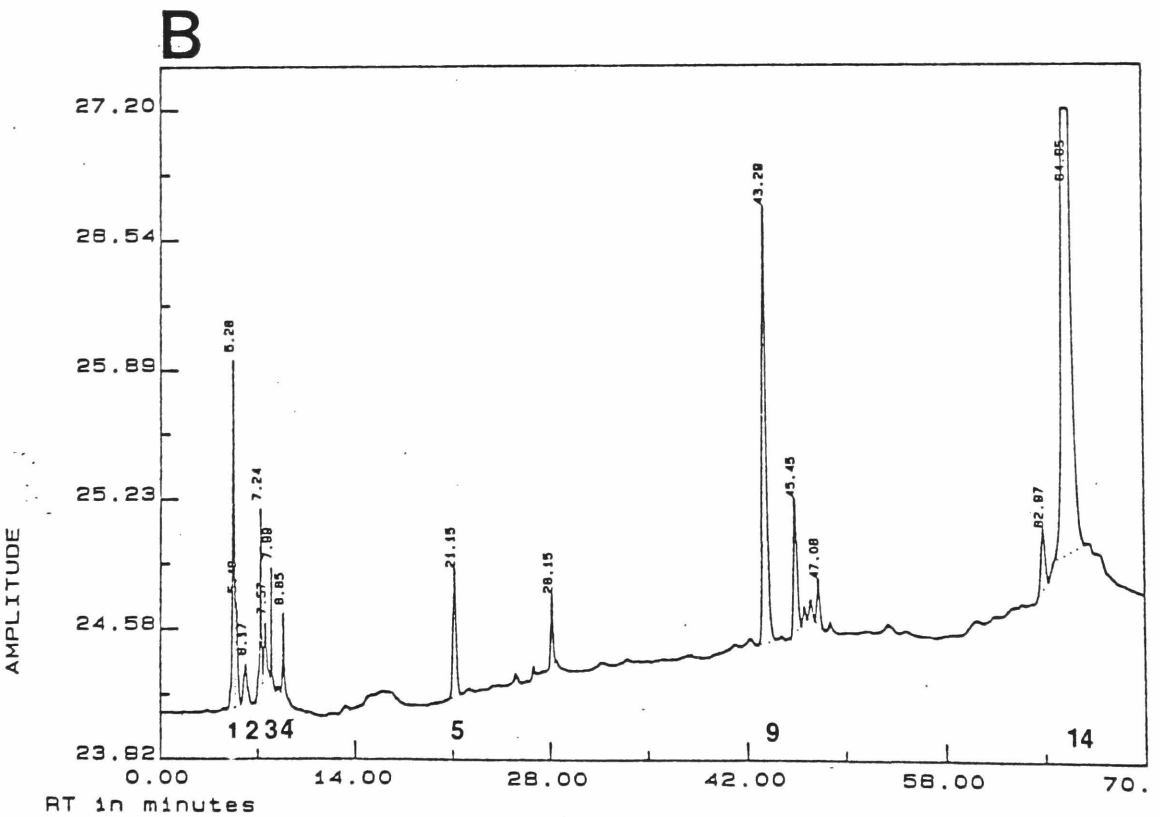

B
27.20
28.54
25.89
25.23
24.58
23.82
AMPLITUDE
0.00
14.00
28.00
42.00
58.00
70.
RT in minutes
6.28
7.24
21.15
28.15
43.20
45.45
47.08
62.87
64.65
1234
5
9
14

FIGURE 9: Radioimmunoassay of S100 proteins from human brain. Competitive inhibition radioimmunoassays were performed with serum 2 from rabbit 616 as described in the text using ^{125}I-S100α as antigen. The ordinate expresses the degree of inhibition as a percentage of the radioactivity bound in the absence of competing protein. The abscissa shows the nanograms of competing protein: bovine S100α (●); H1 (○); H2 (△); H3 (□).

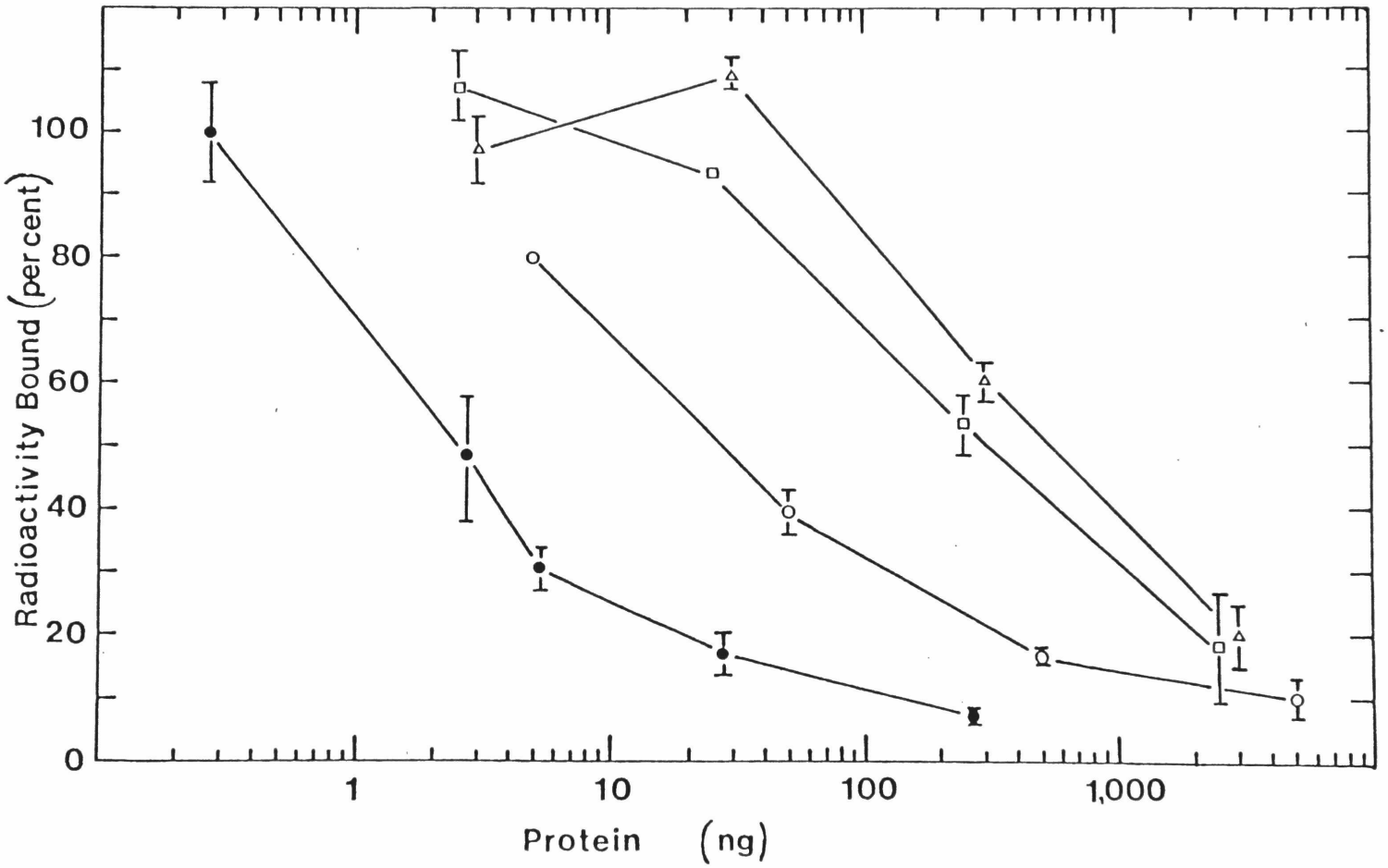

Radioactivity Bound (per cent)
100
80
60
40
20
0
1
10
100
1,000
Protein (ng)

FIGURE 10: Immunological responses of rabbits 512 (○) and 513 (●) to immunization with performic acid oxidized bovine brain S100β. Direct radioimmunoassays were performed using ^{125}I-S100β as antigen according to procedures described in the text. The ordinate expresses the amount of antigen bound as a percentage of the radioactivity added to the assay. The abscissa shows the time in days following the initial injection. The days on which the rabbits received injections are indicated by open arrows, and the days on which sera were collected are indicated by solid arrows.

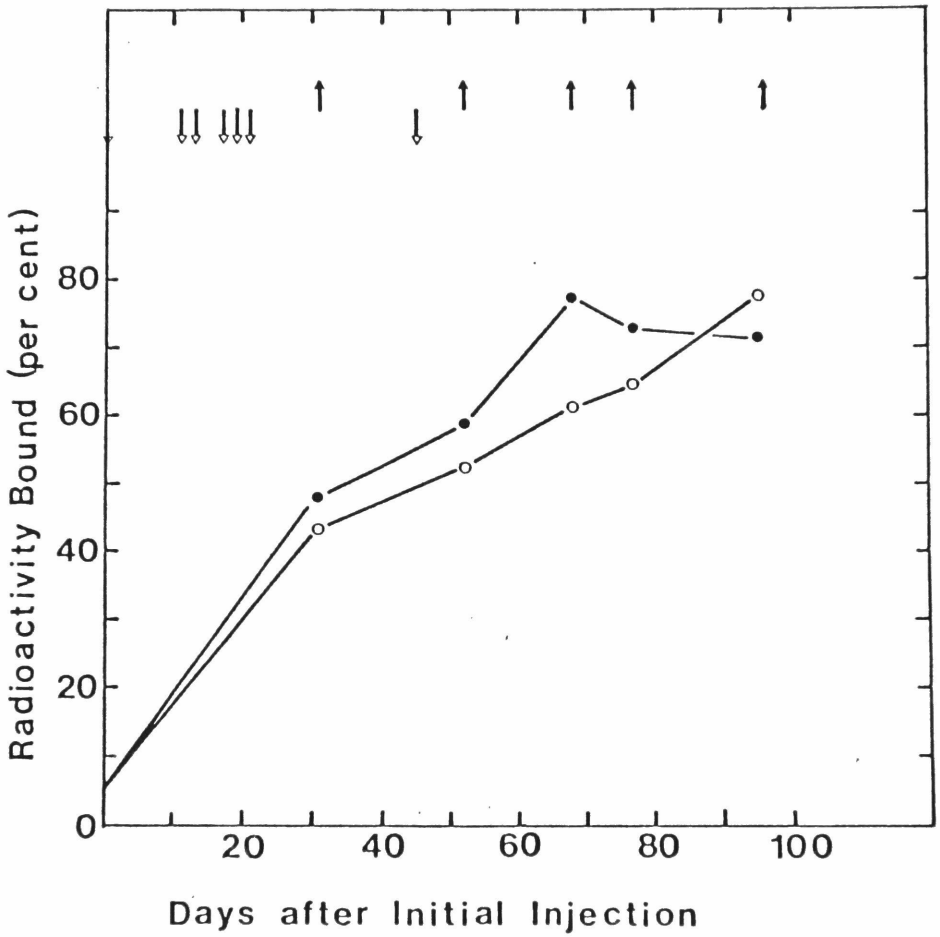

Radioactivity Bound (per cent)
0
20
40
60
80
20
40
60
80
100
Days after Initial Injection

FIGURE 11: Immunological responses of rabbits 615 (●) and 616 (○) to immunizations with performic acid oxidized bovine brain S100α. Direct radioimmunoassays were performed using ^{125}I-S100α as antigen according to procedures described in the text. The ordinate expresses the amount of antigen bound as a percentage of the radioactivity added to the assays. The abscissa shows the time in days following the initial injection. The days on which the rabbits received injections are indicated by open arrows, and the days on which sera were collected are indicated by solid arrows.

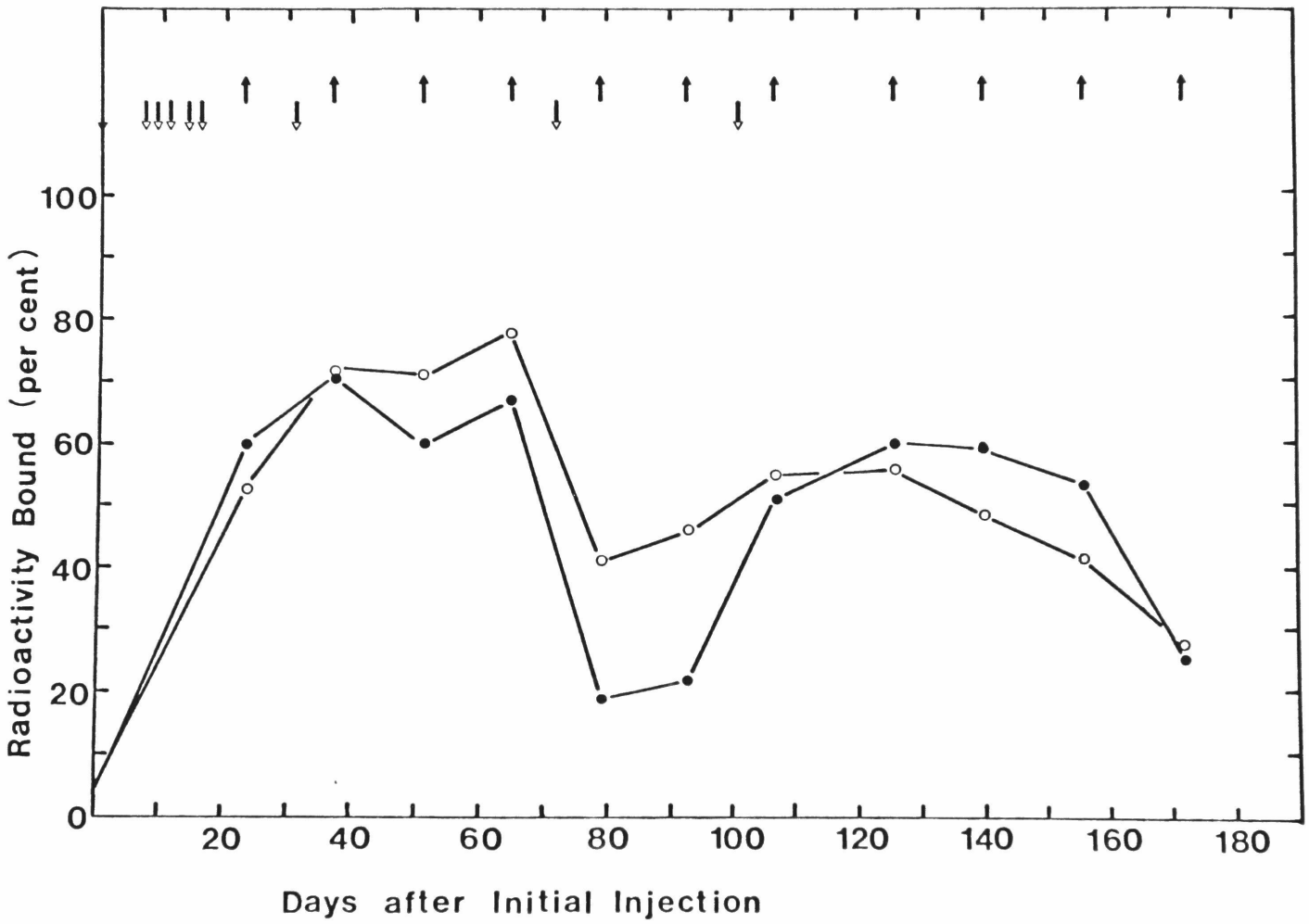

Radioactivity Bound (per cent)
0
20
40
60
80
100
20
40
60
80
100
120
140
160
180
Days after Initial Injection

FIGURE 12: Competitive radioimmunoassays using serum 1 (solid lines) and serum 5 (broken line) from rabbit 512 using ^{125}I-S100β as antigen. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing antigen. The abscissa shows the picomoles of competing proteins: bovine brain S100β (●,▲); bovine brain S100α (○,△); bovine brain calmodulin (■); and rabbit skeletal muscle troponin C (□).

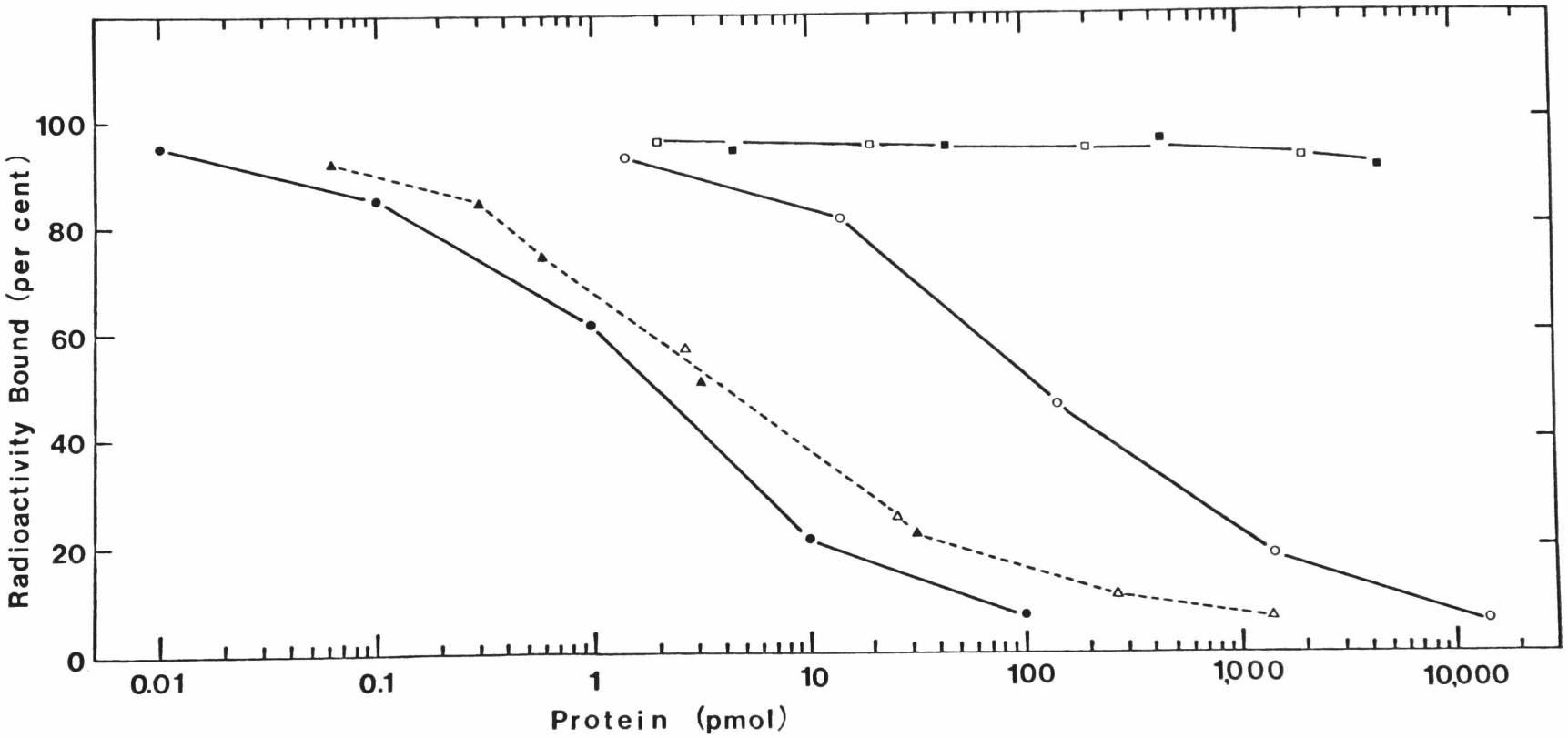

Radioactivity Bound (per cent)
100
80
60
40
20
0
0.01
0.1
1
10
100
1,000
10,000
Protein (pmol)

FIGURE 13: Competitive inhibition radioimmunoassays using serum 4 of rabbit 616 and ^{125}I-S100α as antigen. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing antigen. The abscissa shows the picomoles of competing proteins: bovine brain S100α (●) and bovine brain S100β (○).

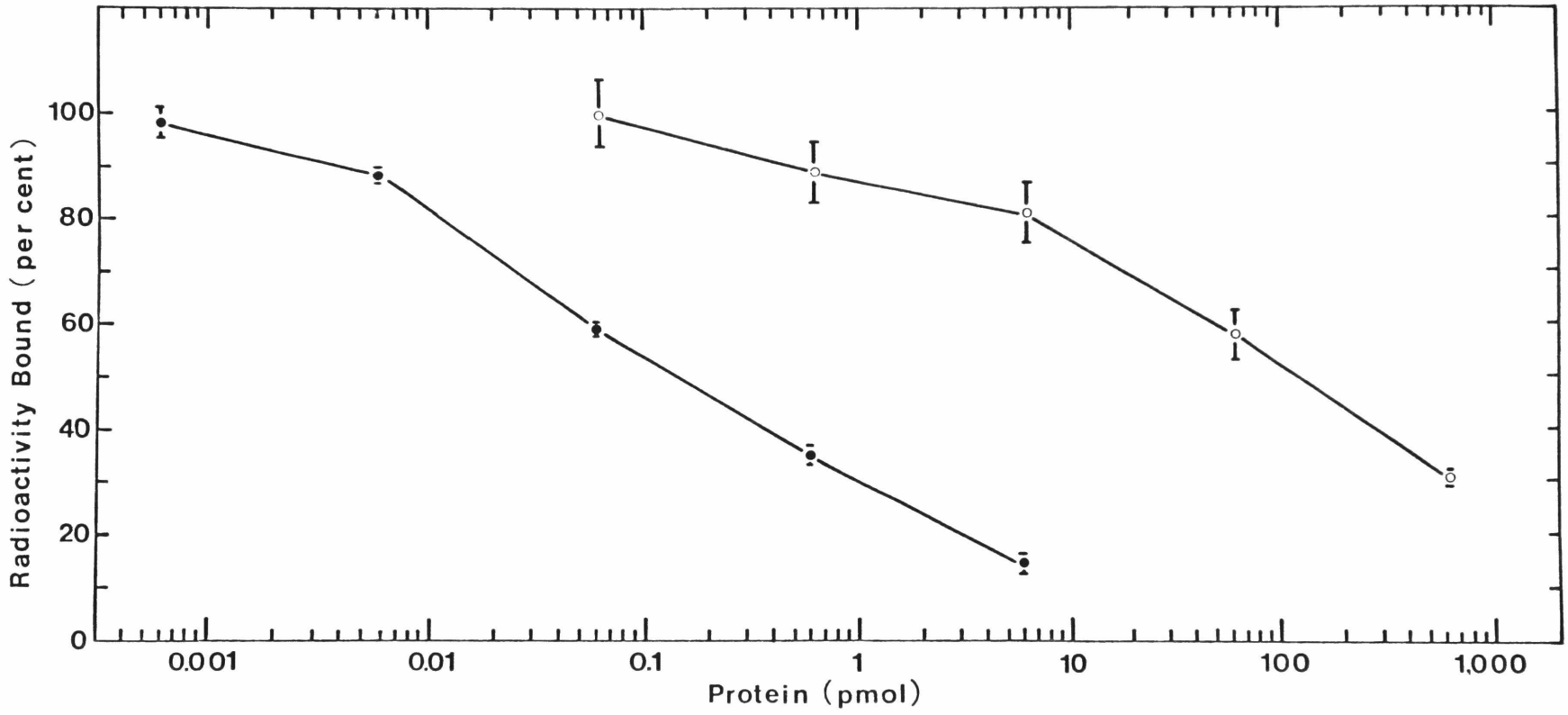

Radioactivity Bound (per cent)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1,000
Protein (pmol)

FIGURE 14: Immunological responses of rabbits 35 (○) and 36 (●) to immunization with bovine brain S100β. Direct radioimmunoassays were performed using ^{125}I-S100β as described in the text. The ordinate expresses the amount of antigen bound as a percentage of the radioactivity added to the assays. The abscissa shows the time in days following the initial injection. Days on which the rabbits received injections are indicated by open arrows, and the days on which sera were collected are indicated by solid arrows.

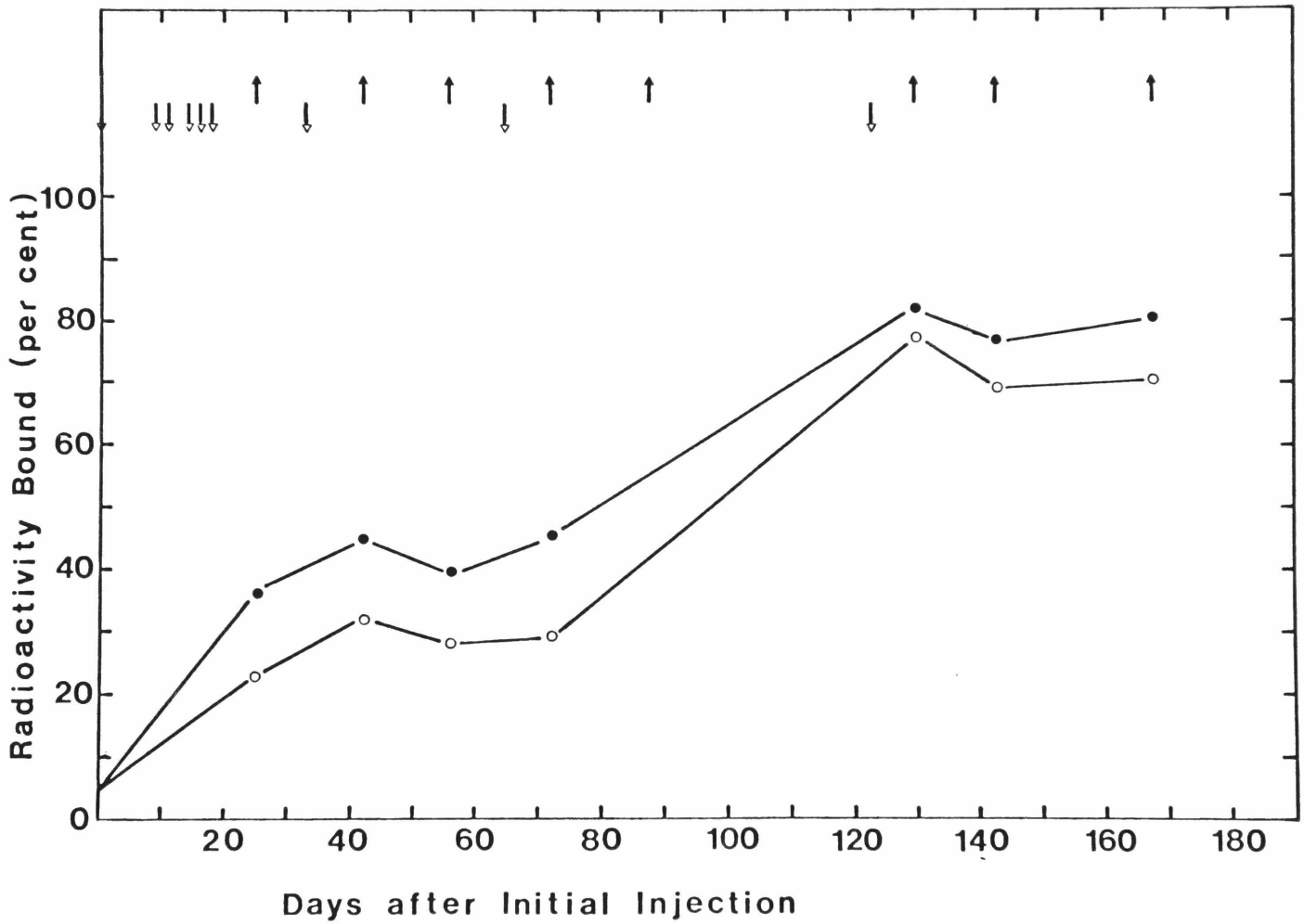

Radioactivity Bound (per cent)
0
20
40
60
80
100
20
40
60
80
100
120
140
160
180
Days after Initial Injection

FIGURE 15: Competitive inhibition radioimmunoassays using serum 1 of rabbit 36 and ^{125}I-S100β as antigen. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing protein The abscissa shows the picomoles of competing antigens: bovine brain S100β (●), and bovine brain S100α (○).

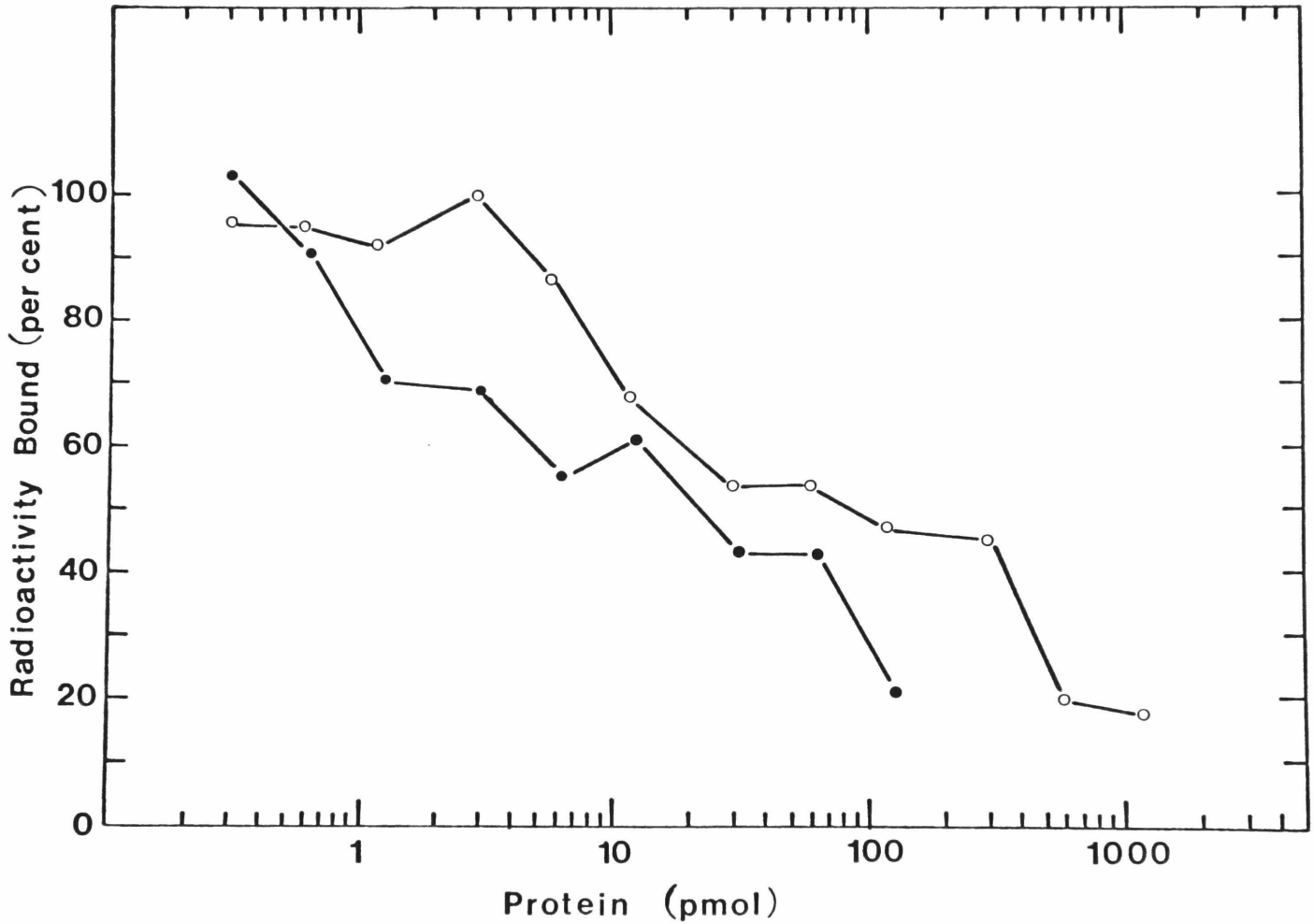

Radioactivity Bound (per cent)
100
80
60
40
20
0
1
10
100
1000
Protein (pmol)

FIGURE 16: Immunological responses of rabbits 13 (●,▲) and 14 (○,△) to immunization with peptide ME13B-ovalbumin conjugates. Direct radioimmunoassays were performed using ^{125}I-S100β (●,○) or ^{125}I-S100α (▲,△) as described in the text. The ordinate expresses the amount of antigen bound as a percentage of the amount of radioactivity added to the assays. The days on which the rabbits received injections are indicated by open arrows, and the days on which sera were collected are indicated by solid arrows.

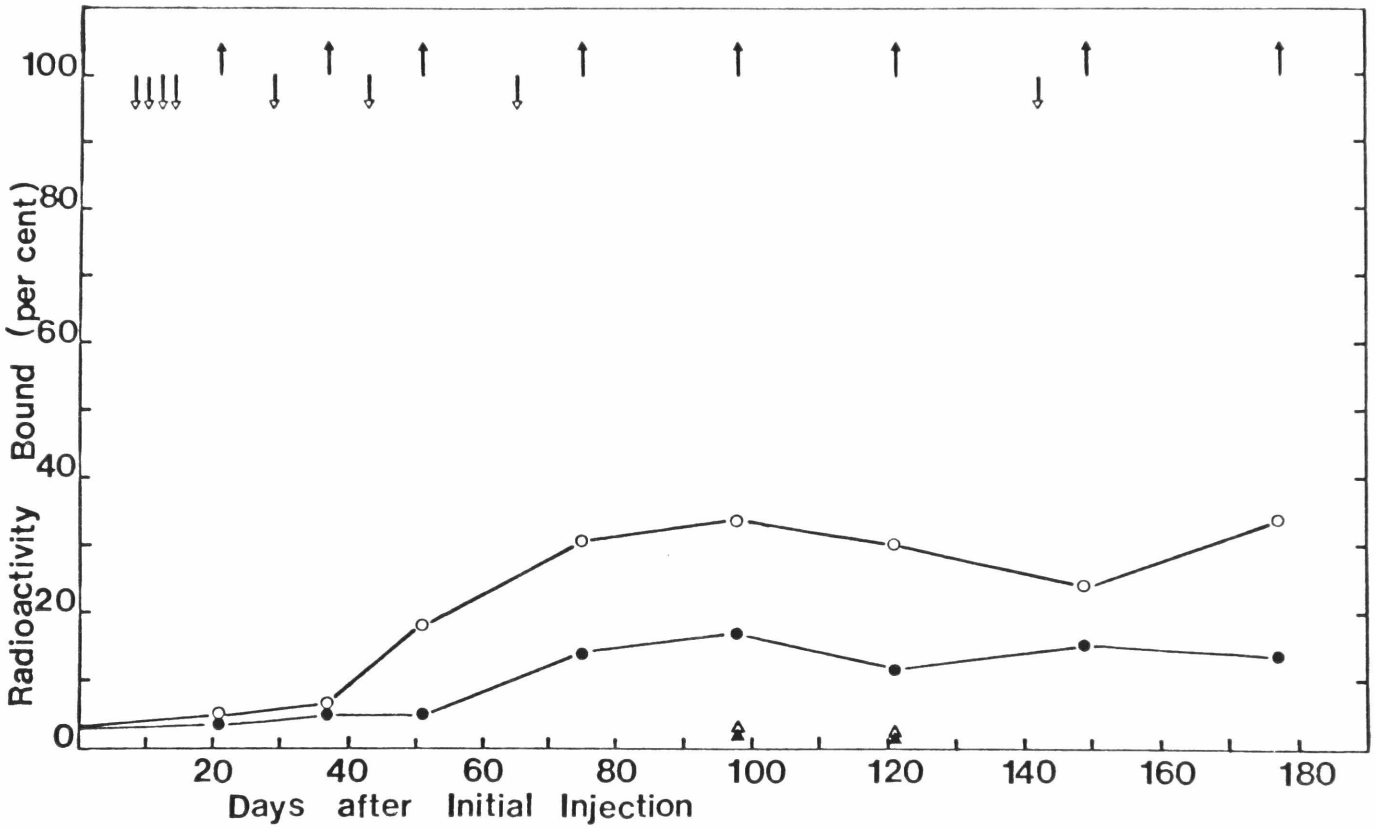

100
80
60
40
20
0
Radioactivity Bound (per cent)
20
40
60
80
100
120
140
160
180
Days after Initial Injection

FIGURE 17: Binding of ^{125}I-calmodulin to immobilized phenothiazines. ^{125}I-calmodulin was incubated with phenothiazine-Sepharose conjugates for 60 minutes at room temperature in buffer containing 10 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol. Following incubation, the unbound radioactivity was removed and the bound radioactivity was eluted as described in the text. The ordinate expresses the amount of radioactivity bound as a percentage of the amount of radioactivity added to each assay. The results are the means of duplicate determinations, and the range of values was <6%.

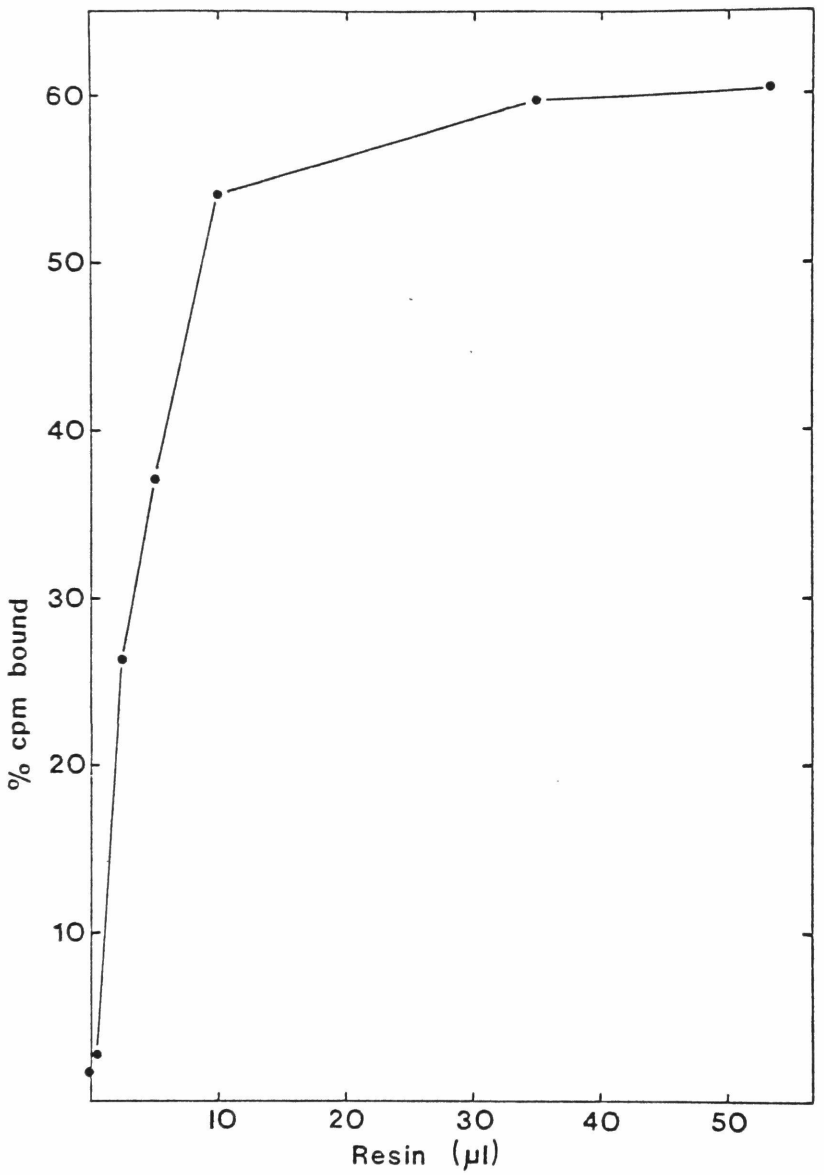

60
50
40
30
20
10
% cpm bound
10
20
30
40
50
Resin (µl)

FIGURE 18: Separation of ^{125}I-calmodulin peptides by reverse phase chromatography. Bovine brain calmodulin was iodinated (180) using lactoperoxidase and digested with trypsin as described in the text. The digest was applied to a column (0.94 x 25 cm) of octadecylsilanyl resin (ODS) equilibrated in 95% (v/v) 0.01 N HCl and 5% (v/v) acetonitrile, and the peptides were eluted by increasing the amount of acetonitrile in the mobile phase. Fractions of 2 ml (1 minute) were collected, and the radioactivity in each fraction was measured. The ordinate shows the amount of radioactivity, and the abscissa shows the retention time in minutes. The arrows indicate the retention time for the iodinated, purified peptides, T-5B and T-1B.

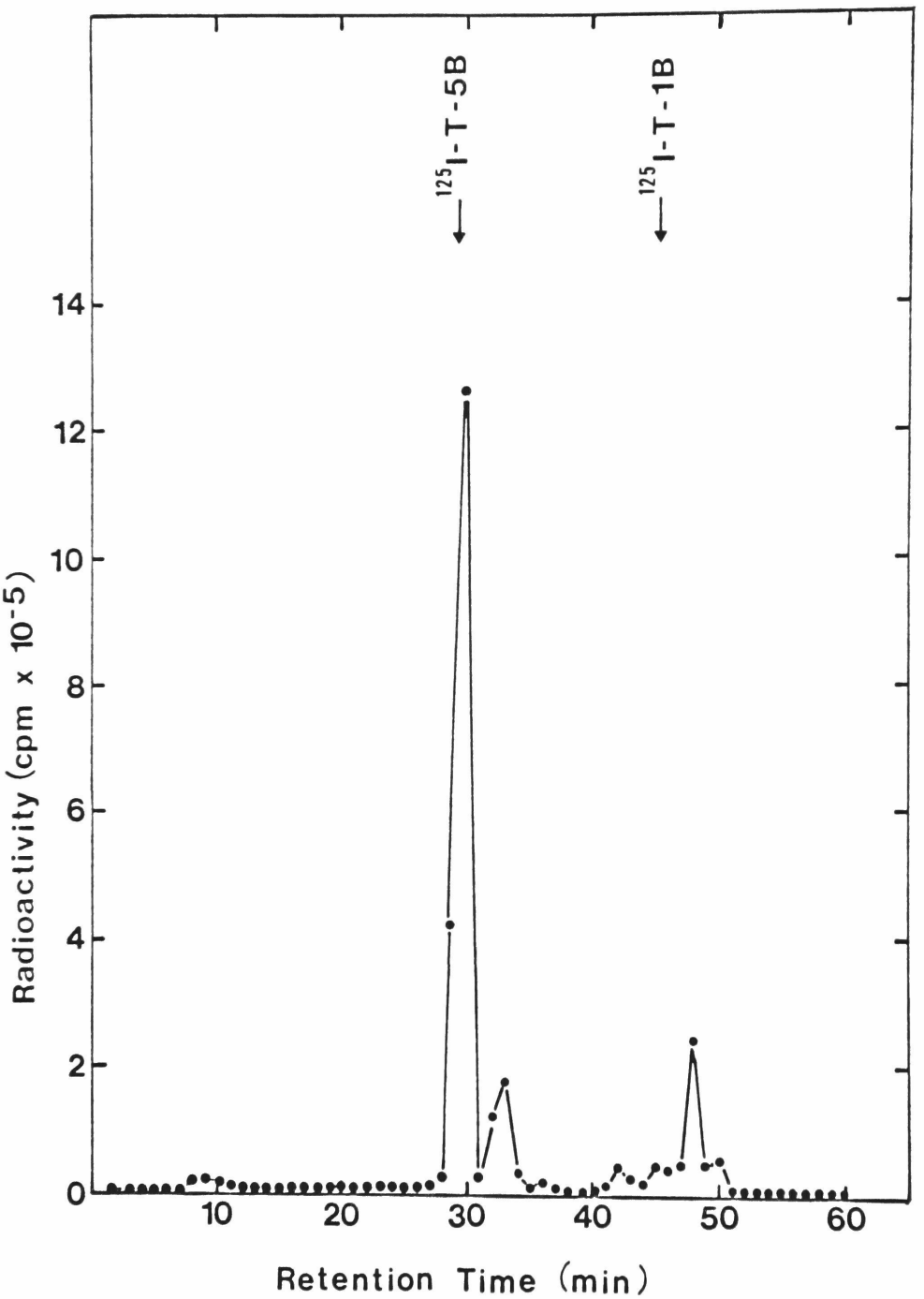
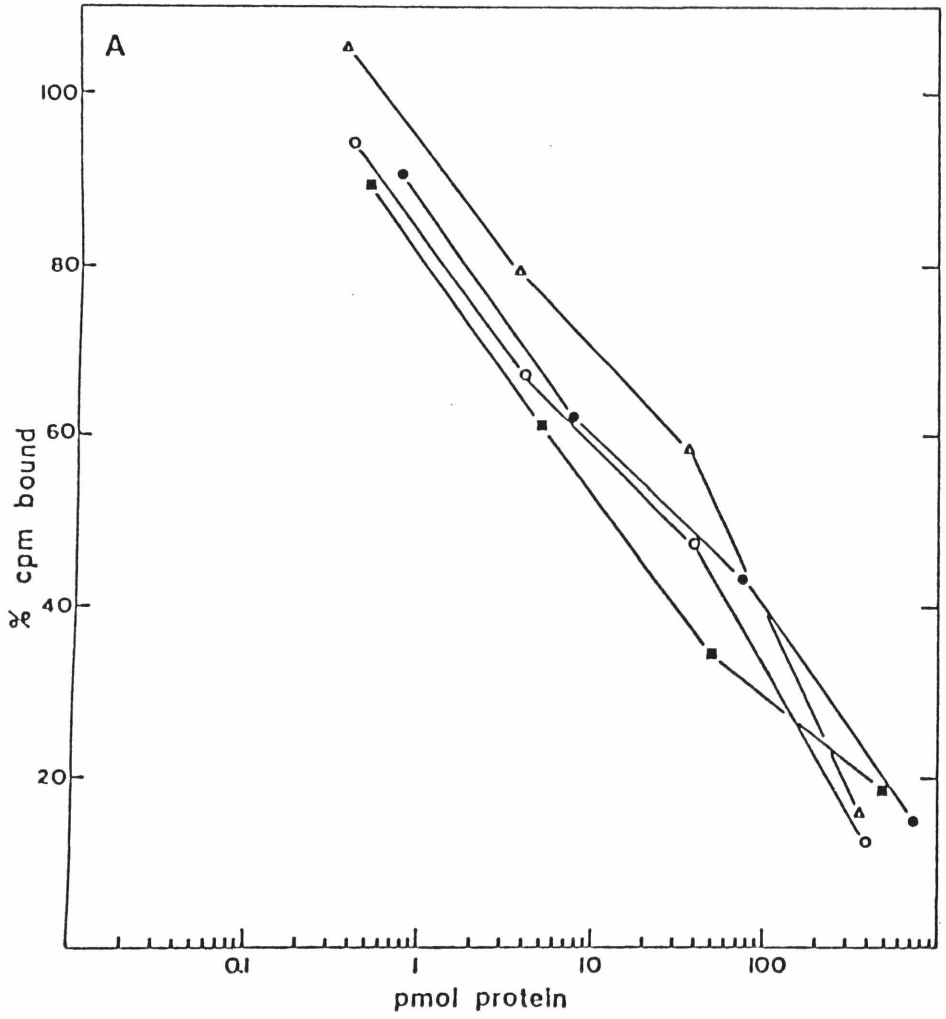

^{125}I-T-5B
^{125}I-T-1B
Radioactivity (cpm x 10^{-5})
0
2
4
6
8
10
12
14
10
20
30
40
50
60
Retention Time (min)

FIGURE 19: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by calcium modulated proteins. The ordinates express the degree of inhibition as a percentage of the radioactivity bound in the absence of competing protein.

A. Competing proteins were bovine brain calmodulin (●); chicken gizzard calmodulin (■); spinach calmodulin (○); and barley calmodulin (△). Results are the means of duplicate determinations, and the range of values was <6%.

B. Competing proteins were: bovine brain calmodulin (●); rabbit skeletal muscle troponin C (○); bovine brain S100β (**x**); and carp parvalbumin (▲). Results are the means of duplicate determinations with the range of values indicated when >2%. Results similar to those shown for parvalbumin were obtained for chicken and rat vitamin D-dependent calcium binding proteins.


A
100
80
60
40
20
% cpm bound
0.1
1
10
100
pmol protein

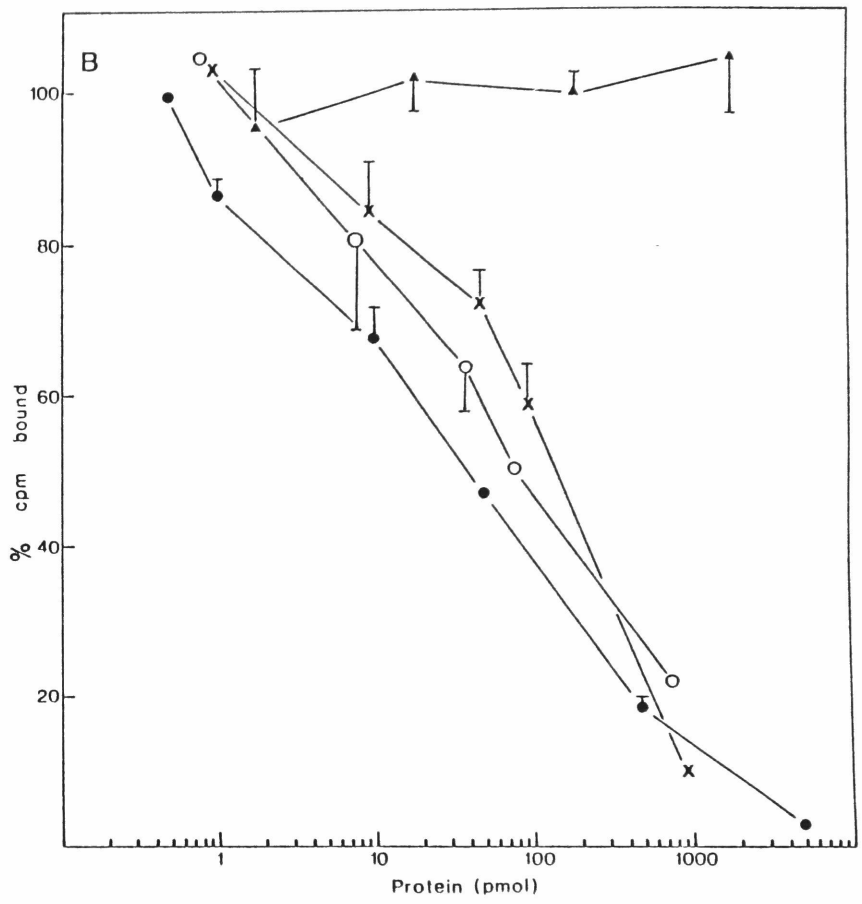

B
100
80
60
40
20
% cpm bound
1
10
100
1000
Protein (pmol)

FIGURE 20: Calcium dependent adsorptionof bovine brain S100β to phenothiazine-Sepharose conjugates. In separate experimetns, purified bovine brain S100β was applied to and eluted from a column (0.65 x 3.0 cm) containing phenothiazine-Sepharose conjugates. Effluents were collected and analyzed by electrophoresis on a 18% (w/v) polyacrylamide gel containing 0.1% (w/v) sodium dodecyl sulfate. In the first experiment (lanes 1-3), the column was equilibrated with calcium buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 2 mM $CaCl_2$); in the second experiment (lanes 4-6), the column was equilibrated with chelator buffer (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 2 mM EGTA). Three milliliters each of the following solutions was passed through the calcium-equilibrated column: calcium buffer, lane 1; calcium buffer containing 0.2 M NaCl, lane 2; chelator buffer containing 0.2 M NaCl, lane 3. The chelator-equilibrated column was eluted with 3 ml each of the following solutions: chelator buffer,lane 4; chelator buffer containing 0.2 M NaCl, lane 5; chelator buffer containing 4 M urea, lane 6.

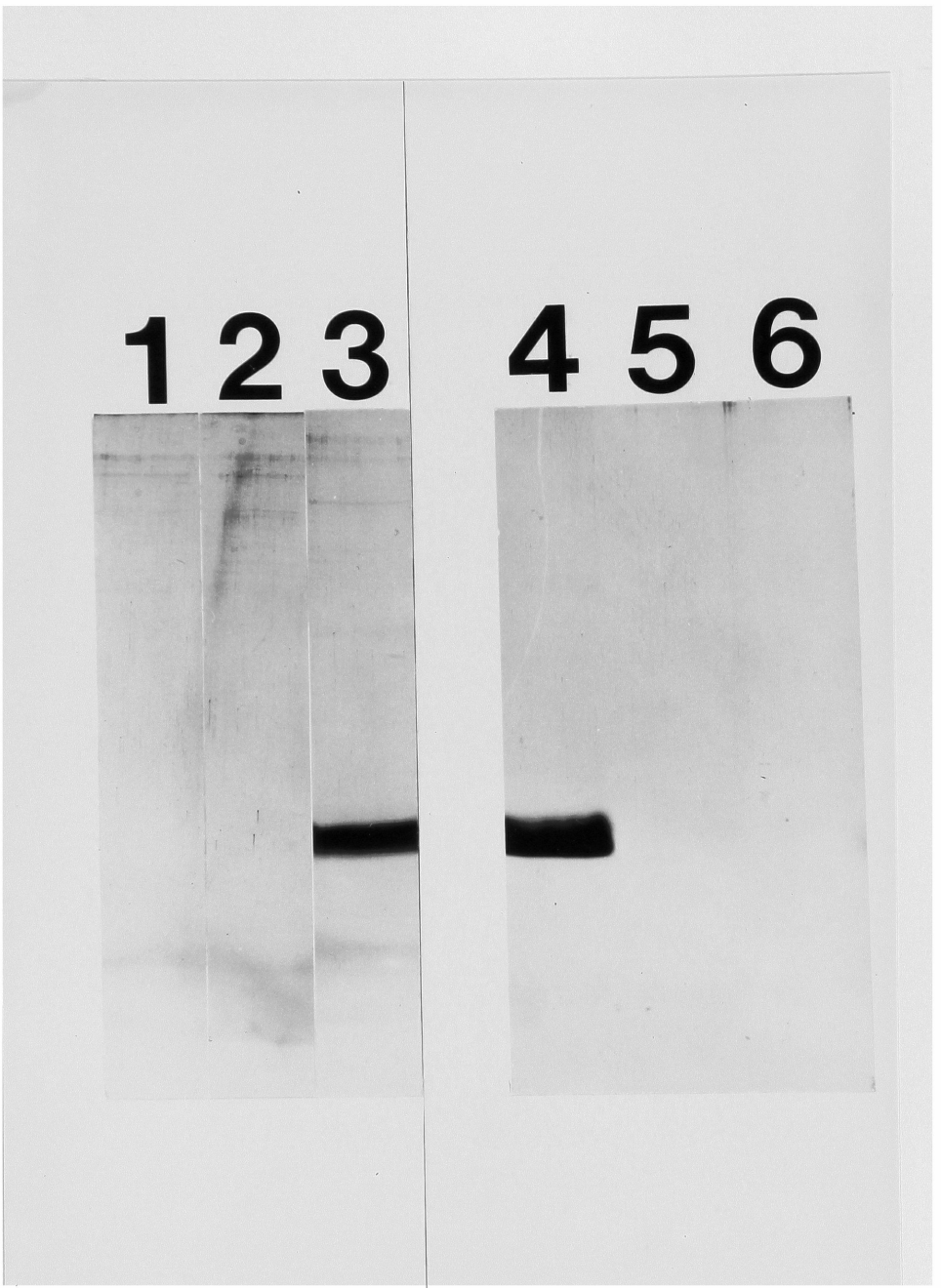

1 2 3
4 5 6

FIGURE 21: Equilibrium binding of chlorpromazine to bovine brain calmodulin using the method of Hummel and Dreyer (178). Bovine brain calmodulin (1.25 nmol) was dissolved in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 9.3 μM chlorpromazine) and applied to a column (0.5 x 30 cm) of BioGel P-6 equilibrated in the same buffer at 0.3 ml/min. The ordinate expresses the absorbance of the effluent at 254 nm expressed as the amplitude of the response of the detector in millivolts. The abscissa shows the retention time (RT) in minutes.

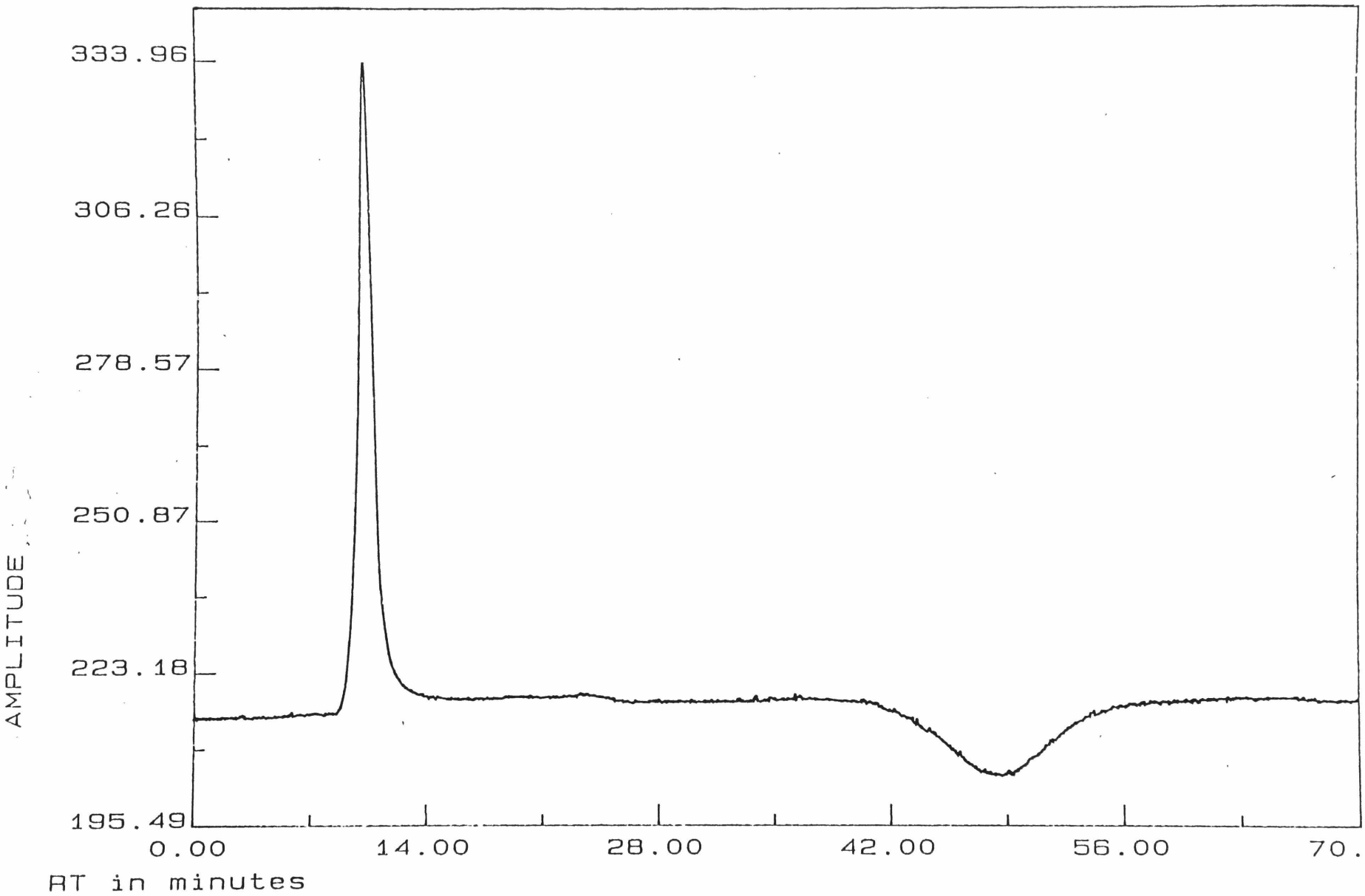

333.96
306.26
278.57
250.87
223.18
195.49
AMPLITUDE
0.00
14.00
28.00
42.00
56.00
70.
RT in minutes

FIGURE 22: External calibration of chlorpromazine binding to bovine brain calmodulin. Samples containing known amounts of chlorpromazine above that in the buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 12.5 μM chlorpromazine) were applied to a column (0.5 x 30 cm) of BioGel P6 equilibrated in the same buffer. The absorbance of the effluent was measured at 254 nm, and the areas of the peaks corresponding to the samples of drug were integrated as described in the text. The areas of these peaks are shown (●). The ordinate expresses the area of the peaks in volt-seconds, and the abscissa shows the nanomoles of chlorpromazine in the samples above that in the buffer. The line shown was calculated by linear regression analysis, and the correlation coefficient was >0.99. An equilibrium binding experiment using 1.25 nmol bovine brain calmodulin was performed as described in the text and in Figure 21. The area of the trough is shown (○) with the range of values from duplicate determinations. The dashed line indicates the interpolated value for the number of nanomoles of chlorpromazine represented by the trough.

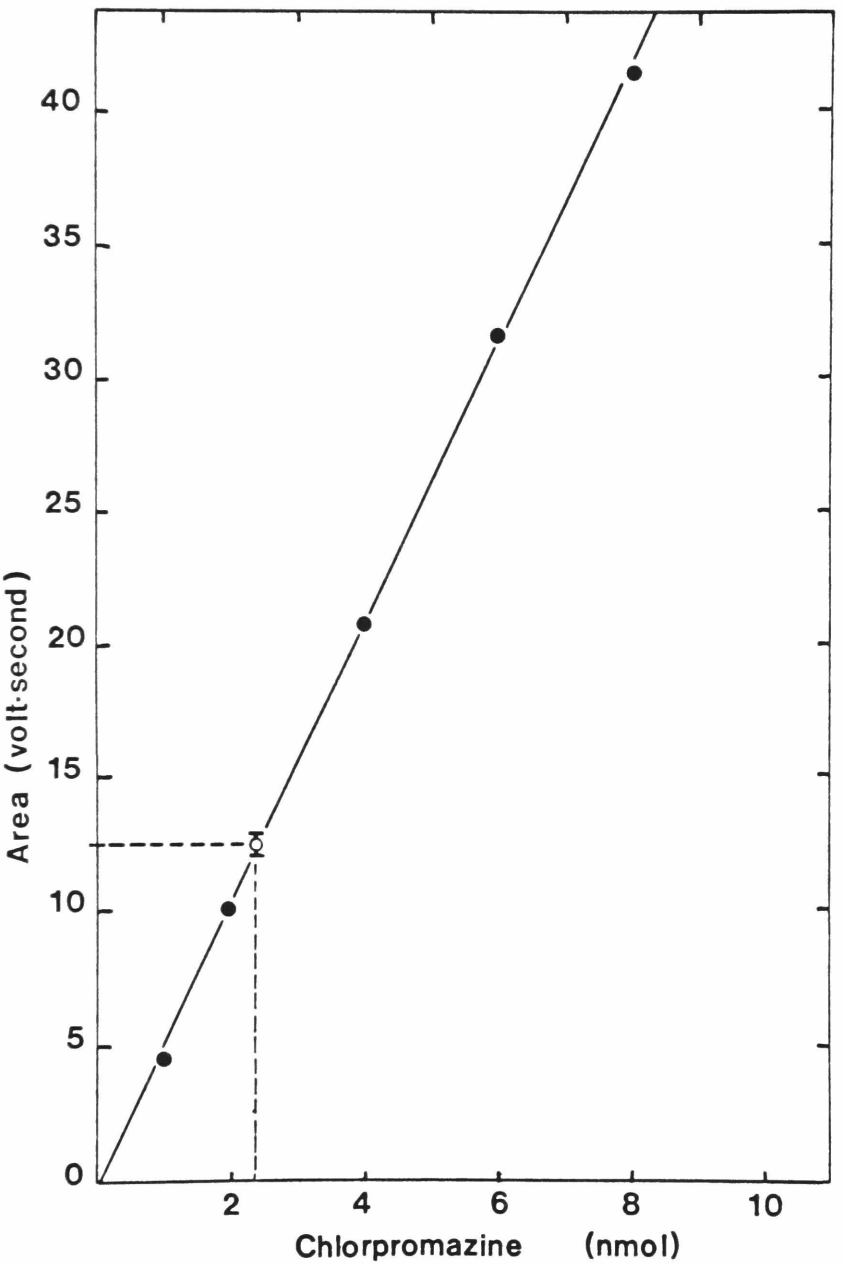

40
35
30
25
20
15
10
5
0
Area (volt·second)
2
4
6
8
10
Chlorpromazine (nmol)

FIGURE 23: Internal calibration of chlorpromazine binding to calmodulin. Five samples of bovine brain calmodulin (1.25 nmol) were dissolved in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 12.5 μM chlorpromazine). An aliquot of excess chlorpromazine (0,1,2,4, or 6 nmol above the 12.5 μM chlorpromazine in the buffer) was added to each sample, and the samples were filtered on a column (0.5 x 30 cm) of BioGel P6 at a flow rate of 0.3 ml/min. The ordinate shows the absorbance of the effluent expressed as the amplitude of the detector response in volts for the five experiments. The abscissa shows the retention time (RT) in minutes. The amount of excess chlorpromazine added to the sample are as follows: curve a, 0 nmol; curve b, 1 nmol; curve c, 2 nmol; curve d, 4 nmol; curve e, 6 nmol.

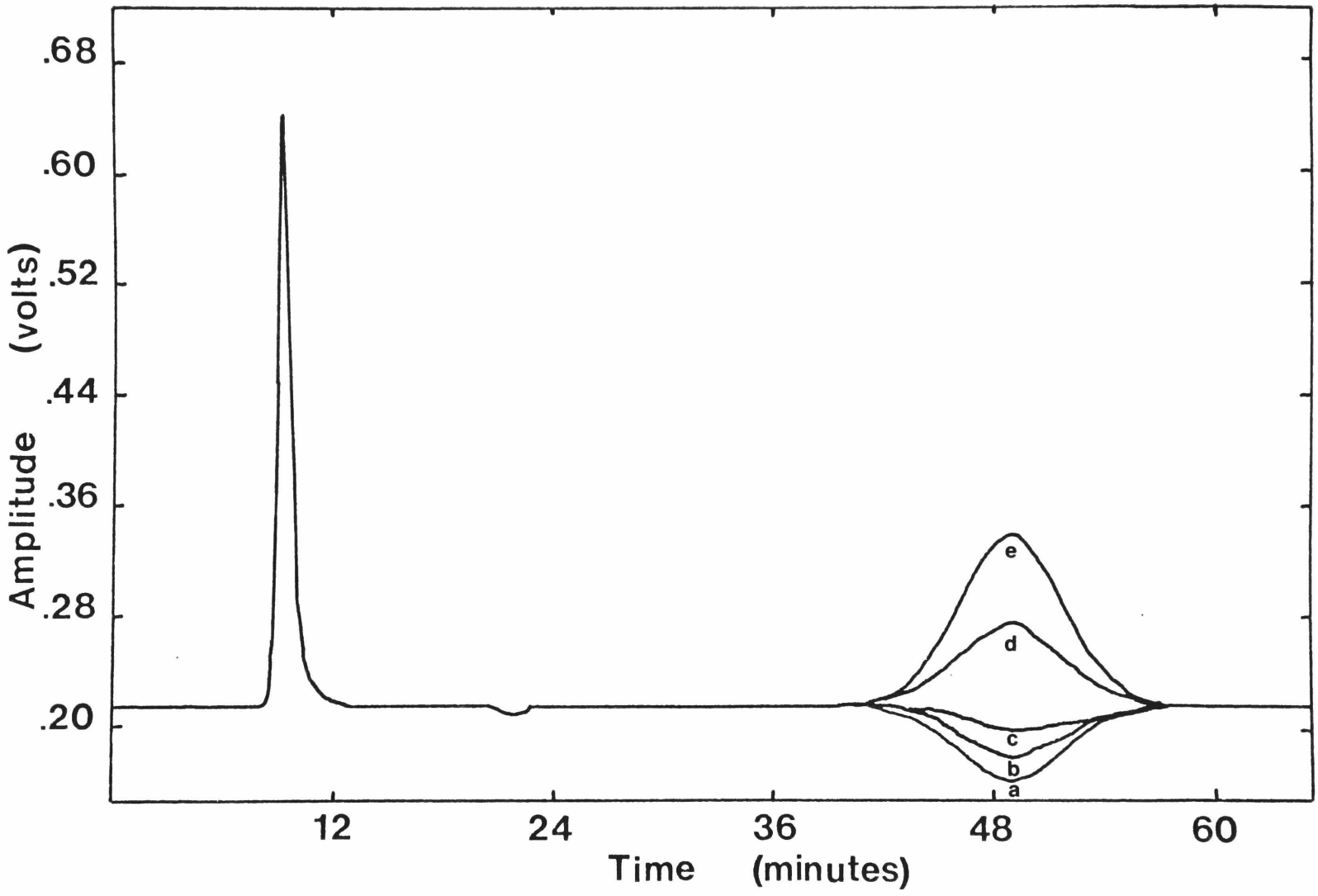

.68
.60
.52
.44
.36
.28
.20
Amplitude (volts)
12
24
36
48
60
Time (minutes)
e
d
c
b
a

FIGURE 24: Graphical representation of the internal calibration of chlorpromazine binding to bovine brain calmodulin. The areas of the troughs (negative areas) or peaks (positive areas) of unbound chlorpromazine for the experiments in Figure 23 are shown (●) as a function of the number of nanomoles of excess chlorpromazine added to each sample. The ordinate expresses the areas in volt-seconds. The solid line was calculated by linear regression analysis, and the correlation coefficient was >0.99. The dashed line indicates the interpolated value for the amount of excess chlorpromazine necessary to bring the area of the trough to zero.

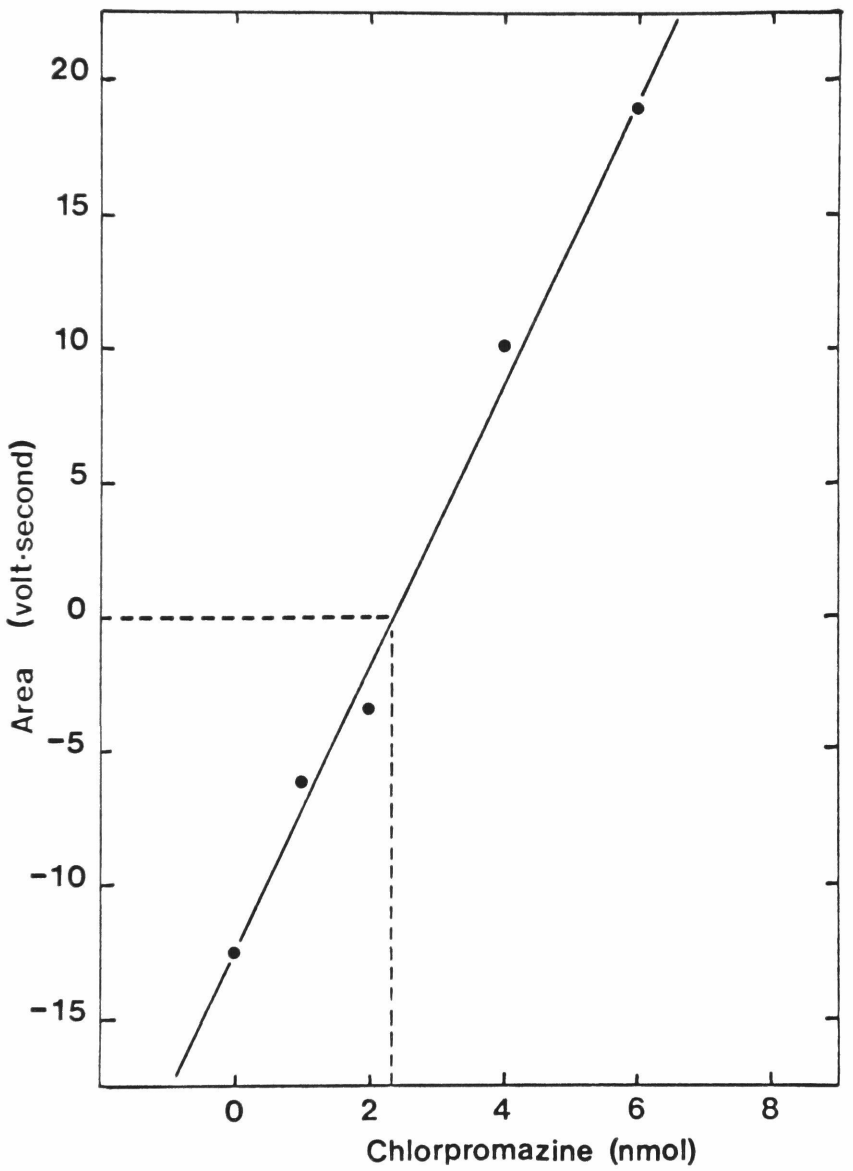

20
15
10
5
0
-5
-10
-15
Area (volt·second)
0
2
4
6
8
Chlorpromazine (nmol)

FIGURE 25: Equilibrium binding of chlorpromazine by bovine brain calmodulin as a function of chlorpromazine concentration. The binding of chlorpromazine to calmodulin was measured using the method of Hummel and Dreyer (178) in buffer containing calcium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$), (●), or in buffer containing magnesium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA), (○). The ordinate expresses the number of moles of chlorpromazine bound per mole of protein. The abscissa shows the concentration of chlropromazine in the buffer. Results shown are the means of values calculated from external and internal calibrations. The range of values is shown when >5%. For experiments in which the binding ratio was less than 0.4 moles of chlorpromazine per mole of protein, the results shown are the means of duplicate determinations calculated from external calibration, and the range of values was <0.02 mole/mole.

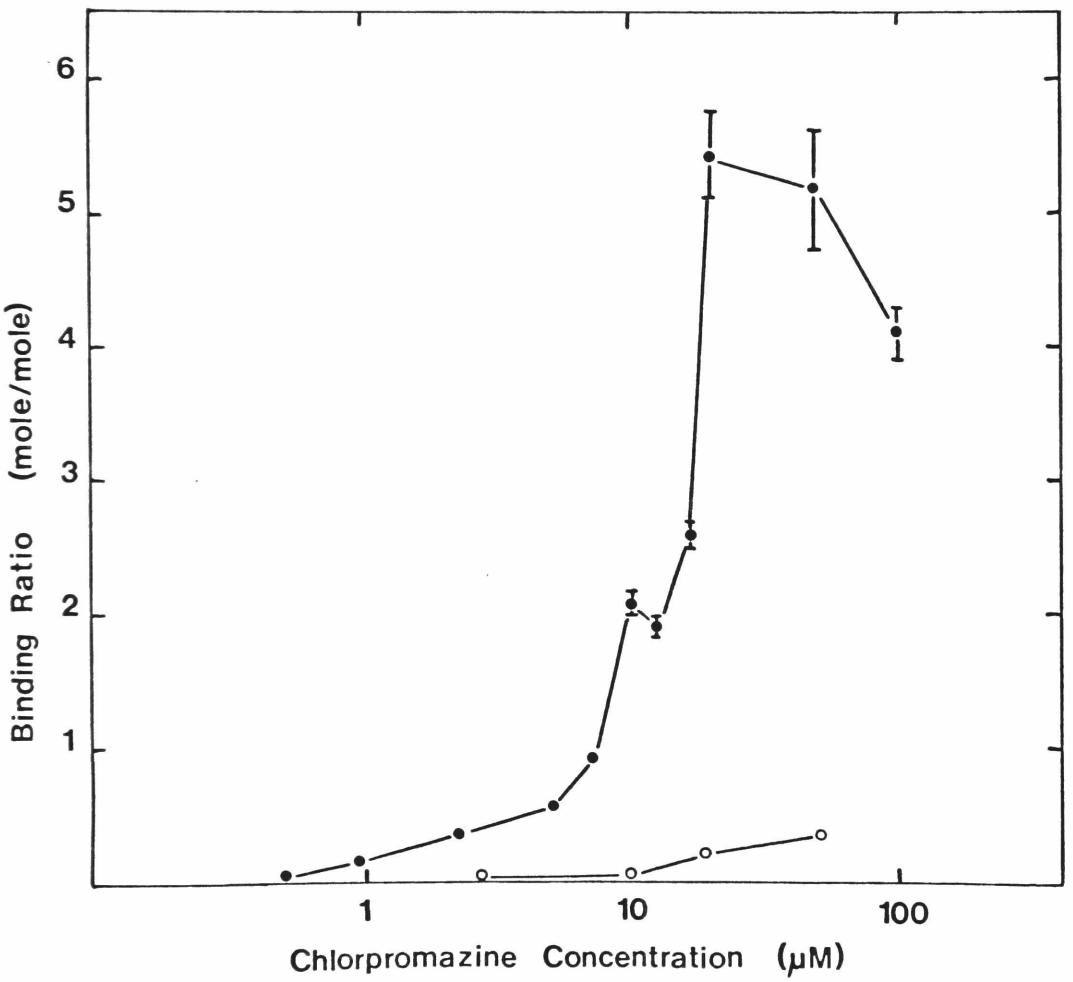

Binding Ratio (mole/mole)
6
5
4
3
2
1
1
10
100
Chlorpromazine Concentration (μM)

FIGURE 26: Separation of peptides of Dictyostelium calmodulin by reverse phase chromatography. Dictyostelium calmodulin was digested with mouse submaxillary gland protease and applied to a column (0.94 x 25 cm) of octadecylsilanyl resin (ODS) equilibrated in 5% (v/v) 0.01 N HCl and 95% (v/v) acetonitrile. The peptides were eluted by increasing the amount of acetonitrile in the mobile phase as described in the text. The ordinate shows the absorbance of the effluent at 215 nm expressed as the amplitude of the detector response in millivolts. The abscissa shows the retention time (RT) in minutes following application of the sample. The numbered peaks (16, 17, 18, 21) were collected and analyzed (Table 5).

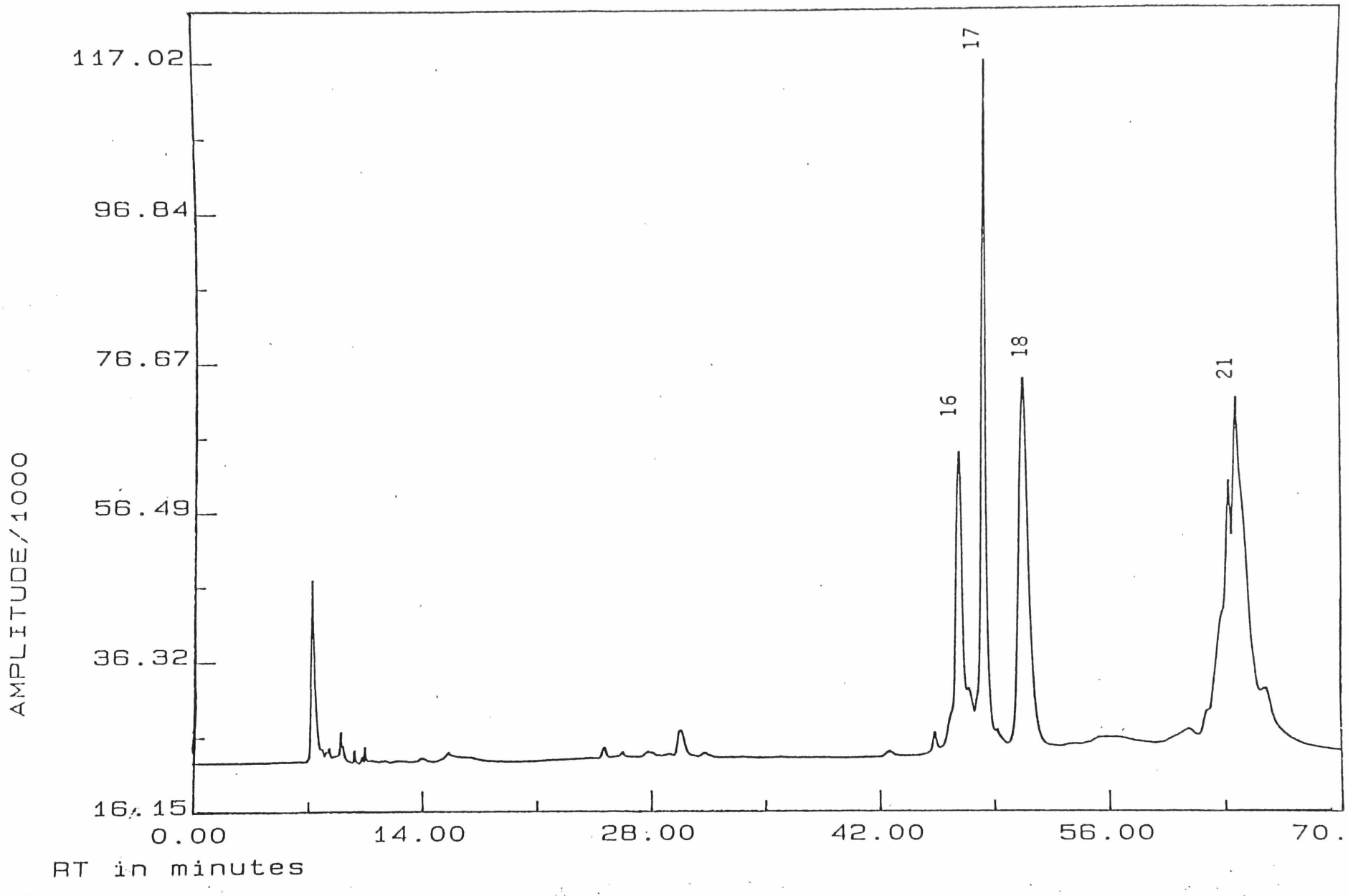

117.02
96.84
76.67
56.49
36.32
16.15
AMPLITUDE/1000
0.00
14.00
28.00
42.00
56.00
70.
RT in minutes
16
17
18
21

FIGURE 27: Binding of chlorpromazine by calcium modulated proteins. Equilibrium binding experiments were performed using the method of Hummel and Dreyer (178) in buffer containing calcium (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$) as described in the text. The number of moles of chlorpromazine bound per mole of protein are shown for CaM, bovine brain calmodulin; TnC, rabbit skeletal muscle troponin C; S100β, bovine brain S100β; S100α, bovine brain S100α; Parv., carp parvalbumin; PAO-CaM, performic acid oxidized bovine brain calmodulin. For each protein, results are shown for three chlorpormazine concentrations: 9-10 μM (left), 45-50 μM (center), and 100 μM (right). The results shown are the means of duplicate determinations with the range of values <0.2 moles of drug per mole of protein.

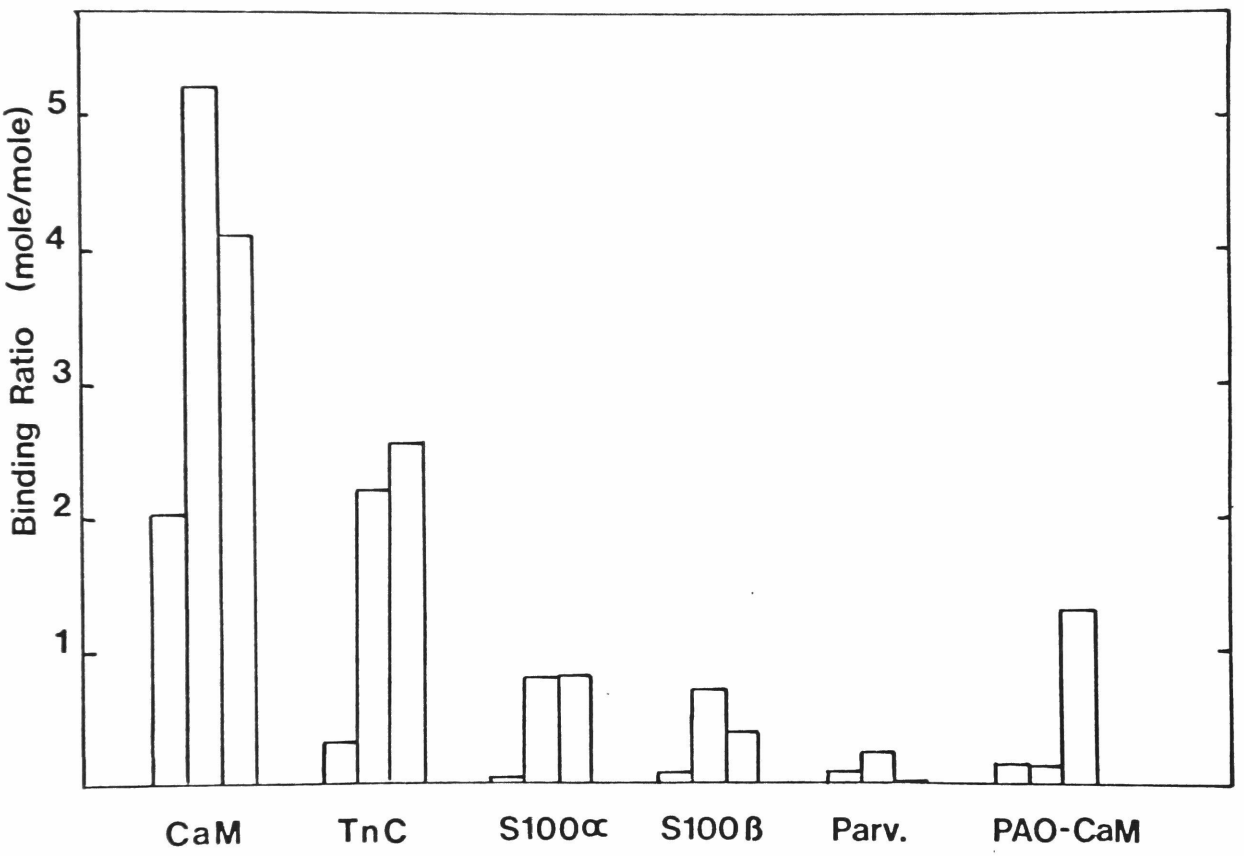

Binding Ratio (mole/mole)
5
4
3
2
1
CaM
TnC
S100α
S100ß
Parv.
PAO-CaM

FIGURE 28: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by digests of calmodulin. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing protein. The abscissa shows the amount of competing protein: bovine brain calmodulin (●), chicken gizzard calmodulin digested with trypsin (▲), and chicken gizzard calmodulin digested with cyanogen bromide (■).

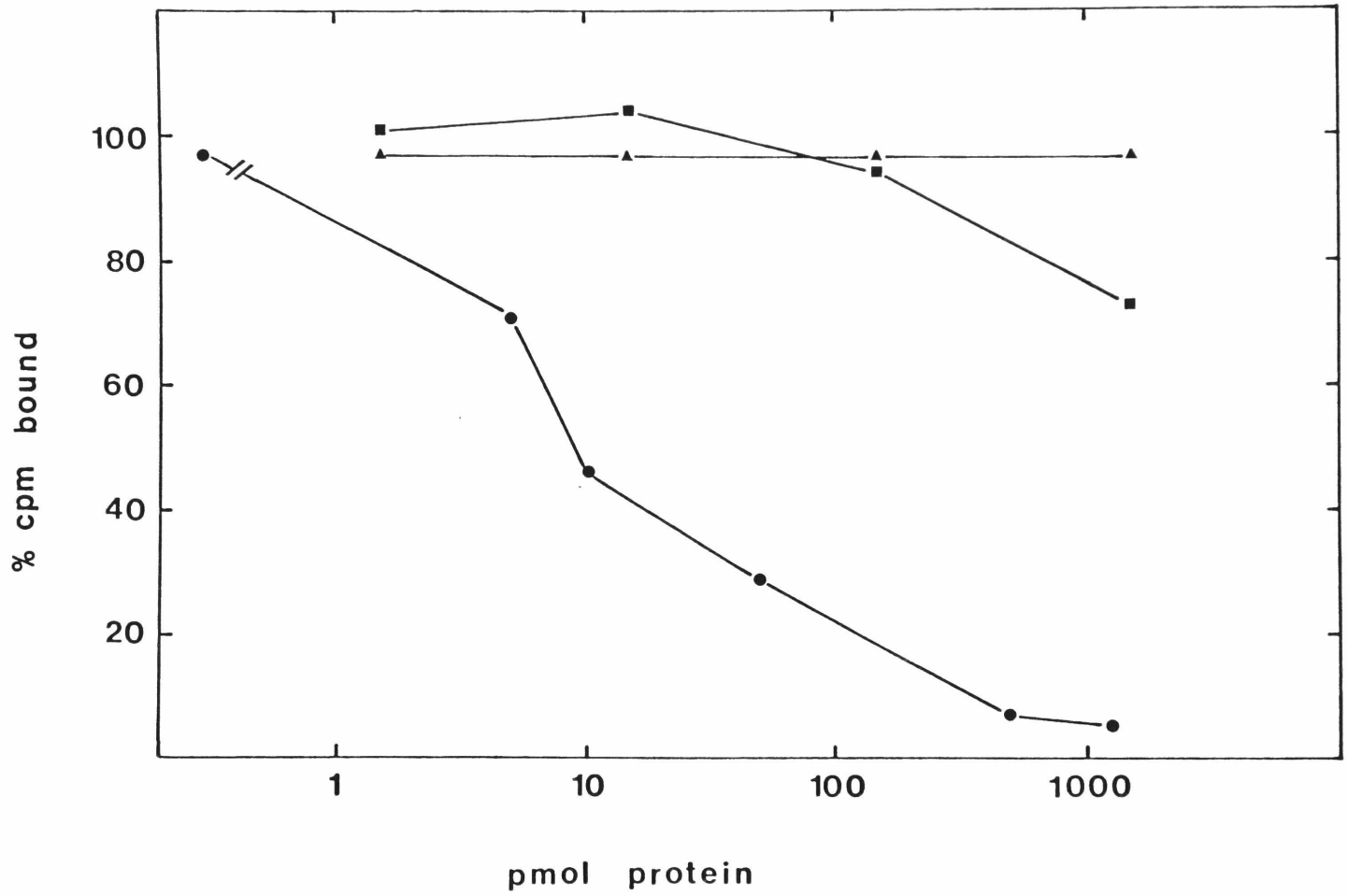

100
80
60
40
20
% cpm bound
1
10
100
1000
pmol protein

FIGURE 29: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by digests of citraconylated calmodulin. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing protein. The abscissa shows the amount of competing protein: bovine brain calmodulin (●); citraconylated bovine brain calmodulin digested with trypsin (○); spinach calmodulin (▲); citraconylated spinach calmodulin digested with trypsin (△). The results shown are the means of duplicate determinations, and the range of values was <6%.

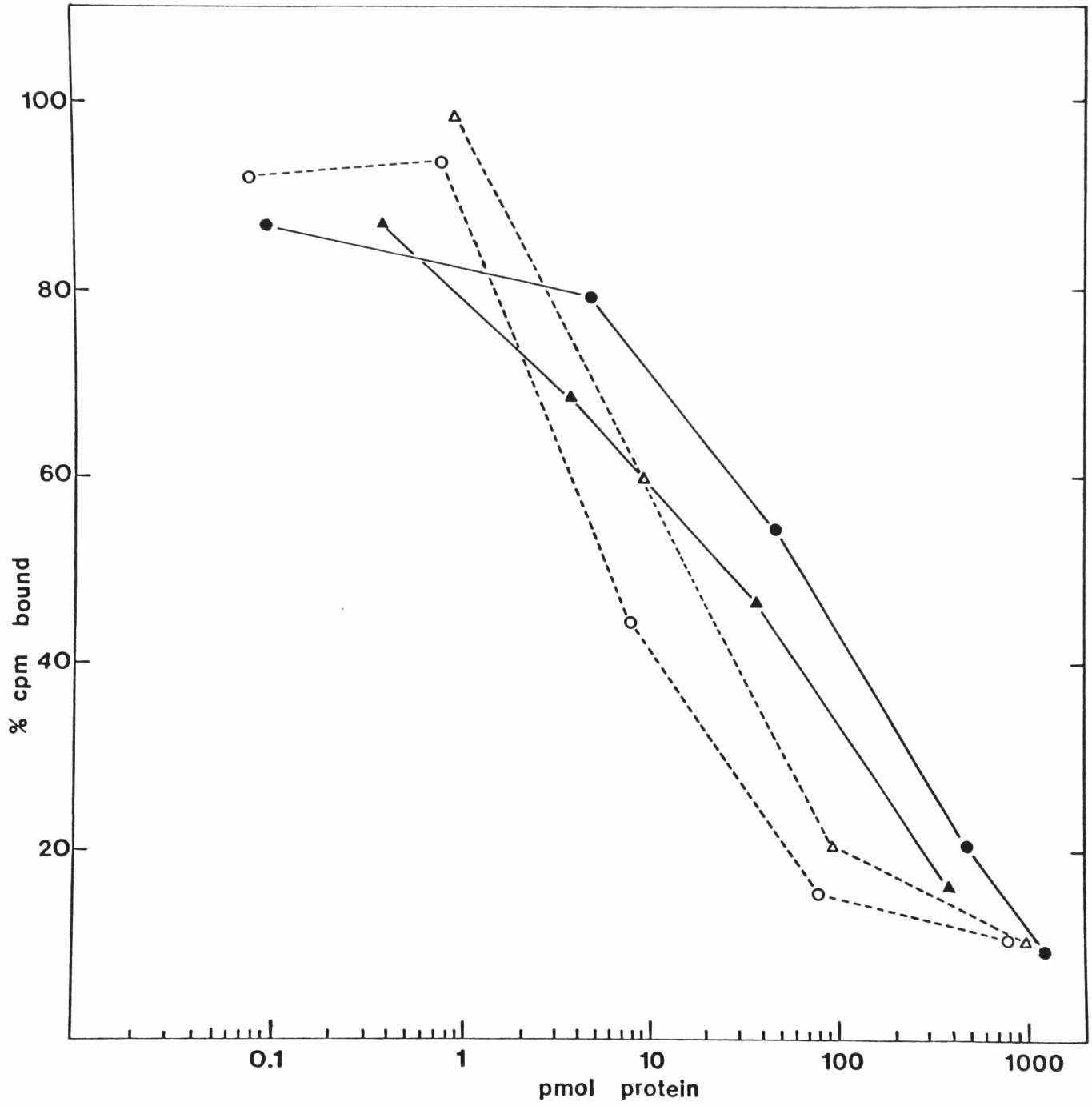

100
80
60
40
20
% cpm bound
0.1
1
10
100
1000
pmol protein

FIGURE 30: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by calmodulin digested with mouse submaxillary gland protease. The ordinate expresses the degree of inhibition as a percentage of the radioactivity bound in the absence of competing protein. The abscissa shows the amount of competing protein: bovine brain calmodulin (●) and bovine brain calmodulin digested with mouse submaxillary gland protease (○). The results shown are the means of duplicate determinations, and the range of values was less than 10%.

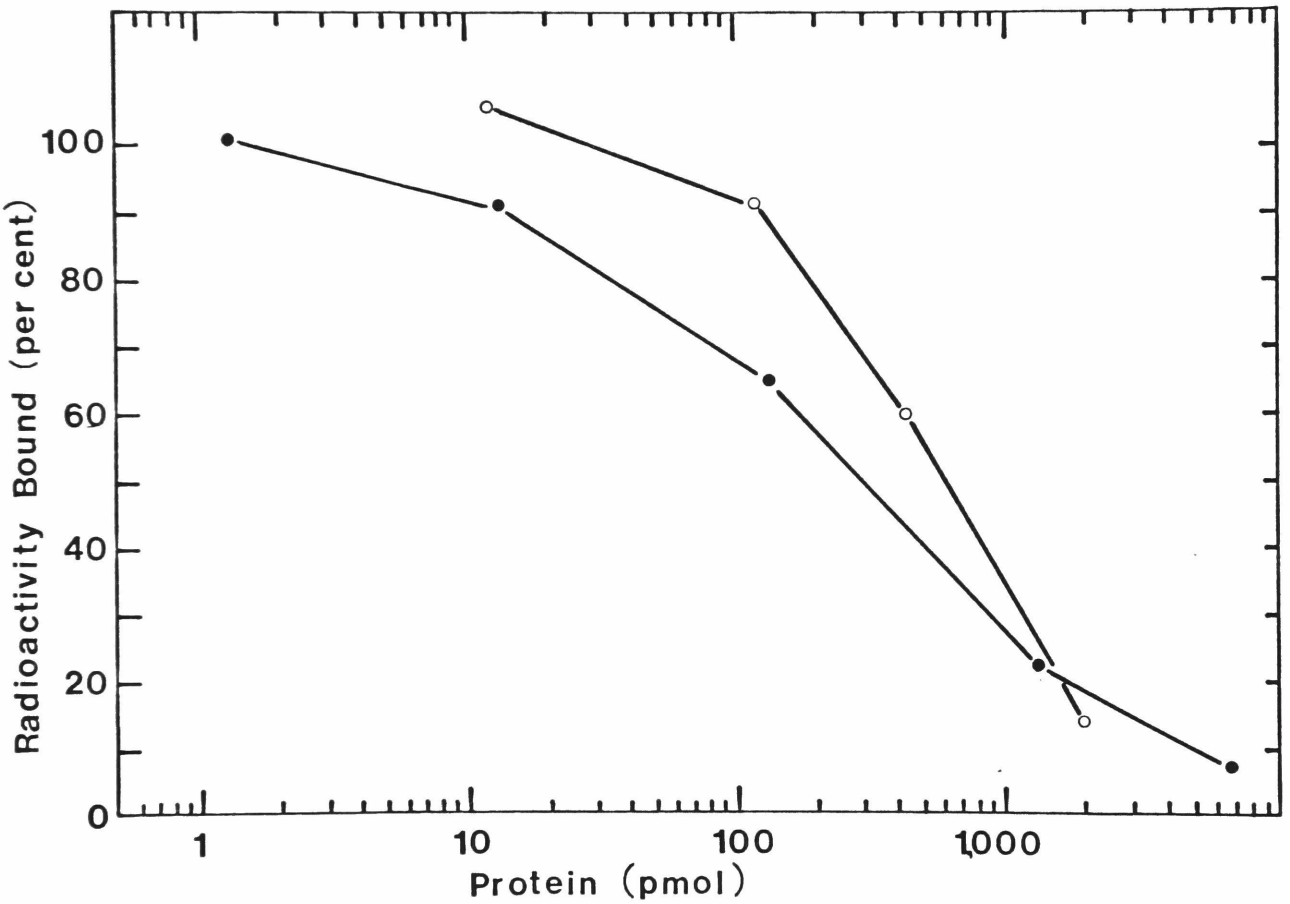

Radioactivity Bound (per cent)
100
80
60
40
20
0
1
10
100
1000
Protein (pmol)

FIGURE 31: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by synthetic peptides. The ordinate expresses the degree of inhibition as a percentage of the amount of radioactivity bound in the absence of competing protein. The abscissa shows the amount of competing protein: bovine brain calmodulin (●); peptide FF25 (■); peptide AF20 (□); and peptide AE21 (▲). A mixture of peptides AE21 (12 nmol) and FF25 (10 nmol) is also shown (○). The results are the means of duplicate determinations, and the range of values was <10%. The amino acid sequences of the synthetic peptides are shown in Table 7.

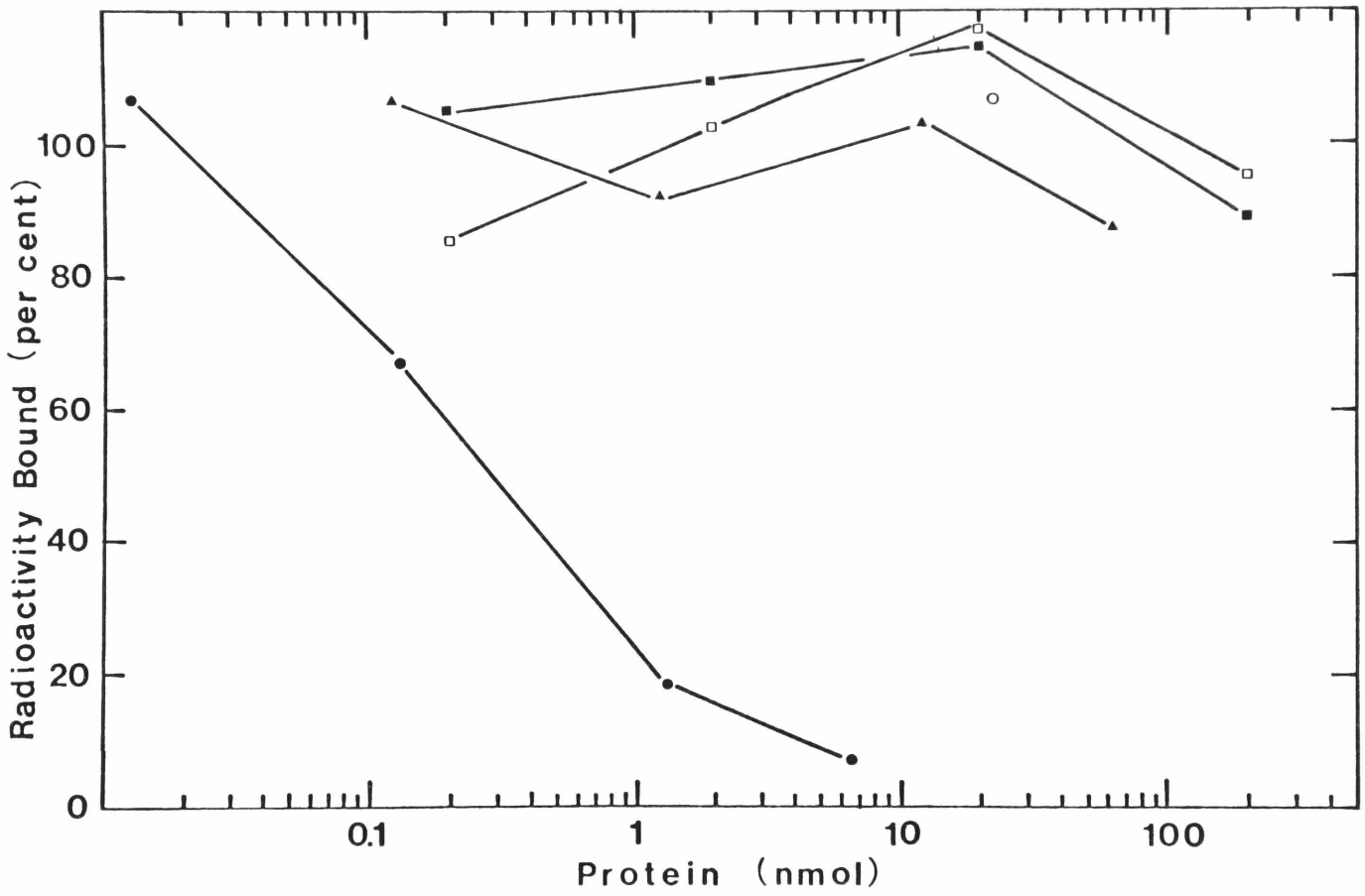

Radioactivity Bound (per cent)
0
20
40
60
80
100
0.1
1
10
100
Protein (nmol)

FIGURE 32: Competitive inhibition of ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates by performic acid oxidized calmodulin. The ordinate expresses the degree of inhibition as a percentage of the radioactivity bound in the absence of competing protein. The abscissa shows the amount of competing protein: bovine brain calmodulin (●), and performic acid oxidized bovine brain calmodulin (■). The results are representative of three separate experiments.

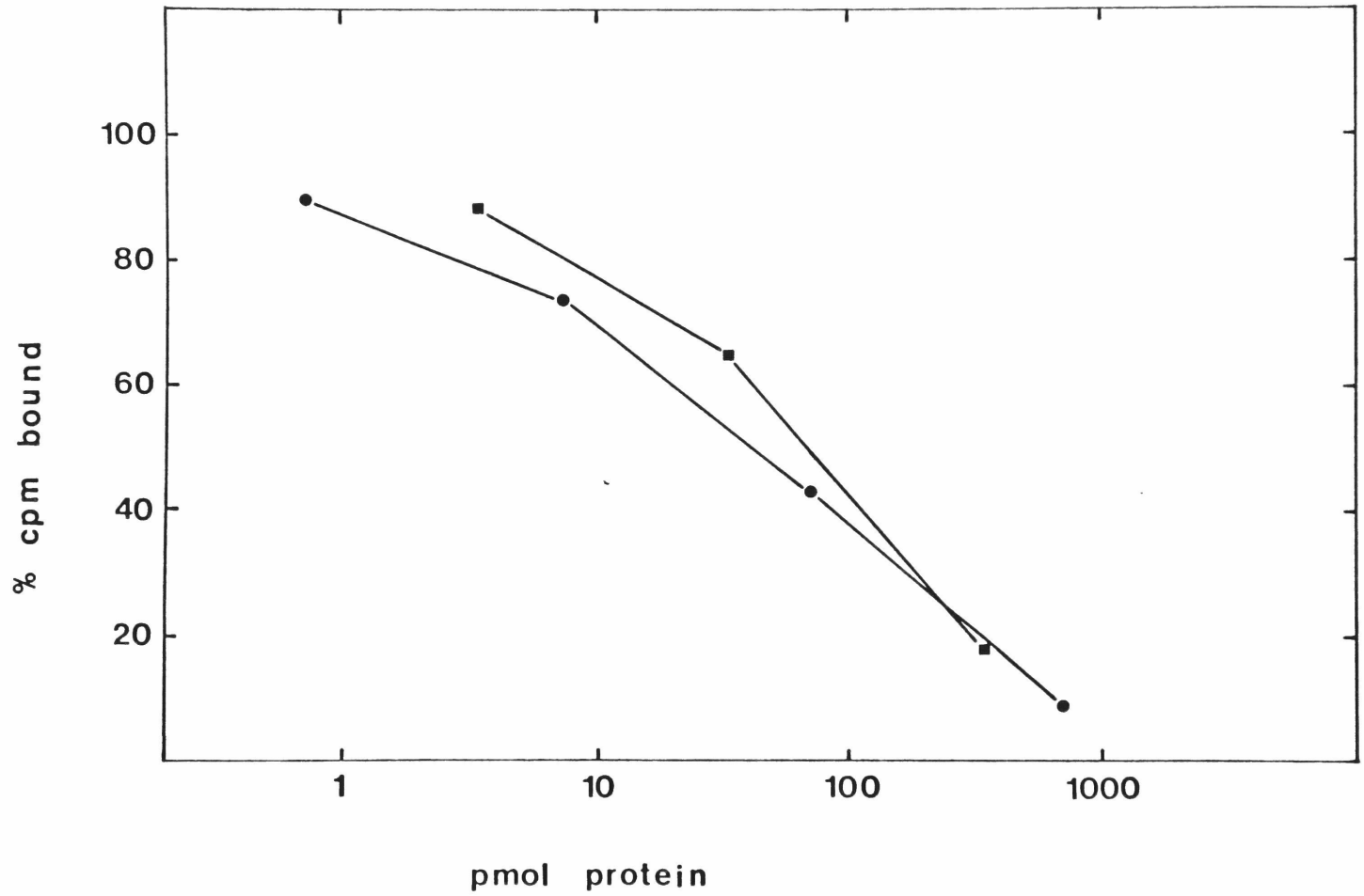

100
80
60
40
20
% cpm bound
1
10
100
1000
pmol protein

FIGURE 33: Activation of cyclic nucleotide phosphodiesterase by chicken gizzard calmodulin (●) and bovine brain calmodulin digested with mouse submaxillary gland protease (○). The ordinate shows the amount of AMP produced during an incubation for 6 minutes at 30 °C with 60 μg of enzyme preparation as described by Watterson et al. (181). The abscissa shows the amount of calmodulin or calmodulin digest added to the assays. The results are the means of duplicate determinations with the range of values as shown.

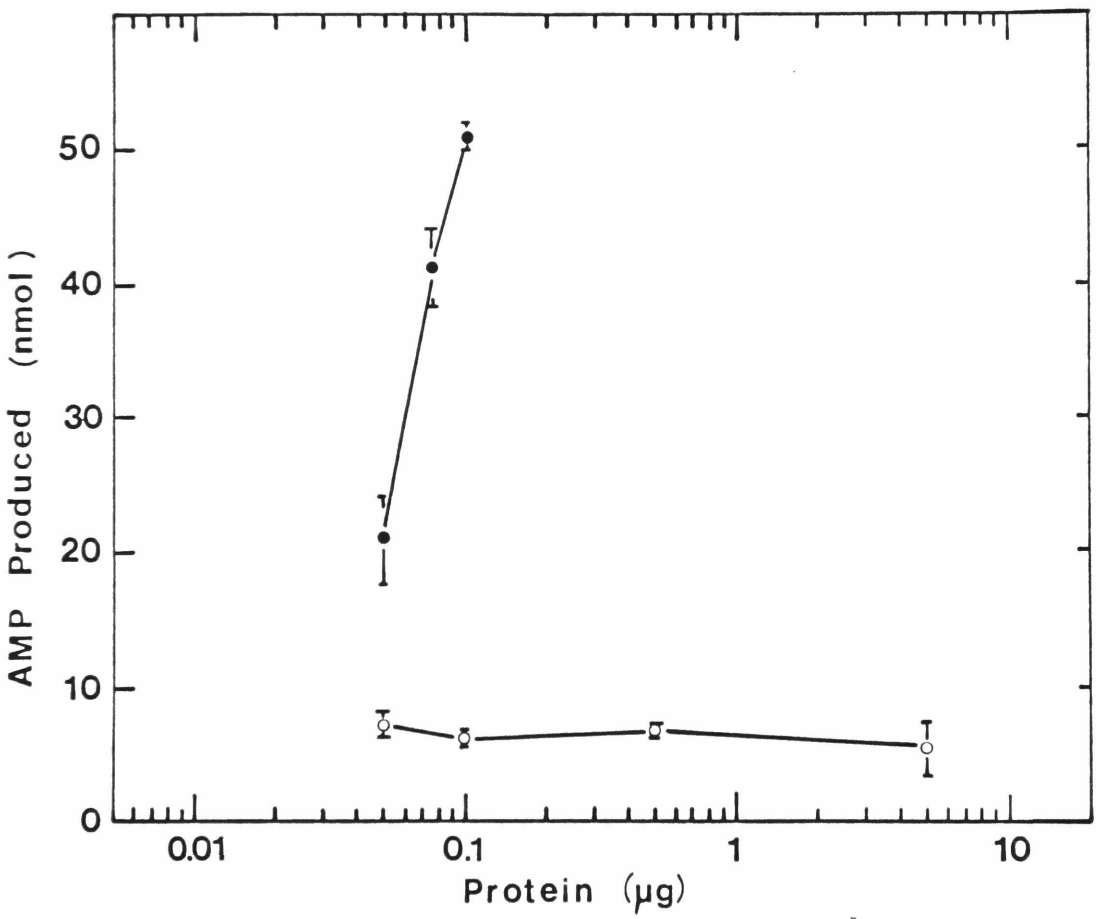

AMP Produced (nmol)
50
40
30
20
10
0
0.01
0.1
1
10
Protein (μg)

FIGURE 34: Effects of trypsin treatment on calmodulin activities. Samples (0.1 mg) of bovine brain calmodulin were treated with various amounts of trypsin for 14 hours at 37 °C as described in the text. The treated protein was tested for its ability to activate cyclic nucleotide phosphodiesterase (○), and to inhibit ^{125}I-calmodulin binding to phenothiazine-Sepharose conjugates (●). The ordinate expresses the activity of each sample as a percentage of the activity of bovine brain calmodulin which was incubated under identical conditions without trypsin. The activities were measured with an amount of protein equal to the amount of control calmodulin necessary to give full enzyme activation (0.3 nmol) or full inhibition of ^{125}I-calmodulin binding to the immobilized phenothiazines. The abscissa shows the amount of trypsin in the digestion. The results shown are the means of duplicate determinations and the range of values was <10%.

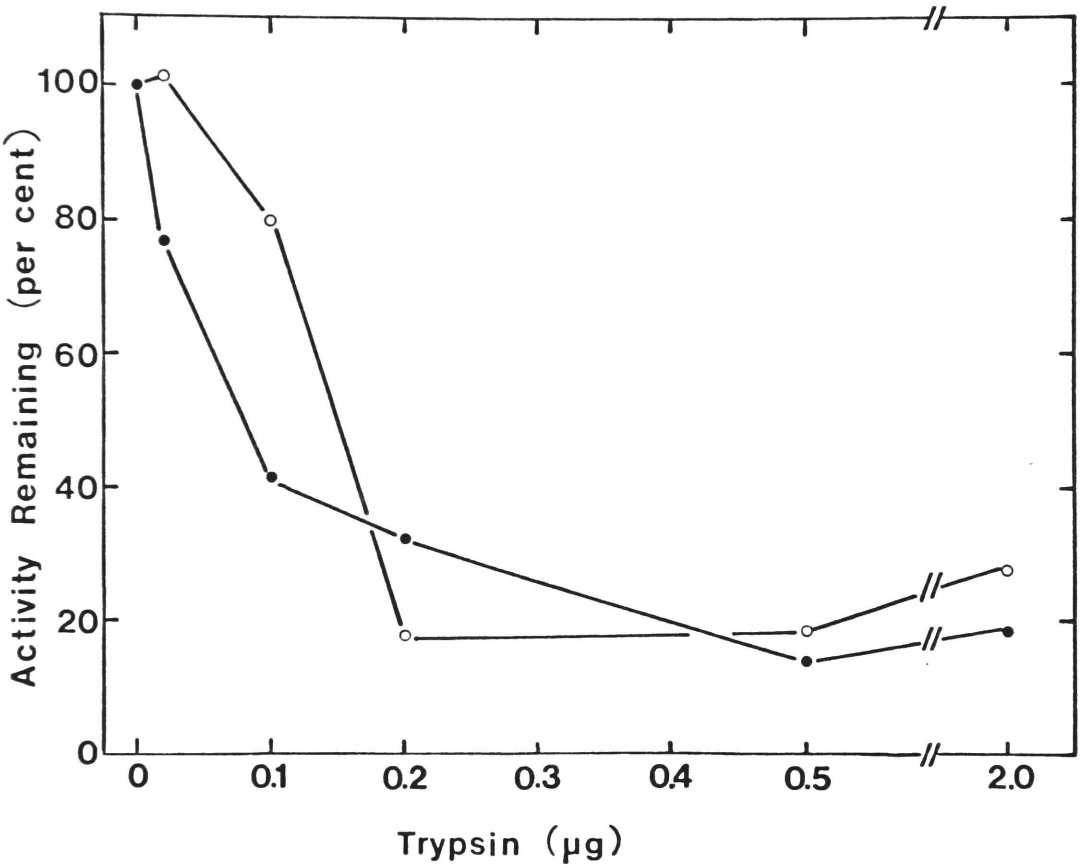
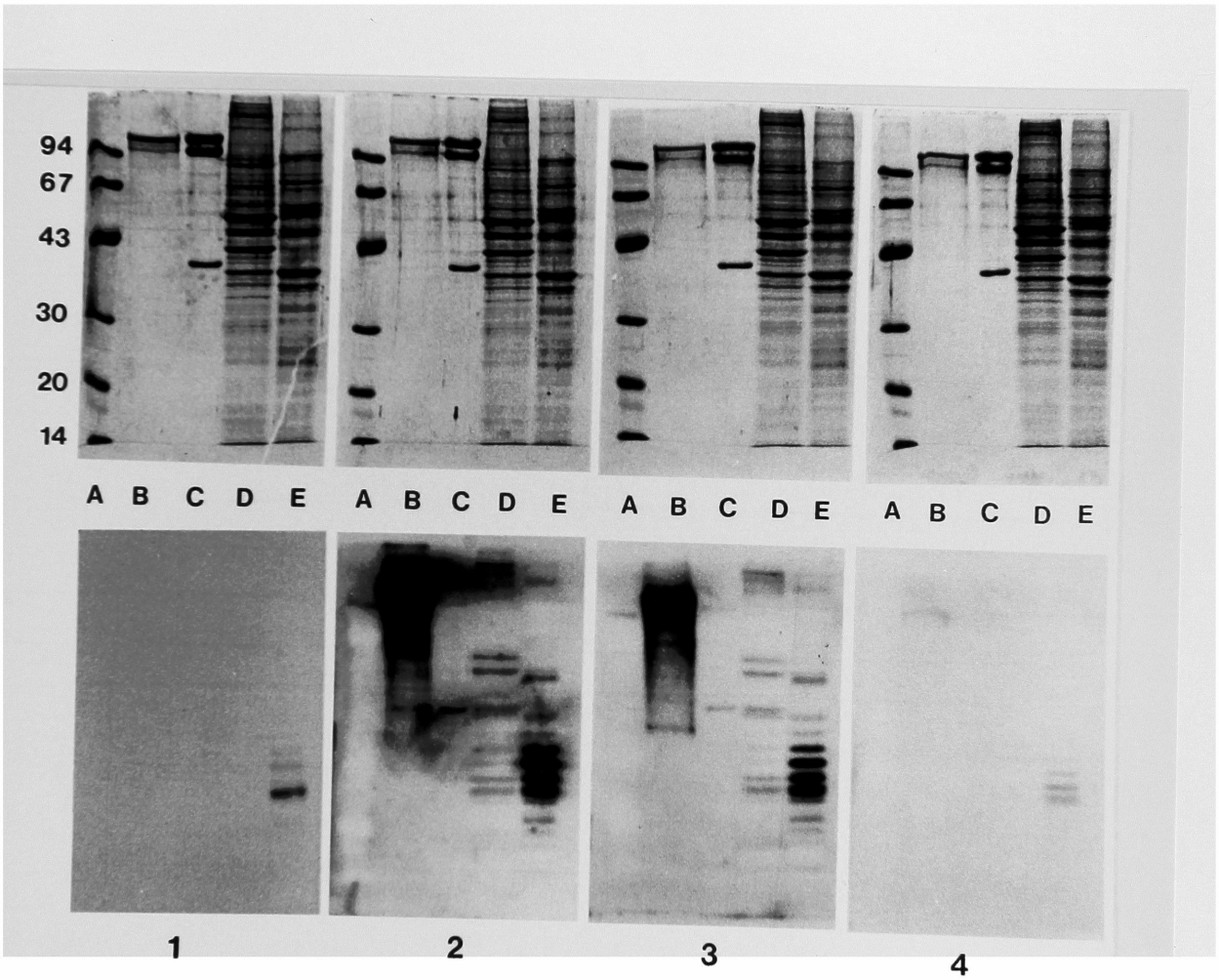

Activity Remaining (per cent)
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
2.0
Trypsin (μg)

FIGURE 36: Effect of chlorpromazine on ^{125}I-calmodulin binding to proteins in a gel overlay experiment. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described in the text on four identical gels with the following samples: A, molecular weight markers (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400); B, chicken gizzard myosin light chain kinase (3 μg); C, rabbit skeletal muscle phosphorylase b kinase (5 μg); D, chicken embryo fibroblast post-microsomal supernatant fraction (13 μg); E, chicken embryo fibroblast microsomal fraction (12 μg). Overlay procedures were performed as described in the text. For each gel, the top panel shows the Coomassie blue staining pattern and the lower panel shows the corresponding autoradiogram depicting the binding of ^{125}I-calmodulin. Before incubation with the gel, ^{125}I-calmodulin was incubated for 30 minutes at room temperature in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl with the following additions: gel 1, 4 mM EDTA; gel 2, 1 mM $CaCl_2$; gel 3, 1 mM $CaCl_2$ and 10 μM chlorpromazine; gel 4, 1 mM $CaCl_2$ and 100 μM chlorpromazine.


94
67
43
30
20
14
A B C D E
A B C D E
A B C D E
A B C D E
1
2
3
4

APPENDIX: Automated Sequencer Determination of Peptides

Peptides were isolated from digests of *Dictyostelium* calmodulin and bovine brain S100β by reverse phase chromatography on octadecylsilanyl resin as described in the text. The peptides were dissolved in water or 0.01 N ammonium bicarbonate, and the protein concentration was determined by amino acid analysis.

An aliquot (1300-2300 pmol) of each peptide was subjected to automated amino acid sequencer determination with an Applied Biosystems 470A instrument using the standard program supplied by the manufacturer. Fractions from the sequencer were dried and dissolved in methanol for analysis. Phenylisothiocyanyl-amino acids were identified by high performance liquid chromatography on a column (0.41 x 25 cm) containing octadecylsilanyl resin (Zorbax ODS, DuPont) operated at 60 °C. The elution solvents consisted of A: 50 mM sodium acetate, pH 5.0 and B: acetonitrile. The PTH-amino acids were eluted at a flow rate of 1.5 ml/min, and the absorbance of the effluent was monitored at 254 nm. The following programmed steps were used: 0 min., %B 10; 8 min., %B 40; 12 min., %B 40; 13 min., %B 55; 16 min., %B 60.

TABLE A1: Automated Sequencer Determinations[1] of Peptides from Bovine Brain S100β

Peptide 58-74: 1780 pmol

Cycle	Residue	pmol
1	Glutamic acid	1345
2	Threonine	308
3	Leucine	400
4	Aspartic acid	305
5	Serine	42
6	Aspartic acid	107
7	Glycine	20
8	Aspartic acid	38
9	Glycine	20
10	Glutamic acid	18

Peptide 80-91: 1350 pmol

Cycle	Residue	pmol
1	Isoleucine	332
2	Threonine	75

Peptide 6-20: 1310 pmol

Cycle	Residue	pmol
1	Alanine	408
2	Valine	218
3	Valine	211
4	Alanine	200
5	Leucine	150
6	Isoleucine	163
7	Aspartic acid	410
8	Valine	137
9	Phenylalanine	-
10	Histidine	-
11	Glutamine	-
12	Tyrosine	-
13	Serine	-
14	Glycine	-

[1] PTH- amino acids obtained at each cycle were identified by liquid chromatography as described in the Appendix. The recoveries of PTH-Serine and PTH-Threonine were variable, and these residues were also identified as their dehydro forms. The last 6 residues of peptide 6-20 were identified qualitatively.

TABLE A2: Automated Sequencer Determination of Peptide 17 from *Dictyostelium* Calmodulin[1]

Cycle	Residue	pmoles	Cycle	Residue	pmoles
1	Histidine	683	19	Isoleucine	119
2	Valine	908	20	Arginine	-
3	Methionine	749	21	Glutamic acid	85
4	Threonine	204	22	Alanine	115
5	Serine	62	23	Aspartic acid	94
6	Leucine	650	24	Leucine	103
7	Glycine	392	25	Aspartic acid	76
8	Glutamic acid	497	26	Glycine	84
9	Lysine	311	27	Aspartic acid	58
10	Leucine	587	28	Glycine	84
11	Threonine	294	29	Glutamine	50
12	Asparagine	366	30	Valine	56
13	Glutamic acid	371	31	Asparagine	44
14	Glutamic acid	333	32	Tyrosine	43
15	Valine	282	33	Glutamic acid	29
16	Aspartic acid	234	34	Glutamic acid	18
17	Glutamic acid	190	35	Phenylalanine	5
18	Methionine	214	36	Valine	12

[1] Peptide 17 (2300 pmoles) was subjected to automated sequence analysis, and the PTH-amino acids obtained at each cycle were identified by liquid chromatography as described in the text. Recoveries of PTH-Serine and PTH -Threonine were variable and were also identified qualitatively as their dehydro forms. PTH-Arginine was identified qualitatively.

ABBREVIATIONS

ACM	acetaminocarboxymethyl
ACP	acyl carrier protein
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCCP	biotin carboxyl carrier protein
Boc	tert-butyloxycarbonyl
cAMP	cyclic adenosine 3'-5' monophosphate
cGMP	cyclic guanosine 3'-5' monophosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
GTP	guanosine 5'-triphosphate
Met(O)	methionine sulfoxide
NAD	nicotinamide adenine dinucleotide
PTH	phenylthiohydantoin
SEM	standard error of the mean
TPCK	L-1-(p-toluenesulfonyl)amido-2-phenylethylchloromethyl ketone
Tris	tris(hydoxymethyl)aminomethane

ABBREVIATIONS FOR AMINO ACIDS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X		Unknown
Y	Tyr	Tyrosine

End