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Alpha$_2$-Macroglobulin: Its Purification and Interactions with Proteases, Lectins, and Cellular Receptors

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$ALPHA_2$-MACROGLOBULIN: ITS PURIFICATION AND INTERACTIONS WITH PROTEASES, LECTINS, AND CELLULAR RECEPTORS

BY

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VITA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

ATEE	Acetyl tyrosine ethyl ester
CD	Circular Dichroism
CGN	Alpha-N-Benzyloxycarbonyl glycine - p-nitrophenyl ester
CLN	Alpha-N-Benzyloxycarbonyl-L-lysine - p-nitrophenol ester
CON A	Concanavalin A
CPM	Counts per minute
DMEM	Delbecco's Modified Eagle Medium
DTNB	5, 5' Dithiobis (2-nitrobenzoate)
DTT	Dithiothreitol
EDTA	(ethylenedinitrilo) - tetraacetate
HEPES	N-2' - Hydroxyethyl piperazine-N' - 2, ethane - sulfonic acid
IEF	Isoelectric Focusing
HSA	Human Serum Albumin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
SBI	Soybean tryspin inhibitor
SDS	Sodium dodecyl sulfate
TAME	Tosylarginine methyl ester
TCA	Trichloracetic acid

CHAPTER 1

INTRODUCTION

Human alpha$_2$-Macroglobulin is a major component of serum and of extravascular fluids (James, 1980). Although the liver is considered to be the primary site of synthesis for circulating alpha$_2$-Macroglobulin, synthesis and secretion have detected in human blood monocytes (Hovi, 1977), human alveolar macrophages, (White, 1980), human lung fibroblasts (Mosher, 1977), human melanoma cells (Morgan, 1984), murine peritoneal macrophages (White, 1981), rat astrocytes (Giebicke-Haerte, 1987), and rabbit spleen cells (Chang, 1981). Alpha$_2$-Macroglobulin is a high molecular weight protease inhibitor capable of inhibiting the majority of known proteases which differ widely in their origins, mechanisms of action, and specificities. Active proteases from all four catalytic classes (Hartley, 1960) (serine, cysteine, carboxyl and metalloproteases) will interact with alpha$_2$-Macroglobulin (Barrett, 1973). Among those enzymes inhibited are the plasma proteases, kallikrein, factor Xa, thrombin, plasmin, and the major neutrophile proteases elastase, cathepsin G, gelatinase, and collagenase, (Harpel, 1970; Savesen, 1983). Alpha$_2$-Macroglobulin also inhibits lysosomal enzymes such as cathepsin B, which is reported to be secreted in large amounts by some tumor cells (Pietras, 1981), and cathepsin D, which is present in elevated levels in osteoarthritic cartilage (Phadke, 1979). This broad range of inhibitory activity makes alpha$_2$-Macroglobulin unique as a plasma protease inhibitor. However, little direct evidence exists demonstrating alpha$_2$-Macroglobulin's role as a regulator of extracellular proteolytic activity (Harpel, 1983). One reason has been the difficulty of

identifying alpha$_2$-Macroglobulin-protease complexes in extracellular fluids because of their rapid removal by cells of the reticuloendothelial system (Imber, 1983).

Alpha$_2$-Macroglobulin's ability to form complexes with all four catalytic classes reflects its unique physical and chemical composition. For clairty, these areas will be discussed individually in the following sections.

A. Physical Characterization

The numerous purification procedures for alpha$_2$-Macroglobulin (Frenoy, 1972; Roberts, 1974; Harpel, 1973; Kurecki, 1979; Virca, 1978; Barrett, 1973; Steinbach, 1965; Song, 1975; Wickerhauser, 1922; Schonenberger, 1958) have included physical characterization of this glycoprotein. A comparison of the literature values for various physical properties of purified alpha$_2$-Macroglobulin indicates large discrepancies between laboratories. The values for the absorption coefficient ($A^{1\%}$, 280) range from 6.9 to 11.1 (Schonenberger, 1958; Hall, 1978; Dunn, 1967; Schultze, 1966; Brown, 1954), while the values for sedimentation coefficient ($S^{o}_{20,w}$) ranges from 18.1 S to 20.6 S (Schonenberger, 1958; Hall, 1978; Jones, 1972; Dunn, 1967). Similarly, the estimates for the molecular weight vary from 620,000 to 820,000 daltons (Frenoy, 1972; Schonenberger, 1958; Hall, 1978; Jaones, 1972; Saunders, 1971). Isoelectric focusing reveals that native alpha$_2$-Macroglobulin can be separated into numerous species, with all species demonstrating trypsin binding activity. The number of species observed as well as the pI of each species differ from laboratory to laboratory. The value of pI of native alpha$_2$-Macroglobulin varies between 4.1 and 6.4 (Schonenberger, 1958; Rosen,

1979; Frenoy, 1974; Back, 1983; Parsons, 1976; Ohlsson, 1976). Frenoy et al (1974) used IEF to resolve native alpha$_2$-Macroglobulin into four species with pI values ranging from 4.97 to 5.50, while Rosen's group (Rosen, 1979) focused native alpha$_2$-Macroglobulin into seven species with their pI's varying between 4.1 and 4.9. Data published on the pI of complexed alpha$_2$-Macroglobulin exhibits similar variability. Parson et al (Parsons, 1976) and Back et al (Back, 1983) indicate that formation of a complex with a protease causes the pI to increase, while Barrett et al., (1979) failed to detect an appreciable change in pI upon complex.

The variability in the values of these physical constants suggests that alteration of protein could occur during purification. Many of these methods contain one or more steps which can lead to a physically or chemically altered product. This appears to have resulted in some variability in published reports of the functions and physical properties of alpha$_2$-Macroglobulin. Failure to minimize activation of Hageman Factor (Cochrane, 1973) or use of primary amines, e.g. ammonium sulphate precipitation represent two major drawbacks of some of these procedures. Nevertheless, investigators have made great strides in determining the mechanism of complex formation.

Complex formation appears to be initiated by proteolytic cleavage of the "bait region" of the alpha$_2$-Macroglobulin molecule. The particular peptide bonds which are cleaved reflect the specificity of the protease used (Sottrup-Jensen, 1981; Hall, 1981). From the data obtained with the four classes of proteases, it has been deduced that the bait region contains the hexapeptide -Arg-Val-Gly-Phe-Tyr-

Glu-(Mortensen, 1981), and that complex formation can be initiated by cleavage of any of the peptide bonds in this sequence. The bait region is located near the middle of four identical 180,000 dalton subunits (Barrett, 1979; Hall, 1978; Sevenson, 1979a). Tetrameric alpha$_2$-Macroglobulin is a 720,000 dalton protein that can be reversible dissociated into two half molecules (Jones, 1972; Harpel, 1973; Gentou, 1968; McConnelly, 1974; Abe, 1972). Reduction of the 360,000 half molecules liberates the two apparently identical subunits with molecular weights of 180,000 daltons. Complex formation with a proteolytic enzyme results in the cleavage of the 180,000 dalton subunit and the appearance of 85,000 dalton fragments as evidenced by SDS-PAGE under reducing conditions (Harpel, 1973). Fragments with molecular weights less than 85,000 were also obtained for native alpha$_2$-Macroglobulin after reduction and performic acid oxidation in the presence of guanidine HCl (Frenoy, 1972; Roberts, 1974). However, it is quite possible that these preparations were either contaminated with or previously exposed to proteases.

The active sites of proteolytic enzymes complexed to alpha$_2$-Macroglobulin remain accessible to low molecular weight substrates and inhibitors; substrates with molecular weights of less than 300 daltons are hydrolyzed at rates which are 80-100% of those obtained with the free enzyme (Barrett, 1973). However, with protein substrates the hydrolytic rates are diminished by 75-00%. Utilizing this unsual property of alpha$_2$-Macroglobulin, investigators infused the serine protease, brinase into animals, to lyse experimentally induced thrombi (Roschlau, 1964). Moreover, clinical invesigations have demonstrated the thrombolytic effect of brinase in man (Fitzgerald, 1979).

Recently, investigators have characterized the interaction of purified human alpha$_2$-Macroglobulin with brinase (Larsson, 1988). Brinase binds to purified alpha$_2$-Macroglobulin with a stoichiometry of 1.7-1.9 moles of enzyme per mole of alpha$_2$-Macroglobulin. Upon saturation, the alpha$_2$-Macroglobulin brinase complex exhibits a considerable amount of proteolytic activity, corresponding to about 25% of that of the free enzyme. This proteolytic activity probably accounts for the thrombolytic effect of brinase in vivo. The active site of the complexed enzyme is not accessible to enzyme inhibitors with molecular weights exceeding 9000 daltons, but smaller inhibitors may cause partial or complete inhibition (Barrett, 1973). On the basis of such observations, Barrett and Starkey proposed that proteolytic enzymes become irreversibly entrapped within an internal space in the alpha$_2$-Macroglobulin molecule during complex formation. Radioactively-labeled proteases do not readily dissociate from the complex, nor can an excess of the enzyme displace the bound label (Barrett, 1973; Saunders, 1971). The entrapment of the protease suggests that alpha$_2$-Macroglobulin undergoes a conformational change on enzyme binding. Electron microscopy (Schramm, 1982) and PAGE (Barrett, 1974) have shown the complex to be more compact that the native molecule.

Some laboratories have demonstrated that the conformation of alpha$_2$-Macroglobulin plasmin appears to differ from the conformation of other alpha$_2$-Macroglobulin protease complexes. I^{125}-alpha$_2$-Macroglobulin-plasmin complexes are cleared from the mouse circulation slower than I^{125}-alpha$_2$-Macroglobulin-methylamie complex (Ney, 1985). Additionally, differential scanning calorimetry was used to probe the conformational change that occurs upon complex formation (Cummings,

1984). Native alpha$_2$-Macroglobulin displayed a single thermal transition characterized by a temperature midpoint (T_m) of 65.8°C, a calorimetric enthalpy (H_c) of 2,550 Kcal/mol and a van't Hoff enthalpy (H_{vh}) of 140 Kcal/mol. The thermodynamic parameters for the alpha$_2$-Macroglobulin-methylamine complex are as follows: T_m = 62.8°C; H_c = 1700 Kcal/mol and H_{vh} = 169 Kcal/mol. When plasmin was complexed to native alpha$_2$-Macroglobulin, the resulting thermogram displays two T_m values, 68.5 $\pm$ .5°C and 77°C. These data suggest that the conformational change induced by alpha$_2$-Macroglobulin-methylamine differs from that of alpha$_2$-Macroglobulin plasmin. Additionally, the electrophoretic mobility of alpha$_2$-Macroglobulin increases following interaction with proteases (Steinbach, 1965; Fine, 1975). In nondenaturing PAGE, alpha$_2$-Macroglobulin plasmin migrated to a position intermediate to that of native alpha$_2$-Macroglobulin and the alpha$_2$-Macroglobulin trypsin complex. This data from conformational and thermodynamic studies is supplemented by data from chemical characterization studies. The protease and inhibitor were subjected to chemical modifications in an attempt to clarify the process of complex formation.

B. Chemical Characterization

Chemical characterization of the protease inhibitor interaction was initially performed using ammonium salts. Treatment of native alpha$_2$-Macroglobulin with ammonium salts produced a form of the inhibitor which has an identical electrophoretic mobility as complexed alpha$_2$-Macroglobulin (Barrett, 1979). Based on electrophoretic mobility, Barrett has designated that native and complexed forms of alpha$_2$-Macroglobulin as 'slow' and 'fast' alpha$_2$-Macroglobulin

respectively (Barrett, 1979). The conversion of 'slow' alpha$_2$-Macroglobulin to 'fast' alpha$_2$-Macroglobulin by ammonium ions was shown to involve cleavage of an internal thiol ester bond. Recent evidence indicates that one labile thiol ester linkage is located in each of the 180,000 dalton subunits (Sottrup-Jensen, 1981a). This internal thiol ester bond which is formed between a carbonyl group of a glutamyl residue and the sulfhydryl group of a cysteinyl residue, is also cleaved during treatment with a alkylamines or proteases (Sottrup-Jensen, 1981a; Van Leuven, 1982a; Sottrup-Jensen, 1981a; Sottrup-Jensen, 1982). During alkylamine treatment, a stoichiometric amount of alkylamine is incorporated into the reactive Glx-residue to form γ-glutamyl-alkylamide with the concomitant appearance of free SH-groups (Sottrup-Jensen, 1981a; Swenson, 1979b). Similarly, the complexes formed between alpha$_2$-Macroglobulin and proteases may involve a covalent bond (Sottrup-Jensen, 1981b; Sottrup-Jensen, 1981a; Salvesen, 1980; Harpel, 1976), however, the extent of covalent binding ranges from 8.3% to 61.2% (Salvesen, 1980).

The formation of covalently bound complexes was further clarified by Wang et al. (Wang, 1981) using native trypsin, anhydrotrypsin, and two active lysyl-blocked trypsin derivatives, methyl-trypin and dimethylamelety-trypsin. His results demonstrated that all enzymes reacted with alpha$_2$-Macroglobulin, but only native trypsin formed covalent complexes which were not dissociable by SDS. Alpha$_2$-Macroglobulin recovered from the complex with anhydrotrypsin contained intact 185,000 molecular weight polypeptide chains, whereas the alpha$_2$-Macroglobulin removed from complexes with proteolytically active enzymes had been cleaved to the 85,000 dalton fragments.

Following removal of the blocking groups from dimethyl-maleyl-trypsin, the enzyme became covalently bound to alpha$_2$-Macroglobulin. Additional results demonstrated that methylamine could react with noncovalent alpha$_2$-Macroglobulin protease complex, but not with covalent complexes. Methylamine treated alpha$_2$-Macroglobulin can still bind trypsin, but these complexes are noncovalent and appear to be formed at a drastically reduced rate. These results suggest that the methylamine binding site and the site of covalent attachment of proteases are identical. It has been proposed that covalent attachment involved the ϵ -amino groups of residues in the protease which attack the internal thiol ester bond in the inhibitor to produce a ϵ -lysyl- γ -glutamyl bond.

The reactivity of the thiol ester linkage has currently been under investigation. In native alpha$_2$-Macroglobulin the thiol ester bond will react with very small nucleophiles such as ammonia, methyl and ethylamine, but not with 1, 3-diaminopropane, indicating that this group is located in a pocket which shields it from even moderately sized solutes (Barrett, 1979). Immediately following exposure of alpha$_2$-Macroglobulin to proteases, this electrophilic group is freed from steric hindrance and becomes much more reactive (Salvesen, 1981), and can covalently attach to putrescine, TLCK-trypsin, and insulin (Sottrup-Jensen, 1981c). Interestingly, alpha$_2$-Macroglobulin, C3 and C4 all contain the reactive B-cysteinyl- γ -glutamyl thiol ester. This thiol ester bond is located in the alpha chain of C3 and C4 and in each of the four identical monomers of alpha$_2$-Macroglobulin (Thomas, 1982). The incubation of small nitrogen nucleophiles or proteases with any one of these three plasma proteins result in a

conformational change concomitant with the cleavage of the thiol ester bond and the appearance of free SH-groups (Salvesen, 1981; Janatova, 1980). The small nitrogen nucleophiles cause alpha$_2$-Macroglobulin to lose its protease binding ability and C-3 and C-4 to lose its hemolytic activity.

A comparison of the amino acid sequences of alpha$_2$-Macroglobulin, C3, and C4 reveals that these proteins share many short stretches of strong sequence homology (Sottrup-Jensen, 1982). Structural studies on alpha$_2$-Macroglobulin indicate that this 720,000 tetramer consists of four identical subunits (Jones, 1972; Barrett, 1979). Alignment of the sequences of one monomer of alpha$_2$-Macroglobulin with murine pro-C3 reveal eight extended regions of sequence similarity. The B-chain region of pro-C3 (residues 1-642) match well with the NH_2-terminal 609 residues of alpha$_2$-Macroglobulin, while extended stretches from the alpha-chain region of pro-C3 (residues 725-1639) correspond well with segments in the COOH-terminal 740 residues of alpha$_2$-Macroglobulin. These regions of similarity show between 19% and 31% sequence identity. If chemically similar residues are included, sequence similarity ranges between 32% and 40%. Not surprisingly, some of the physical and chemical properties alpha$_2$-Macroglobulin, C-3 and C-4 are strikingly similar. The circular dichroism spectra of alpha$_2$-Macroglobulin and C3b and C4b exhibit extended regions of B-sheet conformation and very little alpha-helix (Isenman, 1981; orski, 1981). These data tend to support common ancestry for these proteins.

C. Alpha$_2$-Macroglobulin's Interaction with Receptors

Receptor mediated endocytosis of alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin-protease complex has been studied extensively. Initial reports indicated that cells contain independent receptors for either native alpha$_2$-Macroglobulin or alpha$_2$-Macroglobulin protease complexes, i.e. receptors which bind only complexes or receptors which bind both (Maxfield, 1979; Willingham, 1978; Dickson, 1981; Van Leuven, 1977; Mosher, 1980; Kaplan, 1979a; Van Leuven, 1979; Debanne, 1975; Dickson; Via, 1982; Ohlsson, 1976; Tycks, 1982; Dickson, 1982). Additionally, methylamine treated alpha$_2$-Macroglobulin is also recognized and internalized by fibroblasts (Van Leuvenn, 1981), and macrophages (Kaplan, 1981; Imber, 1981; Fuchs, 1981). The uptake of methylamine and trypsin pretreated alpha$_2$-Macroglobulin was not inhibited by excess amounts of asialoorosomucoid, fucosyl-BSA, mannosyl-BSA, and N-acetyl glucosaminyl-BSA (Imber, 1981). These results indicate that the receptors which mediate the clearance of 'fast' alpha$_2$-Macroglobulin are distinct from those which recognize terminal galactose, fucose, mannose, or N-acetyl glucosamine residues and which mediate the clearance of the corresponding aglycosyl glycoproteins. Covalent modification of the ϵ-amino groups of lysyl residues on alpha$_2$-Macroglobulin block both antibody and receptor binding. This suggests that the receptor recognition site in alpha$_2$-Macroglobulin may reside in the protein portion of the molecule. [Van Leuven (1983), Gonias (1983)] (Marynen, 1982). Another study has demonstrated that the cysteine thiol group, which is liberated by the methylamine reaction, does not participate in receptor mediated uptake of alpha$_2$-Macroglobulin protease complexes [Van Leuven, P. (1982)].

When native alpha$_2$-Macroglobulin is allowed to react with methylamine in the presence of 2,4-dinitrophenyl thiocynate, an "anomalous" "slow" form of alpha$_2$-Macroglobulin is produced as shown by rate electrophoresis. The 2,4-dinitrophenyl thiocynate, which is a specific cyanylating agent for thiol groups, blocks the conformational change of alpha$_2$-Macroglobulin, producing a "slow" form of alpha$_2$-Macroglobulin with its thiolesters cleaved. The addition of trypsin to the cyanylated form overrides the conformational block and converts the cyanylated derivative to the "fast" form of alpha$_2$-Macroglobulin. Trypsin treatment of these cyanylated derivatives results in production of anomalous complexes which exhibit proteolytic activity and are fully inhibited by soybean trypsin inhibitor. Additionally, these anomalous complexes were internalized by fibroblasts, and recognized by a monoclonal antibody (F2B2) directed against the receptor-recognition site on alpha$_2$-Macroglobulin complexes. Interaction of native alpha$_2$-Macroglobulin with a bacterial endoprotease, (Endoprotease Lys-C) resulted in proteolysis of alpha$_2$-Macroglobulin without the hydrolysis of the internal thioester or conformational change (Marynen 1986, Van Leuven, 1986). Treatment of this "proteolyzed form" of alpha$_2$-Macroglobulin with trypsin or methylamine produced the usual conformational change, resulting in exposure of the receptor-binding domain. Limited proteolysis of alpha$_2$-Macroglobulin methylamine with endoprotease Lys C results in the loss of the receptor recognition site. The addition of the monoclonal antibodies F_2B_2 and $F_{12}A_3$ to alpha$_2$-Macroglobulin methylamine protected the receptor site from proteolysis by endoprotease Lys C and permitted the isolation of a fragment which

contains the receptor binding domain. Competitive binding experiments (Marynen, 1986) indicated that the isolated fragment was capable of competing with alpha$_2$-Macroglobulin trypsin complex for the cellular receptor on human fibroblasts. On a molar basis, the fragment appears to bind two orders of magnitude less well to the receptor than the alpha$_2$-Macroglobulin trypsin complex. Additional characterization of the fragment was conducted with SDS-PAGE in the presence and absence of reductant. Under reducing conditions the mobility of the fragment was decreased, indicating the presence of a disulfide bridge. When the I^{125}-labeled fragment was treated with N-glaycanase to remove N-linked carbohydrate, the enzymatically treated fragment revealed an increase in electrophoretic mobility on SDS-PAGE. This data indicates that carbohydrate is present on the fragment. Reduction and alkylation for sequence determination led to the discovery that the carboxyl terminal sequence (-Asp-Leu-Gly-Asn-Ala) of the fragment was identical to the caraboxyl terminus of alpha$_2$-Macroglobulin. Production of this 20,000 dalton fragment results from cleavage of the Lys^{1313} Glu bond. In native alpha$_2$-Macroglobulin, proteolysis at the Lys^{1313}-Glu abond did not result in dissociation of the receptor binding domain until after the thiolester bonds were cleaved by methylamine or trypsin [Van Leuven, 1988]. How the receptor binding site remain associated with native alpha$_2$-Macroglobulin remains to be determined. Van Leuven et al., (1988) have suggested that the stretch of amino acids containing Lys^{1313} are part of the hypothesized hinge region.

Roche et al., (1987) have shown a correlation between receptor recognition site exposure and degree of subunit cleavage of alpha$_2$-

Macroglobulin plasmin complex. Incubation of alpha$_2$-Macroglobulin with a 2.4 molar excess of plasmin results in proteolysis of half the subunits of alpha$_2$-Macroglobulin. Incubation of alpha$_2$-Macroglobulin with trypsin or methylamine leads to cleavage of the four thiolester bonds and exposure of all four receptor recognition sites (Marynen, 1983). Purified alpha$_2$-plasmin complexes, with half of the subunits cleaved, demonstrated about half the in vitro receptor binding of alpha$_2$-Macroglobulin molar ratio produced cleavage of the remaining subunits with the concomitant exposure of the two additional receptor recognition sites. In vivo clearance studies are consistent with this observation; the cleavage of the two remaining subunits by trypsin or plasmin increases receptor binding 2 fold (Roche, et al., 1987).

Recently, cis-dichlorodiammine platinum (II) (cis-DDP) was used to investigate the correlation between thiolester bond cleavage and exposure of the receptor recognition sites (Roche, et al., 1988). Reaction of cis-DDP with native alpha$_2$-Macroglobulin results in extensive cross-linking of the subunits. Interaction of cis-DDP treated alpha$_2$-Macroglobulin with trypsin results in complete subunit cleavage in the bait region without the concomitant hydrolysis of the thiolester bonds or exposure of the receptor recognition site. Removal of the intersubunit crosslinks with diethyldithiocarbamate (DDC) led to the characteristic increase in electrophoretic mobility of the DCC treated protein to the position of alpha$_2$-Macroglobulin trypsin ('fast' form) as well as cleavage of thiol ester bonds and exposure of receptor recognition sites. These results suggest that in human alpha$_2$-Macroglobulin, there is conformational change following bait region cleavage, which normally leads to thiolester bond

cleavage. DDP appears to prevent this conformational change thereby producing a "proteolytically primed form" of alpha$_2$-Macroglobulin.

From this data, it can be concluded that bait region cleavage is insufficient to cause receptor site exposure. Furthermore, these experiments support the hypothesis that receptor recognition site exposure is dependent upon thiol ester bond cleavage and conformational change which occurs concomitantly with proteolysis at the bait region.

D. Tentative Role and Molecular Models for Alpha$_2$-Macroglobulin

The capability of alpha$_2$-Macroglobulin of inhibiting numerous proteases has led to speculations about the involvement of alpha$_2$-Macroglobulin in the regulation of proteolytic enzyme cascades in plasma and interstitial fluids, the control of tumor outgrowth and tissue remodeling, as well as the modulation of inflammatory and immune responses (Starkey, 1977; Hubbard, 1981; Johnson, 1982; Koo, 1982; Steiner, 1987;). Increased levels of alpha$_2$-Macroglobulin have been reported during acute inflammatory tissue injury, pregnancy, immediately after birth, and in some neoplastic states (Northemann, 1983; Truden, 1988). Recent studies have also suggested a role for alpha$_2$-Macroglobulin in the regulation of plasmin activity. In cases of inherited deficiencies of alpha$_2$-Macroglobulin interacted with plasmin only after alpha$_2$-plamin inhibitor was totally consumed. However, in recent studies, Harpel (Harpel, 1981) has been able to detect alpha$_2$-Macroglobulin-plasmin complexes when plasmin was added to plasma at levels well below those required to saturate alpha$_2$-plasmin inhibitor. Also, the partitioning of plasmin between its complexes with alpha$_2$-plasmin inhibitor and alpha$_2$-Macroglobulin

depended on whether plasmin was added to plasma directly or whether plasmin was generated endogenously by activation with urokinase. The mechanisms which regulate the distribution of plasmin between the two inhibitors have not been ascertained. Steiner et al., (Steiner, 1987) conducted kinetic experiments to determine the association rate of Lys_{77}-plasmin and Val_{442}-plasmin with $alpha_2$-Macroglobulin. The association rates were 1.34×10^5 $M^{-1}S^{-1}$ and 1.75×10^{-5} M^{-1} S^{-1} for Lys_{77}-plasmin and Val_{442}-plasmin respectively. The antifibrinolytic agents, ϵ -aminocaprioc acid and histidine-rich glycoprotein had little effect on the rate of association of plasmin with $alpha_2$-Macroglobulin. ϵ -Aminocaproic acid, at a concentration of 1.0 mM was not inhibitory, while the presence of 12 uM histidine rich glycoprotein slightly reduced the association rate to 0.31×10^5 M^{-1} S^{-1}. In contrast, work by Lijnen (Lijnen, 1980) has shown that these antifibrinolytic agents greatly reduce the association of plasmin with $alpha_2$-plasmin inhibitor to a rate comparable to that measured for the reaction of plasmin with $alpha_2$-Macroglobulin. Thus, HRG and other antifibrinolytic agents, could possibly function to regulate the distribution of plasmin between $alpha_2$-Macroglobulin and $alpha_2$-plasmin inhibitor. The role of $alpha_2$-Macroglobulin as modulator of the immune system is currently under investigation. Wilson et al., (1983) have shown that macrophages produce compliment C3 and C5, and secrete a variety of mediators such as ciliary cyskinesia substance. $Alpha_2$-Macroglobulin protease complexes appear capable of suppressing these functions (Wilson, 1983). Experiments with murine T cells have indicated that T cell proliferation can be specifically augmented by macrophages exposed to an $alpha_2$-Macroglobulin antigen conjugate e.g.

alpha-galactosidase covalently bound to alpha$_2$-Macroglobulin (Osada, et al., 1987). Hubbard et al., have shown that T-cell response is suppressed when alpha$_2$-Macroglobulin-trypsin complexes are added to human mixed lymphocyte cultures (Hubbard, 1981).

In order to understand the effects of alpha$_2$-Macroglobulin-trypsin complex on the cellular immune system, one needs to consider earlier observations that low molecular weight substrates are readily degraded by alpha$_2$-Macroglobulin protease complexes (Largmann, et al., 1977). In addition, these findings have been extended by the observation that alpha$_2$-Macroglobulin trypin complexes can degrade polypeptide hormones. For example, alpha$_2$-Macroglobulin trypsin can convert proinsulin to insulin, while free trypsin will degrade the prohormone to inactive products (Largmann, 1977). Thus, the alpha$_2$-Macroglobulin-trypsin complex may exert its effects on the immune response by modifying or degrading polypeptide modulators.

Other investigators have examined the interaction of alpha$_2$-Macroglobulin trypsin complex with Interleukin-2, the sole proliferative stimulus for the cloned interleuken-2-dependent murine cell line, CTLL-20 (Teodorescu, M. and Barth, W., 1986). In order to determine whether the suppression of the T-cell response to interleukin-2 by alpha$_2$-Macroglobulin-trypsin complex is due to degradation of the interleukin-2, the following experiments were performed. Aprotinin (trypsin inhibitor), trypsin, methylamine treated alpha$_2$-Macroglobulin, alpha$_2$-Macroglobulin trypsin complex or alpha$_2$-Macroglobulin trypsin complex plus aprotinin were added to culture medium containing interleukin-2. Incubation of native alpha$_2$-Macroglobulin, aprotinin, or methylamine-alpha$_2$-Macroglobulin did not

result in any loss of IL-2 activity when measured by [^{3}H] TdR uptake by the CTLL-20 cells. In contrast, alpha$_2$-Macroglobulin-tryspin complex inactivated interleukin-2 at a rate one-sixth of that of free trypsin. Aprotinin, a low molecular weight (MW6500) inhibitor of trypsin was capable of preventing the inactivation of interleukin-2 by alpha$_2$-Macroglobulin-trypsin complex. Therefore, these observations suggest that the in vitro immunosuppressive effects of alpha$_2$-Macroglobulin-trypsin complex is due to its ability to degrade IL-2.

A number of laboratories have documented the binding of alpha$_2$-Macroglobulin to ions and proteins which lack protease activity. These studies implicate possible novel roles for this protease inhibitor. For example, alpha$_2$-Macroglobulin has been shown to bind to platelet-derived growth factor which is the principle mitogen in serum for cells of mesenchymal origin (Huang, 1984; Ross, 1978). Platelet-derived growth factor also acts as a powerful chemoattractant protein for inflammatory cells and for cells involved in wound repair (Deul, 1982; Seppa, 1982; Senior, 1983). These properties of chemotaxis and mitogenesis suggest that PDGF may be important in normal inflammation and repair as well as mediator in the abnormal process of atherosclerosis. Attempts to measure platelet-derived growth factor in human plasma resulted in the discovery of a specific plasma binding protein for platelet derived growth factor. PAGE and immunoprecipitation of platelet-derived growth factor-alpha$_2$-Macroglobulin complex helped establish alpha$_2$-Macroglobulin as the specific binding protein. 8 M urea, 1 M acetic acid, 0.1 M NaOH or 1% $NaDodSO_4$ failed to dissociate the alpha$_2$-Macroglobulin from the platelet derived growth factor while 2-mercaptoethanol successfully

dissociated the complex. N-ethylmaleimide and iodoacetamide completely blocked the binding of platelet derived growth factor to alpha$_2$-Macroglobulin. These sulfhydryl blocking reagents have no effect on the binding of proteases to alpha$_2$-Macroglobulin (Salvesen, et al., 1981). Methylamine inhibits the binding of proteases to alpha$_2$-Macroglobulin, but does not inhibit the formation of plasma derived growth factor - alpha$_2$-Macroglobulin-platelet derived complex. The physiological role for this alpha$_2$-Macroglobun-platelet derived growth factor remains unclear. Similarity in the amino acid sequence between platelet derived growth factor and p28 SIS, the transforming protein of simian sarcoma virus, suggests that viral and other cellular transformation events may be mediated by growth factor-like proteins. Therefore, alpha$_2$-Macroglobulin may be involved in regulating expression of platelet derived growth factor-like molecules into the extracellular space or may simply act as a scavenger of PDGF not locally bound to injured vessel walls. The binding of PDGF to alpha$_2$-macroglobulin may result in rapid clearance of the complex and thus limit PDFG's ability to stimulate both cell migration and cell division. Similarly, streptococci belonging to serological groups, A, B, and C have been shown to interact with alpha$_2$-Macroglobulin (Chhatwal, 1987). The alpha$_2$-Macroglobulin binding protein from group A streptococci has been purified by affinity chromatography and HPLC. It exhibited a molecular weight of 78,000 daltons on SDS-PAGE and possessed no proteolytic activity. Incubation of the purified binding protein with native alpha$_2$-Macroglobulin resulted in a change in conformation in the alpha$_2$-Macroglobulin similar to that observed as a result of complex formation with proteases. The reversibility of this

complex leads to speculation that the conformational change effected by the streptococcal binding protein proceeds via a different mechanism than that followed by alpha$_2$-Macroglobulin protease complexes. The biological significance of this data remains to be elucidated. Other investigators have examined the interaction of histone H3 and poly-L-lysine with native alpha$_2$-Macroglobulin, alpha$_2$-Macroglobulin methylamine, and alpha$_2$-Macroglobulin trypsin complex (Gonias, 1984). Under the conditions and concentrations utilized, histone H-3 exhibited the ability to precipitate alpha$_2$-Macroglobulin trypsin and alpha$_2$-Macroglobulin-methylamine, but not native alpha$_2$-Macroglobulin. Solutions containing poly-lysine and all the aforementioned forms of alpha$_2$-Macroglobulin failed to form precipitates. In addition to histone H-3, alpha$_2$-Macroglobulin binds to aspartate aminotransferase (Boyd, 1968) and encephalitogenic basic rotein (McPherson, 1970). These reactions are reversible but their in vivo significance remains unclear. Aside from proteins, divalent cations exhibit binding to alpha$_2$-Macroglobulin. Parisi (1970) and Giroux (1975) have shown that alpha$_2$-Macroglobulin is a major serum zinc binding protein.

The interaction of hystidyl residues in alpha$_2$-Macroglobulin with Zn^{++} was recently examined (Roche, 1987). Preincubation with Zn^{++} results in the precipitation of methylamine treated alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complexes but not native alpha$_2$-Macroglobulin. Treatment of the alpha$_2$-Macroglobulin protease complexes or alpha$_2$-Macroglobulin methylamine with the histidine specific acylating reagent, diethylpyrocarbonate, prevented the zinc induced precipitation. Other experiments have demonstrated

that the same number of histidine residues are modified by diethylpyocarbonate in alpha$_2$-Macroglobulin protease complex as in native alpha$_2$Macroglobulin. Inhibition of precipitation by diethylpyrocarbonate could be reversed by hydroxylamine treatment. Experiments were performed to determine whether the precipitation of alpha$_2$-Macroglobulin-methylamine by Zn^{++} and histone H-3 is mediated by a similar mechanism. The histone H-3 induced precipitation of alpha$_2$-Macroglobulin-methylamine and acylated alpha$_2$-Macroglobulin-methylamine was identical. These results suggest that histone H-3 precipitation of alpha$_2$-Macroglobulin-methylamine does not involve histidyl residues.

A few investigators have presented models of alpha$_2$-Macroglobulin which are consistent with the large body of known structural and functional properties of native and complexed alpha$_2$-Macroglobulin. Based on computerized electron microscopy Feldman et al. (1985) have presented a model of native alpha$_2$-Macroglobulin which depicts alpha$_2$-Macroglobulin as a hollow cylinder. This cylinder is composed of two identical functional halves with three two fold axis of symmetry. In electron micrographs, native alpha$_2$-Macroglobulin appears as the cyrillic letter  . Each half is made up of a ring with four arms (two per monomer) projecting outward. The other side of the ring makes contact with the second functional half. Reaction with the protease results in movement of the one long arm per monomer (two arms per functional half). This movement causes compacting of the arms with increased curvature of the arms. Furthermore, the authors postulate that the thiolester bond is located near the hinge of the trap arm of each subunit. Cleavage of the thiolester following

the interaction of alpha$_2$-Macroglobulin with protease or methylamine allows the trap arms to swing inward, thereby causing a conformation change. The proximity of the thiolester bond is consistent with the fluorescence energy transfer experiments of Ponchon (Ponchon, 1983), which concluded that the thiolester sites are in close proximity to the protease binding sites.

Studies on the stoichiometrics of complex formations have yielded conflicting results (Howell, 1983; Ponchon, 1981; Gonias, 1982). Feldmann's model of alpha$_2$-Macroglobulin has attempted to reconcile some of these differences. Small proteases such as trypsin and papain bind to alpha$_2$-Macroglobulin in a 2:1 protease-to-inhibitor ratio. Large proteinases such as plasmin bind in a nearly 1:1 ratio. This could occur because protrusion of one of the domains of plasmin across the hollow core into the second protease binding site in alpha$_2$-Macroglobulin could sterically prevent binding of a second proteinase molecule. Lastly, this model attempts to predict the location of the receptor-recognition sites of alpha$_2$-Macroglobulin. Since the trap arms are the only moving part of this model, the authors envision the location of the receptor recognition site to be at the base of the trap arms, near its hinge. There would be one recognition site present on each subunit (one recognition site present on each subunit; one per trap arm and two per half molecule). Interaction with proteinases or methylamine would result in exposure of these areas. Immunoelectron microscopy essentially supports Feldman's model except for the postulated location of the receptor recognition site. The use of monoclonal antibodies demonstrates that the receptor recognition domains are located at the tips of the

cyrillic letter . In native alpha$_2$-Macroglobulin these recognition domains appear obscured physiologically and immunologically, as opposed to electron micrographs which suggest they are quite accessible. Therefore, the simple movement of the trap arms cannot explain expression of the receptor recognition site at the extremities of the long arms. A change, more complex than slight movement of the two trap arms is therefore needed.

Bretaydiere et al., have proposed an alternative model (Bretaydiere, 1988). Computer processing of their electron micrographs indicate that the structure of native alpha$_2$-Macroglobulin resembles the shape of a padlock. These authors suggest that the 'open' and 'closed' form of the cyrillic letter are in fact the same structure viewed from the side and the end, respectively. Consequently, they propose that native alpha$_2$-Macroglobulin consists of the juxtaposition of two protomers. One protomer is shaped like a distorted S and the other is its reversed image. Each protomer is composed of two covalently linked subunits. The noncovalent interactions between the protomers would take place at the apex where the ends meet, and near the bottom, where the two strands overlap. Protease treatment of native alpha$_2$-Macroglobulin would result in rearrangement of the protomers to form the cyrillic letter  . This conformational change may be envisioned by a shift of the binding domain from the bottom of the protomer toward the middle, with a concomitant opening of the four arms.

Additional studies are necessary to elucidate the exact structure, function, and mechanism for alpha$_2$-Macroglobulin. My work will assist this endeavor by providing a mild three step procedure to

purify large quantities of alpha$_2$-Macroglobulin. Previous procedures utilized harsh techniques or failed to minimize activation of endogenous proteases. My experiments will examine the interaction between alpha$_2$-Macroglobulin, proteases, and ions using a variety of physical, chemical and enzymatic procedures to further clarify the mechanism of action of alpha$_2$-Macroglobulin. Circular dichroism, ultraviolet difference spectra, gel filtration, and hydrophobic interaction chromatography will be employed to investigate the conformational change which accompanies complex formation. A DTNB titration of alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex will be undertaken to assess changes in the sulfhydral titer upon complex formation. Carbohydrate studies will evaluate changes in the exposure of oligosaccharide chains and examine their involvement in receptor mediated endocytosis of alpha$_2$-Macroglobulin by cultured cells. These studies are to provide additonal knowledge on alpha$_2$-Macroglobulin's structure and function.

CHAPTER II

EXPERIMENTAL

A. Materials

1. Chromatography

a. Molecular Sieve

Sepharose 4B, Sepharose CL-6B, Sephacryl S-300, Sephadex G-25, and Sephadex G-75 were obtained from Pharmacia Fine Chemicals. A HPLC column, TSK-G300 SW was purchased from Toyo Soda.

b. Ion Exchange

CM-32 cellulose and DE-32 cellulose were products of Whatman Chemical Company. CM-Sephadex DEAE-Sephadex, and Sulphopropyl-Sephadex was supplied by Pharmacia. Hydroxyl apatite (regular and spherical) were obtained from Nutritional Biochemicals.

c. Hydrophobic Interaction

Ethyl, butyl, and octyl agarose were procured from Pierce Chemical Company.

d. Affinity

Concanavalin A was purchased from Sigma Chemical Company and Pharmacia Fine Chemicals. Wheat germ lectin was obtained from P. L. Biochemicals. Affigel ovalbumin and affigel blue were products of Bio-Rad Laboratories. Cyanogen bromide was supplied by Aldrich Chemical Company. A turkey ovomucoid resin was a gift of Dr. Richard Schultz. Alpha-methylmannoside was purchased from Sigma Chemical Company

2. Inhibitors

Soybean trypsin inhibitor was purchased from Worthington.

Sodium Heparin was obtained from the Upjohn Company. Benzamidine·HCl, tosyl-L-lysine chloromothyl ketone, and L-1-p-tosylamino-2-phenylethyl chloromethyl ketone were products of Aldrich Chemical Company. Egg white proteinase inhibitor was a gift from Dr. Susan Buktenica.

3. Isoelectric Focusing Reagents

Ampholytes were purchased from Brinkmann Instruments and Pharmacia Fine Chemicals. Lysine and arginine were products of Sigma. Electrode filter wicks were purchased from DeSaga. Chromatography paper MN-214 was obtained from Macheney Nageland Company. Precast polyacrylamide slabs for IEF were purchased from LKB.

4. Reagents for Active Site Titration

The titrant p-nitrophenyl p'-guanidino benzoate·HCl was purchased from Sigma. Dimethyl formamide and acetonitrile were supplied by Mallinckrodt.

5. Anti-human whole sera, anti-human fibrinogen, and anti-human IgM, anti-human alpha$_2$-Macroglobulin serum were obtained from Miles. Agar immunodiffusion plates were supplied by Hyland. Ion Agar was a product of Consolidated Laboratories.

6. Materials for alpha$_2$-Macroglobulin Purification

Bentonite was obtained from Fisher Scientific. Concentration membranes were a product of Amicon. Acrodisc filters (0.2 micron) were purchased from Gelman, while 0.45 micron filters were purchased from Millipore. Dialysis tubing was obtained from Fisher Scientific.

7. Cell Culture Material

DMEM, virus screened fetal calf serum, penicillin-streptomycin

sulfate, and L-glutamine were purchased from Grand Island Biological Company. Lux polystyrene tissue culture petri dishes and multiplates were obtained from Microbiological Associates.

8. Labeling Reagents

$Na^{125}I$ was purchased from Amersham-Searle. Chloramine-T and lactoperoxidase were products of Sigma Chemical Company. The enzymobead kit for radioiodination was obtained from Bio-Rad Laboratories.

9. Electrophoresis

For flat electrophoresis and PAGE, the acrylamide, methylbis-acrylamide, Photo-Flow, TEMED, Alcian Blue and Bromophenyl Blue were obtained from Eastman Kodak Company. SDS was a product of Sigma. Coomassie Brilliant Blue was obtained from Schwarz Mann. Molecular weight standards were a product of Pharmacia Fine Chemicals. Periodic acid and cross-linked hemocyanin were products of Sigma.

10. Enzymes

Trypsin and alpha-chymotrypsin were products of Worthington. Papain was purchased from Sigma. Sialidase was supplied by Miles Laboratory.

11. Substrates

TAME was obtained from Sigma. CLN was a product of Aldrich Chemical Company. CGN was procured from Sigma.

12. Reagents for Sialic Acid Determination

Thiobarbituric acid and sialic acid were obtained from Sigma. Butanol was a product of Mallinckrodt.

13. Calibration Standards (Circular Dichroism and Fluroscence)

Standardization of the Jasco was performed using d-10 Camphorsulfonic Acid supplied by Durrum Instruments Company. Fluorescent standardization required Rhodamine, which was a product of Perkin Elmer.

14. Miscellaneous

Methylamine was purchased from Mallinckrodt. Zinc chloride, zinc acetate and magnesium chloride were obtained from Aldrich. All other chemicals used in this work were analytical grade and were obtained from commercial sources. All these reagents were used without further purification. A Gilson Mixograd gradient maker was purchased from Gilson.

B. Methods

1. Enzyme Assays for the Detection of Alpha$_2$-Macroglobulin

The determination of alpha$_2$-Macroglobulin concentrations was based on the ability of SBI to block the hydrolysis of TAME by free trypsin, but not by alpha$_2$-Macroglobulin bound trypsin. Stock solutions of trypsin (0.02 mg/ml-in 0.001 N HCl), SBI (0.5mg/ml in 0.05 M Tris-HCl, pH 8.10), and TAME (0.01 M in distilled water) were prepared and stored in the refrigerator until needed. The assay was initiated by adding 0.05 ml of sample and 0.1 ml of trypsin to 2.6 ml of a 0.05 M Tris-HCl, pH 8.10 buffer. The excess (unbound) trypsin which remained after reaction with alpha$_2$-macroglobulin in the sample was then inactivated by the addition of 0.1 ml of SBI. The activity of the alpha$_2$-macroglobulin bound trypsin was then measured by the adding of 0.3 ml of TAME and monitoring the hydrolysis products at 247

nm. The rate of hydrolysis of the substrate was found to be proportional to the amount of alpha$_2$-Macroglobulin trypsin complex present in the reaction. Samples were also assayed in the absence of trypsin in order to determine to what degree alpha$_2$-Macroglobulin may have been pre-exposed to a trypsin-like activity and to determine the extent of spontaneous hydrolysis of substrate. From time to time assays were performed to check the activities of the stock solutions of trypsin, SBI and TAME.

In some cases enzymes other than trypsin were utilized in the proteinase binding assay. Replacement of chymotrypsin for trypsin necessitated changing the substrate to ATEE. Its hydrolysis was measured as described by Schwert and Takenaka.

Papain activity was measured by the method of Bajkowski and Frankfater. Papain and alpha$_2$-Macroglobulin were preincubated for 30 minutes in 25 mM NaAc-2 mM DTT 10 mM EDTA, pH 4.0. Unbound papain was inhibited by an excess of egg white proteinase inhibitor. The activity of bound papain was then measured by adding either CGN or CLN and following the appearance of the p-nitrophenol product at 326 nm.

2. Column Chromotography

a. General Comments

Column chromatography was performed at 4°C in jacketed columns supplied by Pharmacia Fine Chemicals, unless otherwise stated. All columns were equipped adjustable with end adapters except for the 9 mm diameter columns for which adapters were not available.

Sephadex G-25, Sephadex G-75 superfine, DEAE Sephadex, Sulfopropyl-Sephadex and CM-Sephadex were preswollen as per the manufacturer's instructions and then treated to remove fine particles.

After degassing the resin and packing the chromatography column, the gel bed was equilibrated with two or three bed volumes of degassed buffer before use. In the case of DEAE-Sephadex, CM-Sephadex, or Sulphopropyl-Sephadex, the resin was washed until the desired pH was attained.

The flow rate of the column was maintained with either a Cheminert Metering Pump or a LKB peristaltic pump. The effluent of the column was monitored either with a Beckmann DB-G spectrophotometer containing a flow cell (0.3 ml capacity and 1 cm light path - Beckmann Instruments) or a LKB Uvicord II Fractionator. Fractions were collected with a Gilson Microfractionator.

b. Precycling CM-Cellulose

Whatman CM-32 microgranular cellulose was precycled prior to use according to the manufacturer's instructions. The cation exchanger was poured into a beaker containing fifteen volumes of 0.5 N NaOH (15 ml of NaOH per 1 g. of dry exchanger). The resin was stirred although care was taken that it not be too vigorous or too prolonged. The resin remained in contact with the sodium hydroxide for at least thiry minutes, but not more than 2 hours. After aspirating off the supernatant liquor, the exchanger was washed in a buchner funnel with distilled water until the effluent was pH 8.0. The resin was next washed with 0.5 N HCl for at least thirty minutes but not more than 2 hours, and the supernatant removed by aspiration. The acid wash was then repeated. The aspirated resin was then added to a 10 times concentration of column equilibration buffer. While slowly stiring, the exchanger was then titrated to the correct pH with the base component of the buffer. The resin was allowed to settle, after which

the supernatant was removed by aspiration and replaced with a sufficient volume of equilibration buffer so that the slurry volume was 115% of the settled volume. The slurry of CM-cellulose was then poured into a chromatography column washed with the equilibration buffer until the pH and conductivity of the effluent was the same as that of the starting buffer.

c. Purification of Concanavalin A

Concanavalin A (Pharmacia Fine Chemicals) was purified by affinity chromatography on an affi-gel ovalbumin column. Eight ml of an affigel ovalbumin resin was washed with a 0.02 M Hepes - 1.0^{-6} uM $MnCl_2$ 1.0^{-6} uMM $CaCl_2$ and adjusted to pH 7.4. The flow rate was 10 ml/hour. Following sample application, the resin was washed with equilibration buffer until the absorbance of the effluent at 280 nm dropped to zero. To ensure that all the non-binding protein was eluted, the affinity resin was washed with the Hepes buffer for one additional bed volume. The bound protein was then eluted by the addition of buffer containing 0.5 M alpha-methylmannoside.

d. Purification of Trypsin

Trypsin was purified according to the method of Ryan (1975). A turkey ovomucoid-Sepharose column having a bed volume of 100 ml was equilibrated with 0.1 M Tris·HCl 0.1 M NaCl 0.12 M $CaCl_2$, pH 8.0 at a flow rate of 24 ml per hour. Fifteen mg of trypsin were applied to the column and the column was washed with buffer until all non-binding protein had been eluted. Trypsin was then eluted with a solution containing 0.2 M KCl HCl pH 2.0.

e. Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography was performed with ethyl, butyl, and octyl agarose. Minicolumns were constructed from dispo pipettes, glass wool, polypropylene tubing, and screw clamps. The bed volume of the columns was maintained at 1.1 ml. The resins were all washed with at least four bed volumes of equilibration buffer at a flow rate of 10 ml/hour. The equilibration and elution buffers utilized for each resin are listed below:

Resin Number	Type of Resin	Equilibration Buffer
1	Ethyl Agarose	0.05 M NaPhosphate 0.1 M NaCl, pH 7.40
2	Ethyl Agarose	0.02 M NaAcetate 0.1NaCl, pH 5.29
3	Butyl Agarose	0.05 M NaPhosphate 0.1MNaCl, pH 7.40
4	Butyl Agarose	0.02 M NaAcetate 0.1MNaCl, pH 5.29
5	Octyl Agarose	0.05 M Tris·HCl, pH 8.10
6	Octyl Agarose	0.05 M Tris·HCl 0.3 M KCNS 0.3M $BaCl_2$, pH 8.10
7	Octyl Agarose	0.1 M Na Citrate - 10% ethylene glycol, pH 5.50
8	Octyl Agarose	0.02 M NaAcetate, pH 5.20.

Resin Number	Sequential Elution Buffers
5	0.05 M Tris·HCl 50% Glycerol, pH 8.10
	0.05 M Tris·HCl 50% Glycerol, pH 9.10
6	0.05 M Tris·HCl 0.3 M $BaCl_2$ 0.3 M KCNS 50% Glycerol, pH 8.10
7	0.05M Tris-0.3 M $BaCl_2$ 0.3 M KCNS, pH 8.10
	0.05M Tris-0.3 M $BaCl_2$ 0.3 M KCNS 50% ethylene glycol, pH 8.10
8	0.02 M NaPhosphate, pH 7.10
	0.02 M NaPhosphate pH 9.25
	0.02M NaPhosphate 10% Glycerol, pH 9.25
	0.02M NaPhosphate 50% Glycerol, pH 9.25

Fractions were collected with a Gilson Microfractionator, and subsequently assayed for the presence of alpha$_2$-Macroglobulin using the trypsin binding assay.

3. Absorbance Measurements

The absorbitivity of solutions was ascertained using either a CGA/McPherson Model 707 double beam recording spectrophotometer, a Beckman DB-G grating double beam recording spectrophotometer or a Perkin Elmer Model 310 double beam recording spectrophotometer. The cell compartment of each spectrophotometer was thermostated at 25 $\pm$ 0.2°C. Spectrophotometer cells which had a path length of 1 cm were composed of either quartz or silica (Beckmann Instruments, or Pyrocell Manufacturing Company, Inc.).

4. pH Measurements

Measurements of pH were performed either on a Corning Model 12 or Model 135 Research pH Meter with a number 476051 Corning

combination electrode.

5. Protein Determination

Protein was determined using one or more of the following procedures. When solutions did not contain other constituents expected to absorb in the far UV, protein concentrations were quantitated by absorbance measurements at 210 nm by assuming an absorption coefficient of 20.5 for a 1 mg/ml solution (Tombs, 1957). Absorbance measurements at 280 nm were used to quantitate solutions of highly pure proteins of known absorption coefficients. Protein concentrations were also determined with a Bio-Rad protein determination kit according to manufacturers instructions. BSA was chosen as the protein standard.

6. Purification of alpha$_2$-Macroglobulin

a. Preparation of Mg - Bentonite

Mg - Bentonite was prepared according to the method of Dunn and Hitchborn (1965). Bentonite was suspended in 0.01 M $Na_2HPO_4KH_2PO_4$ 0.01 M $MgSO_4$, pH 7.4 and stirred for 2 hours. The resulting Mg bentonite was then purified by differential centrifugation. After centrifugation at 4°C for 1 minute at 400g, the pellet was discarded. The supernatant was then centrifuged at 17000g for 15 minutes at 4°C., after which the pellets were saved and resuspended in 1.0 mM $MgSO_4$ 1.0 mM $Na_2HPO_4KH_2PO_4$, pH 7.4, and stirred for 2 hours. The entire centrifugation procedure is repeated prior to using the bentonite.

b. Addition of Mg-Bentonite to Citrated Plasma

In order to limit the activation of endogenous protease citrated plasma was placed in plastic labware and allowed to contact only

plastic utensils. A mixture of protease inhibitors were also added to the outdated citrated plasma. These were 0.005 M Benzamidine, 0.01 mg/ml SBI, and 2 units/ml Sodium Heparin. Four volumes of cold Mg-Bentonite was then slowly added to one volume of citrated plasma (room temperature) while gently stirring with a plastic rod. This mixture was allowed to sit for 10 minutes at room temperature and then stirred briefly prior to centrifugation. The Mg-Bentonite was removed by centrifugation at 17,000 g at $4^{o}C$ and the supernatent filtered, concentrated to one third the original volume of the plasma, and dialyzed against 0.02 M Na Barbital 0.1 M NaCl 10 uM ZnAcetate, pH 7.4.

c. Chromatography on Sepharose CL-6B

The Mg-Bentonite treated plasma from the previous step was applied to a CL-6B column having a bed volume of 1951 ml, equilibrated with 0.02 M NaBarbital 0.1 M NaCl 10 uM ZnAcetate, pH 7.40 at a flow rate of 100 ml/hour. Fractions were collected and assayed as described in a preceeding section. The fractions containing $alpha_2$-Macroglobulin were pooled and dialyzed against 0.02 M NaAcetate, pH 5.29.

d. Ion Exchange Chromatography

The dialyzed fractions from the previous step were applied to a CM-cellulose column having a bed volume of 40 ml), which had been prewashed as described by the manufacturer and pre-equilibrated with 0.02 M NaAcetate, pH 5.29 at a flow rate of 24 ml/hour. Following elution of all non-binding proteins, the $alpha_2$-Macroglobulin was eluted with 0.02 M NaAcetate 0.1 M NaChloride, pH 5.20.

7. Analysis of Purified Alpha$_2$-Macroglobulin

a. Electrophoresis

Gel electrophoresis was used to monitor the purification of alpha$_2$-Macroglobulin and to analyze the subunit structure of alpha$_2$-Macroglobulin. Polyacrylamide gel electrophoresis was performed on both cylindrical gels and slabs. To assess the purity of alpha$_2$-Macroglobulin, increasing amounts of native alpha$_2$-Macroglobulin (5 ug to 200 ug) were subjected to discontinuous PAGE according to the method of Davis. Native and complexed forms of alpha$_2$-Macroglobulin were run on slab gels under non-reducing or reducing-denaturing conditions according to the method of Laemmlin (1970). With some samples the denaturation procedure was modified slightly. These samples were first acidified to pH 3.0 prior to the addition of SDS. After a 15 minute incubation period in the SDS, the pH of the mixture was adjusted to 7.0 followed by the addition of the reductant. Upon completion of the electrophoresis, the gels were stained for proteins, or glycoproteins, or were assayed for trypsin binding activity when SDS was not used.

Coomassie Brilliant Blue was used to detect protein, while Periodic Schiff (Segnest) was employed to indicate the presence of glycoprotein. In order to assay for alpha$_2$-Macroglobulin, the gels were sliced into 1.5 mm sections and placed into 0.5 ml of 0.05 M Tris· HChloride, pH 8.1 for a period of one hour. During this incubation period, the acrylamide sections were periodically vortexed, after which aliquots were taken for the trypsin binding assay as previously described.

b. Isoelectric Focusing

Isoelectric points were determined by isoelectric focusing in polyacrylamide tube gels using a Hoeffer-type electrophoresis apparatus or in thin layers of either polyacrylamide or Sephadex G-75 SuperFine using a DeSaga/Brinkmann double chamber flat bed electrophoresis apparatus. Both systems required dialyzing the samples against distilled water prior to focusing. The amount of sample focused ranged from 20 ug to 120 ug. Prefocusing of the gel was allowed to take place prior to sample application. The focusing was carried out in the cold room at 4°C. A circulating pump attached to the apparatus provided additional cooling. Typically, the ampholyte concentration ranged from 3% to 5%. The power source utilized for both instruments was a Beckman Duostat.

The method of Eder (1972) was followed when IEF was performed in the Hoeffer type cell. The only modification of the procedure was to wash the gel tubes with nitric acid and coat them with a .5% solution of Photoflow 200. The polyacrylamide gels contained ampholytes which focus between pH 3.0 and pH 10.0. A current of 1 ma per tube was applied to the gels for 15 minutes. After layering the sample in the gel tube, the current was restored. A current of 1 ma per tube was maintained until a maximal voltage of 400 V. was reached, which requires approximately 2 hours. To ensure isoelectric equilibrium, this voltage was then maintained for another 5 hours. On completion of IEF, the gels were either cut into 1.5 mm segments for assay of trypsin binding activity or were fixed and stained for protein detection.

The DeSaga instrument allows IEF to be run on two types of support media, e.g., Sephadex or polyacrylamide. The IEF in both medias was run according to the manufacturer's instructions. When Sephadex was used, a slurry of swollen G-75 superfine was poured on to an acid washed 20 cm x 20 cm plate. The gel was distributed with a glass rod with gentle back and forth movement until a uniform gel layer was produced. The gel was dried at room temperature until fine cracks appeared on all four edges of the plate. At this point, the gel has lost 20 - 25% of its original water content. Filter wicks (DeSaga) were saturated in their respective electrolyte solutions, e.g., Anode: 2 $\underline{N}$ H_2SO_4 and Cathode: 0.4 $\underline{N}$ ethylene diamine. The saturated wicks were placed on the gel and platinum electrodes were placed over the wicks. The recommended voltage and time of IEF varied with the range of the ampholyte used. The conditions used are as follows:

pH Range of Ampholytes	Voltage and Time
2 - 10	4 hours at 200 V. and 2-4 hours at 400 - 800 V.
	OR
	16 hours at 200 V. and 1-2 hours at 400 - 800 V.
4 - 6	4 hours at 250 - 300 V. and 2-4 hours at 400 - 1000 V.
	OR
	16 hours at 250 V. and 1-2 at 400 - 1000 V.

Samples were applied in the form of a thin streak. Approximately, 10 to 40 ul of the protein solution was applied to the edge of a glass coverslip. The coverslip was then brought in contact with the gel layer without disturbing the gel. The protein samples were applied to the gel at various locations.

Upon completion of IEF the gel layer was blotted with MN-chromatography paper and the paper prints stained for protein. To assay for alpha$_2$-Macroglobulin, a small segment of gel was removed by scraping and added to assay buffer. Following centrifugation, an aliquot of the supernatant was assayed for trypsin binding activity.

Paper prints were stained for proteins in the following way: The prints were first dried and then subjected to 3 consecutive 30 minute washes with 10% TCA. The TCA was removed by washing the paper with a mixture of methanol:water:acetic acid (60:133:20) for 2-5 minutes.

The print was stained for 10 minutes with a 0.2% solution of Coomassie Brilliant Blue G250 made up in methanol:water:acetic acid (50:50:10). The paper was then washed consequentively with methanol:water:acetic acid (33:66:10) until the background was white.

The IEF was also performed on precast polyacrylamide slabs (LKB). The procedure followed was identical to the Sephadex thin layer IEF except that the samples were applied both with filter wicks as well as by streaking.

c. Immunological Analysis

The purity of the alpha$_2$-Macroglobulin preparation was verified by two immunological techniques, immunodiffusion and immunoelectrophoresis. Both procedures utilized an ion agar solution which was prepared according to the method of Campbell (1963). A stock ion agar solution was made by adding the following reagents

to a beaker: 10.0 g Ion Agar

8.5 g NaCl

1.0 g Sodium Azide

37.5 g Glycine

Distilled water to 1 liter.

The beaker was heated until the agar turned clear. Care was taken not to burn the agar. This solution was poured into test tubes and stored in the refrigerator. When the agar solution was needed, the test tube was placed in boiling water until the gel liquefied. This ion agar solution was poured into either petri dishes or on clean (oil free) microscope slides resting on a level tray. A sample well template for the Ouchterlony technique was used to determine the location of the

wells. Cork borers and/or a 13 gauge needle were used to cut wells into the agar, after which the agar plug was removed by suction. In some cases commercial immunodiffusion plates were used. The antigen and antibodies were aliquoted into the wells with care so that the wells were not overfilled. The following commercial antibodies were used:

anti-human whole sera

anti-human fibrinogen

anti-human alpha$_2$-Macroglobulin

anti-human IgM

The slide or the petri dish were inserted into a plastic container along with a moistened paper towel or wet sponge. The plastic container was covered and kept at room temperature for 24 to 48 hours, after which the agar was washed with saline and placed in the refrigerator for a day. In order to prepare the plates for permanent record, the agar was soaked in saline for 24 hours. During this time the saline was changed twice. Next, the plates were washed with distilled water, covered with moistened filter paper, and allowed to air dry. The stain and destain solutions for immunodiffusion plates were made by mixing the following reagents:

Amido Black Stain	Destaining Solution
1.9 g Amido Black	100 ml Glacial Acetic Acid
500 ml Methanol	700 ml Methanol
200 ml Glacial Acetic Acid	200 ml Distilled Water
500 ml Distilled Water	

The dry agar was stained with the Amido Black solution for 30 to 45 minutes. The agar was washed with the destaining solution until all background dye was removed. Alternatively, samples of alpha$_2$-Macroglobulin were run on disc gels according to the procedure of Davis (1964). A gel cutter was constucted from two pieces of 1/4" thick plexiglass, which were bolted together and bored out such that the i.d. of the bore was smaller than the o.d. of the gel. This difference in size produces a gap for the razor blade to fit in. The gels were then sliced lengthwise. One half of the disc gel was stained with the Amido Black solution, while the other half was imbeded in a 2% ion agar solution. (See immunodiffusion section for reagents). Two troughs (1.5 mm) were cut in the ion agar parallel to the acrylamide gel. The procedure followed was identical to the immunodiffusion previously described.

d. Digestion of Lipid for Pi Determination

An aliquot of purified alpha$_2$-Macroglobulin was subjected to a lipid extraction and subsequent lipid digestion. The lipid was extracted with a solution containing chloroform, methanol, and water according to the procedure of Blight. The lipid extract and an aliquot of native alpha$_2$-Macroglobulin were digested with 70% perchloric acid as indicated by the method of Bartlett. Bartlett's ANSA reagent and ammonium molybdate were employed to detect the presence of inorganic phosphate.

8. Active Site Titration of Trypsin

Trypsin was titrated by the method of Chase and Shaw (1970). The reaction was initiated by the addition of 10 ul of 10 mM p-nitrophenol-p'-guanidino benzoate·HCl to a cuvette containing trypsin

at a concentration of 10 uM. The colorimetric burst was measured at 410 nm in 0.1 M sodium veronal buffer at pH 8.30. The extinction coefficient of the nitrophenol under these conditions is 16,595.

9. Circular Dichroism Spectroscopy

Measurements were performed with a Durrum-Jasco ORD/CD/UV-5 Spectropolarimeter at room temperature. The cell compartment was continuously purged with nitrogen. The cuvettes had a path length of 1 cm. and were made of quartz (Beckmann Instruments, Inc.). The results were expressed as mean-residue ellipticities. The mean residue weight used for the calculation of mean-residue ellipticities was 115. The instrument was calibrated prior to use with a 0.1% solution of d-10-camphorsulfonic acid (Durrum Instrument Company). The trypsin used for complex formation was affinity purified according to the method of Ryan (1975). Measurement of the C.D. spectra for native $alpha_2$-Macroglobulin and $alpha_2$-Macroglobulin trypsin complex required four individual runs, each scanning between 209 nm. and 290 nm. For each run the volume was kept constant. The constituents of each run are as follows: Run 1 = 0.02 M Na Phosphate, pH 7.40

Run 2 = 0.02 M Na Phosphate, pH 7.40 + $alpha_2$-M

Run 3 = 0.02 M Na Phosphate, pH 7.40 + trypsin

Run 4 = 0.02 M Na Phosphate, pH 7.40 + $alpha_2$-M and trypsin

10. Fluorescence Measurements

Fluorescence measurements were performed with either an Aminco-Bowman ratio spectrophotofluorometer equipped with a Houston Instruments Model 2000 X-Y recorder or a Perkin Elmer MPF-44B. Quartz cuvettes (Beckmann Instruments) with a pathlength of 1 cm were

utilized for all measurements. The cell compartment of the Perkin Elmer was thermostated at 25°C. All solutions were filtered and degassed prior to use. Rhodamine and the Raman spectra of distilled water were used to standardize the MPF-44B. The dark current was used as the base line for all spectra. Excitation spectra were performed to determine the optimum wavelength for excitation with the emission monochromator set at a wavelength of 330 nm. When emission spectra were measured, the excitation monochromator was set at 288 nm. Affinity purified trypsin was used for the experiments performed with the Aminco-Bowman. Emission spectra were also measured for alpha$_2$-Macroglobulin treated with alpha-chymotrypsin and papain.

11. Iodination of Alpha$_2$-Macroglobulin

Alpha$_2$-Macroglobulin was iodinated by three different techniques. Initially, alpha$_2$-Macroglobulin was iodinated by the method of Hunter and Greenwood using Chloramine-T and Na ^{125}I. Alternatively, the alpha$_2$-Macroglobulin was labeled using lactoperoxidase by the method of J. Marchalonis (1969). The Bio-Rad enzymobead kit was also utilized for labeling. Following each procedure, the iodinated protein was purified on a Sephadex G-25 column. Prior to use the glass column was silanized (See Silanization of Glass). Additionally, fractions from the Sephadex columns were collected in plastic test tubes.

The labeled proteins were refrigerated after purification and generally used within 3 to 6 days. The radioactivity of protein samples stored for any period of time was corrected for the known decay rate of ^{125}I.

12. Analysis of Radiolabeled Alpha$_2$-Macroglobulin

The iodinated alpha$_2$-Macroglobulin was analyzed by several techniques. The precipitability of the labeled protein by TCA and Concanavalin A was examined. The precipitation of alpha$_2$-Macroglobulin by TCA was initiated by mixing 5 ul of I^{125}-alpha$_2$-Macroglobulin (0.13 ug/ul) and 5 ul of 20% TCA in a hand blown glass microfuge tube which had been silanized prior to use. The mixture was vortexed and allowed to incubate on ice for 30 minutes. Following centrifugation at 6000 rpm for 5 minutes, a 5 ul aliquot of the supernatant was removed for gamma counting. The remainder of the supernatant was aspirated off with a spinal tap needle, after which the precipitation was counted in the gamma counter.

Alternatively, Concanavalin A was used to precipitate iodinated alpha$_2$-Macroglobulin protease complex. Aliquots of alpha$_2$-Macroglobulin (native or complexed), buffer, and Concanavalin A were placed in hand blown silanized microfuge tubes. Increasing amounts of Concanavalin A were added to a constant amount of alpha$_2$-Macroglobulin. The final volume of all microfuge tubes was maintained at a constant volume of 0.3 ml by the addition of the appropriate volumes of buffer (0.2 M NaPhosphate 10 uM $CaCl_2$ 10 uM $MgCl_2$, pH 7.20).

All microfuge tubes contained labeled as well as unlabeled alpha$_2$-Macroglobulin because the preipitability of the alpha$_2$-Macroglobulin from solution was monitored by radioactivity as well as with the trypsin binding assay. Aliquots of alpha$_2$-Macroglobulin buffer, and Concanavalin A were mixed, vortexed, and allowed to incubate for 40 minutes at room temperature. The tubes were

centrifuged at 6000 rpm for 6 minutes. Aliquots from the supernatant were subjected to gamma counting or assayed for trypsin binding activity.

The trypsin binding activity of I^{125}-alpha$_2$-Macroglobulin was also examined. Alpha$_2$-Macroglobulin was iodinated by three different methods (See Iodination), but non-radioactivive iodine was utilized in place of I^{125}. The labeled protein was purified on a calibrated Sephadex G-25 column. Fractions containing alpha$_2$-Macroglobulin were pooled, concentrated, and assayed in order to determine the specific activity of the iodinated alpha$_2$-Macroglobulin.

13. Silanization of Glass

A 10% (u/v) solution of Dimethyldichlorosilane in toluene was used to rinse or dip glass columns or microfuge tubes. Next the glassware was rinsed with methanol and blown dry with nitrogen.

14. Cell Culture and Receptor Mediated Uptake by Cells

All cells were maintained in a Forma Scientifica CO_2 incubator in an atmosphere of 5% CO_2 at 37^{o}C. All cells were incubated in DMEM. This media was supplemented with fetal calf serum (10%), penicillin (1000 units/ml), streptomycin sulfate (1000 ug/ml) and L-glutamine before use. All cell cultures used in a given experiment were plated at the same time and with the same known number of cells. Fibroblast cultures were confluent monolayers when used for uptake experiments. Prior to receptor binding study, the supplemented media was removed and replaced with DMEM containing only 4 mg/ml HSA. The cells were allowed to incubate in this media for 90 minutes prior to the start of the uptake experiment. The I^{125}-alpha$_2$-Macroglobulin and unlabeled alpha$_2$-Macroglobulin for the experiment were allowed to warm to 37^{o}C

for 50 minutes. All experiments were performed in a shaker water bath at 37°C. The experiment was initiated by the addition of iodinated alpha$_2$-Macroglobulin to the cells. The experiment was terminated by removing the media and washing the cells 5 times with cold PBS. Cellular radioactivity was extracted by adding formic acid to the cells and placing the multiplate in a shaker for 25 minutes at room temperature. Microscopic examination of the plates was done to confirm total cell removal. Aliquots from the acid solubilized cells were counted in a Picker Pace Gamma Counter.

15. Detection of Carbohydrate - Anthrone Sulfuric Acid Reaction

This reaction is generally used to detect the presence of hexoses. Initially, four mls of a .2% solution of anthrone in concentrated sulfuric acid was added to 2 mls of a standard (10-100 ug hexose/ml) or an unknown. The mixture was carefully agitated and allowed to sit at room temperature for a few minutes. The presence of carbohydrate was indicated by the development of a blue-green color. The absorbances of the solutions were then measured at 620 nm.

16. Enzymatic Cleavage of Sialic Acid

Vibrio Cholerae neuraminidase was incubated with native alpha$_2$-Macroglobulin or alpha$_2$-Macroglobulin-Trypsin complex in a 0.05M NaAcetate 0.15 M NaChloride 8.0 mM $CaCl_2$, pH 5.20 buffer. The reaction mixture was incubated at 37°C for 26 hours in a shaker water bath. At various times aliquots were removed, boiled for 2 minutes, and assayed for free sialic acid according to the method of Papermaster (1975).

17. Chemical Cleavage of Sialic Acid

Sialic acid was cleaved off native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex according to the procedure of Spiro (1960). Prior to chemical cleavage of scialic acid, the alpha$_2$-Macroglobulin trypsin complex was chromatographed on a G-25 sephadex column with a bed volume of 19 ml. Native and complexed alpha$_2$-Macroglobulin were incubated in 0.1 N H_2SO_4 at 80°C. for 1 hour. At zero minutes and 60 minutes aliquots were removed and assayed for free sialic acid by the method of Papermaster. Samples of sialic acid were also treated with 0.1 N H_2SO_4 in order to determine the extent to which sialic acid is destroyed by acid treatment.

18. Formation of Methylamine Alpha$_2$-Macroglobulin and Methylamine I^{125} alpha$_2$-Macroglobulin

Prior to formation of methylamine alpha$_2$-Macroglobulin, the pH of the methylamine solution (40% in water) was adjusted to pH 7.40 with 4 N HCl. The reaction was initiated by mixing the following reagents:

2.0 ml alpha$_2$-Macroglobulin (0.41mg/ml) in 0.2 M NaPhosphate, pH 7.40

0.2 ml I^{125}-alpha$_2$-Macroglobulin in 0.2 M NaPhosphate, pH 7.40

0.2 ml Methylamine solution, pH 7.40

After a sixteen hour incubation period, the reactants were dialyzed against 0.0125 M Na Barbital 10 uM $CaCl_2$ 10 uM $MACl_2$, pH 7.40. The activity of the methylamine alpha$_2$-Macroglobulin was determined using the trypsin binding assay.

19. Ultraviolet Difference Spectra

Difference spectra of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin enzyme complex were obtained on a Perkin Elmer Model 310

spectrophotometer using a .9 cm tandem cell (Pyrocell) specifically designed for difference spectroscopy.

All samples were filtered and degassed prior to their addition to the tandem cell. Extreme care was taken when 1 ml of each protein solution was pipetted into its respective compartment of the tandem cell. The temperature of the cell was maintained at 25°C. A spectra from 300 nm to 215 nm was then taken of the unmixed alpha$_2$-Macroglobulin and protease solutions. Following inversion of the tandem cell, the spectra of the alpha$_2$-Macroglobulin protease complex was taken from 250 nm to 215 nm. The spectra of the alpha$_2$-Macroglobulin and trypsin was obtained in a 0.05 M NaPhosphate, pH 5.20 buffer, while the spectra of alpha$_2$-Macroglobulin and papain was obtained in a 0.05 M NaP 1.0mM EDTA 3.3mM DTT pH 4.0 buffer. Papain was activated prior to complex formation. After shaking the bottle of papain, 0.250 ml of papain were removed and diluted 1:10 with 0.001 NHCl. Following centrifugation, the absorbance at 210 nm. was taken. An appropriate aliquot of papain was added to the 0.05 M NaPhosphate 1.0 mM EDTA 3.3 mM DTT, pH 4.0 buffer prior to delivery to the cuvette.

20. H_2O_2 Treatment of alpha$_2$-Macroglobulin

H_2O_2 was allowed to react with native alpha$_2$-Macroglobulin and I^{125} - alpha$_2$-Macroglobulin. The reaction was initiated by mixing the following reagents:

2.5 ml alpha$_2$-Macroglobulin (0.41mg/ml) in 0.2 M NaPhosphate, pH 7.40

0.2 ml I^{125} alpha$_2$-Macroglobulin in 0.2 M NaPhosphate, pH 7.40

0.3 ml H_2O_2 (30%)

After thirty minutes an additional milliliter of H_2O_2 was added to the reaction. After 3.5 hours the reactants were dialyzed against 0.0125 M Na Barbital 10 uM $CaCl_2$ 10 uM $MACl_2$, pH 7.40.

21. Titration of alpha$_2$-Macroglobulin With Proteases

Alpha$_2$-Macroglobulin was titrated with trypsin and chymotrypsin to determine the stoichiometry of binding. The complex formation was monitored fluorometrically by exciting the proteins at 288 nm and observing the emission at 330 nm. Previously, it was demonstrated that protease binding caused the intensity of fluorescent emission at 330 nm to increase. Increasing amounts of affinity purified trypsin or chymotrypsin were pipetted into a fluorometric cell containing alpha$_2$-Macroglobulin at a concentration of 0.29 uM.. Appropriate amounts of 0.05 M Tris·HCl, pH 7.40 buffer were added to the cell to maintain the volume at 3.0 ml. The cell holder was thermostated to maintain the temperature at 25°C.

22. DTNB Titration of Alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin Protease Complex

The method of Ellman (1959) was followed in order to determine the sulfhydryl content of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex. DTNB (0.01 M) was dissolved in a 0.1 M NaP, pH 8.00 buffer, which was then flushed with nitrogen. The protein sample (0.1ml) was added to 2.9 ml of the DTNB solution. The reaction was monitored at 412 nm until an end point was reached.

23. Lectin Binding Studies

The interaction of alpha$_2$-Macroglobulin with lectins was examined using free lectin in solution or lectin conjugated to an insoluble porous matrix (Sepharose). Concanavalin A and wheat germ

lectin were both coupled to cyanogen bromide activated Sepharose according to the method of Cuatrecasas. The conditions used to affix the Concanavalin A to the activated Sepharose are as .pa indicated below:

Coupling Conditions for Con. A

Resin	Temperature (^{o}C)	Buffer	pH
1 (Commercial)	Unknown	Unknown	11.0
2	Room	0.1 M $NaHCO_3$	9.0
3	4	0.1 M $NaHCO_3$	9.0
4	Room	0.0125 M Na Barbital	8.0
5	Room	0.0125 M Na Barbital	8.0

For each resin two hundred mgs of Concanavalin A were added to one hundred mls (settled volume) of activated Sepharose. For resin number 4, equimolar amounts of alpha-Macroglobulin-methylmannoside and Concanavalin A were mixed together prior to the addition to the activated Sepharose. The coupling mixture was stirred overnight at the indicated temperature after which the resin was placed in a buchner funnel and washed with 500 mls of each of the following buffers:

1) Buffer used for coupling
2) 0.1 M Na Borate - 1 M NaCl pH 8.50
3) 0.1 M Na Acetate - 1 M NaCl pH 4.10

The absorbance at 280 nm of a sample of the first wash was used to determine the extent of coupling. Following the third wash, the gel was defined and placed in a beaker containing the coupling buffer without any blocking reagents. The active sites were allowed to neutralize themselves by leaving the resin coupling buffer for at

least two weeks. The binding capacity of each of the five columns was determined. Column 5, which had the greatest binding capacity was prepared a second time, but methylamine was used to block any residual active groups remaining on the gel. In addition, the coupling conditions for resin 5 were then used to prepare a wheat germ affinity column. The binding of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex to .pa both affinity resins was examined at a variety of pH's e.g.,

Protein	Buffer	Resin
Native alpha$_2$-Macroglobulin	0.0125 M Na Barbital 10 uM $CaCl_2$ 10 uM $MnCl_2$, pH 7.40	Con A
Native alpha$_2$-Macroglobulin	0.1 M NaCitrate 10 uM aCl_2 10 uM $MnCl_2$, pH 5.50	Con A
Alpha$_2$-Macroglobulin Trypsin Complex	0.0125 M NaBarbital 10 uM $CaCl_2$ 10 uM $MnCl_2$, pH 7.40	Con A
Alpha$_2$-Macroglobulin Trypsin Complex	0.1 M Na Borate 10 uM $CaCl_2$ 10 uM $MnCl_2$, pH 6.0	Con A
Alpha$_2$-Macroglobulin Trypsin	0.1 M Na Citrate 10 uM $CaCl_2$ 10 uM $MnCl_2$, pH 5.5	Con A

In some experiments elution was effected with alpha-methylmannoside for the Con A affinity columns. When soluble lectins were used for

precipitating native and complexed forms of alpha$_2$-Macroglobulin, the reactions were done in the following buffers:

Protein	
Native alpha$_2$-Macroglobulin	0.0125 M Na Barbital 10 uM $CaCl_2$ 1.0 uM $MnCl_2$, pH 7.4
Alpha$_2$-Macroglobulin Trypsin	0.0125 M Na Barbital 10 uM $CaCl_2$ 1.0 uM $MnCl_2$, pH 7.4
Native alpha$_2$-Macroglobulin	0.05 M NaAcetate 1.0 uM $CaCl_2$ 10 uM $MnCl_2$, pH 5.29

Preliminary studies were performed to examine the effects of time and temperature on the precipitation of alpha$_2$-Macroglobulin by the respective lectins. From these studies it was determined that Concanavalin A and wheat germ precipitations could be conducted at room temperature. The incubation period was 60 minutes for the wheat germ reaction and 30 minutes for the Concanavalin A reaction.

24. Gel Filtration of Native Alpha$_2$-Macroglobulin and Alpha$_2$-Macroglobulin Protease Complex

Chromatography of native and complexed forms of alpha$_2$-macroglobulin was performed on a 1.6 x 3.6 cm column of Sepharose CL-6B in a Pharmacia K-16 chromatographic column. The column was equilibrated and eluted with 0.1 M Na Pyrophosphate buffer, pH 8.0, at a flow rate of 24.0 ml/hr. The resin was calibrated with blue dextran, potassium dichromate, thyroglobulin, albumin, B-galactosidase, and xanthine oxidase. Seive coefficients were calculated for the proteins according to the relationship

$$= \frac{\text{(Ve) Blue dextran - (Ve) protein}}{\text{(Ve) blue dextran - (Ve) potassium dichionate}}$$

Molecular weights of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin-trypsin complex (2:1) were then estimated from the standard curve relating the sieve coefficients to the log of the molecular weight of the protein standards.

25. Effect of Metal Ions on Trypsin Binding Activity

During the development of the purification scheme, the observation was made that metal ions were capable of altering the ability of alpha$_2$-Macroglobulin protease complex to hydrolyze the substrate TAME. The trypsin binding assay was modified by adding native alpha$_2$-Macroglobulin and the divalent cation initially, followed by the inversion of the cuvette. Trypsin, SBI, and TAME were added in an identical manner as described previously (Enzyme Assays for Detection of alpha$_2$-Macroglobulin. The final concentration of divalent cations in the cell was as follows:

Divalent Cation	Concentration (M)
$MgCl_2$	4.6×10^{-3}
$MgCl_2$	4.6×10^{-4}
$MgCl_2$	4.6×10^{-5}
$MgCl_2$	4.6×10^{-6}
$MgCl_2$	4.6×10^{-7}
Divalent Cation	**Concentration (M)**
$ZnCl_2$ or Zn Acetate	6.1×10^{-4}
$ZnCl_2$ or Zn Acetate	6.1×10^{-5}
$ZnCl_2$ or Zn Acetate	6.1×10^{-6}
$ZnCl_2$ or Zn Acetate	6.1×10^{-7}

The following controls were performed:

1. Magnesium chloride or zinc chloride were assayed in the presence of trypsin, but in the absence of alpha$_2$-Macroglobulin.

2. The metal cations and native alpha$_2$-Macroglobulin were assayed in the presence and in the absence of trypsin.

3. Following completion of each assay, the pH of the cuvette's contents was determined.

CHAPTER III

RESULTS

A. Purification

The purification scheme developed for alpha$_2$-Macroglobulin was designed to utilize mild conditions as well as minimize exposure of alpha$_2$-Macroglobulin to endogenous proteases which might be activated during blood clotting. Consequently, citrated plasma was utilized as the starting material, and plastic labware was employed to avoid activation of the blood coagulation cascade. As an added precaution, Benzamidine, SBI, and Sodium Heparin were added to the citrated plasma. The first step in the purification utilized bentonite to remove fibrinogen and lipoproteins. The recovery of the alpha$_2$-macroglobulin in this step was not reproducible due to impurities in the bentonite and to variations in the size of the bentonite particiles. This problem was eliminated by using Dunn and Hitchborn's procedure for preparing Mg-bentonite (see Methods section). Figures 1 and 2 are elution profiles obtained following chromatography of plasma samples treated with either unpurified bentonite or Mg-bentonite. It is evident from a comparison of these figures that the Mg-bentonite was more effective in eliminating proteins which co-eluted with alpha$_2$-Macroglobulin. As shown in Table 1, preparations of alpha$_2$ .pa -macroglobulin after Mg-bentonite treatment and chromatography on Sepharase CL-6B were greater than 90% pure. The overall yield was 74%. Ion exchange chromatography was then attempted in order to further purify alpha$_2$-Macroglobulin and resolve it into its various isoenzymic forms. To this end, alpha$_2$-Macroglobulin was chromatographed on a number of anion and cation exchangers using a

FIGURE 1 Chromatography of bentonite treated citrated plasma on Sepharose CL-6B in 0.02 M Na Barbital 10 uM Zn Acetate, pH 7.40 at a flow rate of 100 ml/hr. Eight ml fractions were collected and assayed for trypsin binding activity (·····) and absorance at 280 nm (·■·■··).

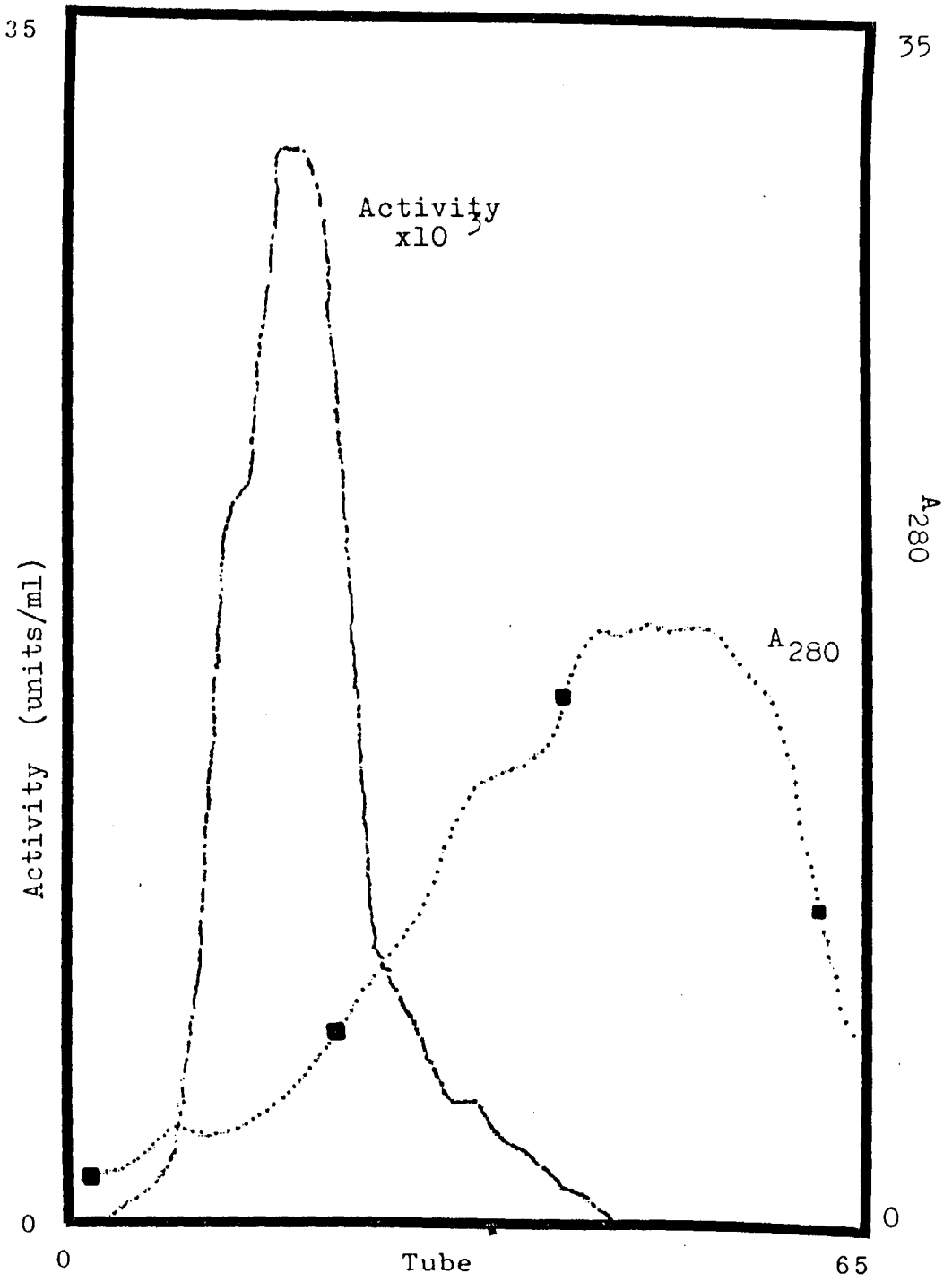

35
35
Activity
x10
Activity (units/ml)
A_{280}
A_{280}
0
0
0
Tube
65

FIGURE 2 Chromatography of Mg-bentonite treated citrated plasma on Sepharose CL-6B in 0.02 M Na Barbital 0.1 M NaCl 10 uM Zn Acetate, pH 7.40 at a flow rate of 100 ml/hr. Eight ml fractions were collected and assayed for trypsin binding activity (··o·o·) and absorbance at 280 nm (··o··o··). This elution profile is one representative sample of the twenty columns run.

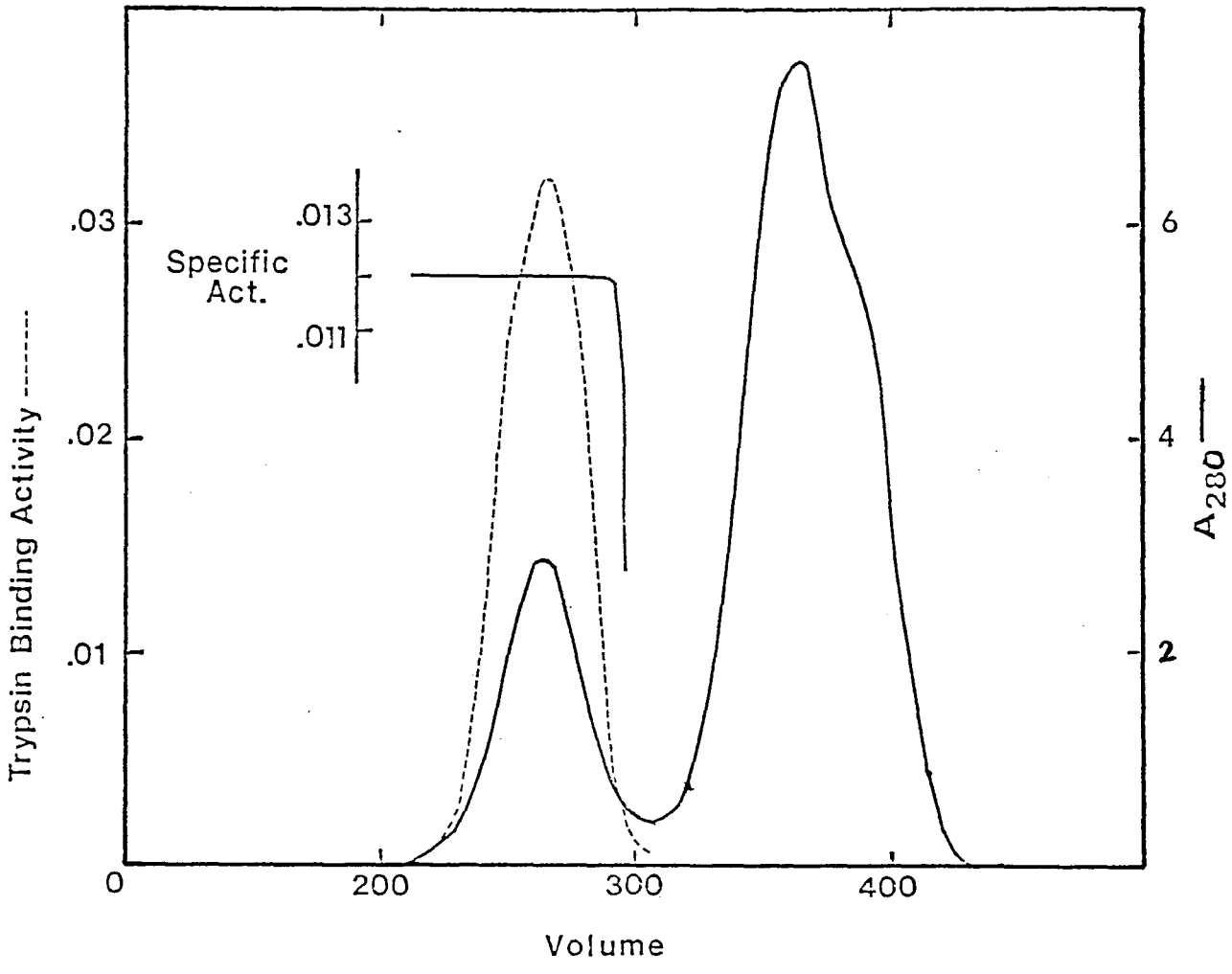

Trypsin Binding Activity
.03
.02
.01
0
200
300
400
Volume
Specific Act.
.013
.011
6
4
2
A_{280}

Table 1

Summary of the Purification of Alpha$_2$-Macroglobulin

Step	Activity	Protein	Specific Act.	Purification Factor	Yield
	Units	GM	units/$A_2$80		%
Plasma	4.85	16.5	.00032		100
Bentonite	3.83	1.4	.0025	8	79
Sepharose	3.6	.31	.0125	40	74

variety of salt and/or pH gradients. The best results were obtained with a Whatman CM-cellulose resin, preequilibrated with a 0.2 M NaAcetate buffer, pH 5.29. After applying the protein in the equilibration buffer, the inhibitor was eluted with 0.02 M NaAcetate 0.1 M NaCl, pH 5.20. The alpha$_2$-Macroglobulin was separated into three peaks whose relative proportions were 75: 20:5 (Fig. 3).

I next tested various conditions for storing the purified protein. Alpha$_2$-Macroglobulin was dialyzed against 0.02 M NaPhosphate buffer, pH 7.40, and then either lyophilyzed or frozen. Prior to freezing some samples had glycerol added to a final concentration of either 10% or 20%. The trypsin binding activity was then measured before and after storage. The results are presented in Table II. Since all the aforementioned procedures for storage resulted in a decrease in trypsin binding activity, alpha$_2$-macrogloulin was sterile filtered and stored in a sterile test tube at 4^{o}C. Under these conditions the trypsin binding activity was stable for three to four months.

FIGURE 3 Ion exchange chromatography of alpha$_2$-Macroglobulin obtained from the Sepharose Cl-6B column. The precycled CM-Cellulose resin was equilibrated with 0.02 M Na Acetate, pH 5.29 at a Flow rate of 24 ml/hr. Following elution of all non-binding proteins, the alpha$_2$-Macroglobulin was eluted with 0.02 M Na Acetate 0.2 M NaCl, pH 5.20. Three ml fractions were collected and assayed for trypsin binding activity (---) and absorbance at 280 nm (——). This elution profile is one representative sample of the twenty columns run.

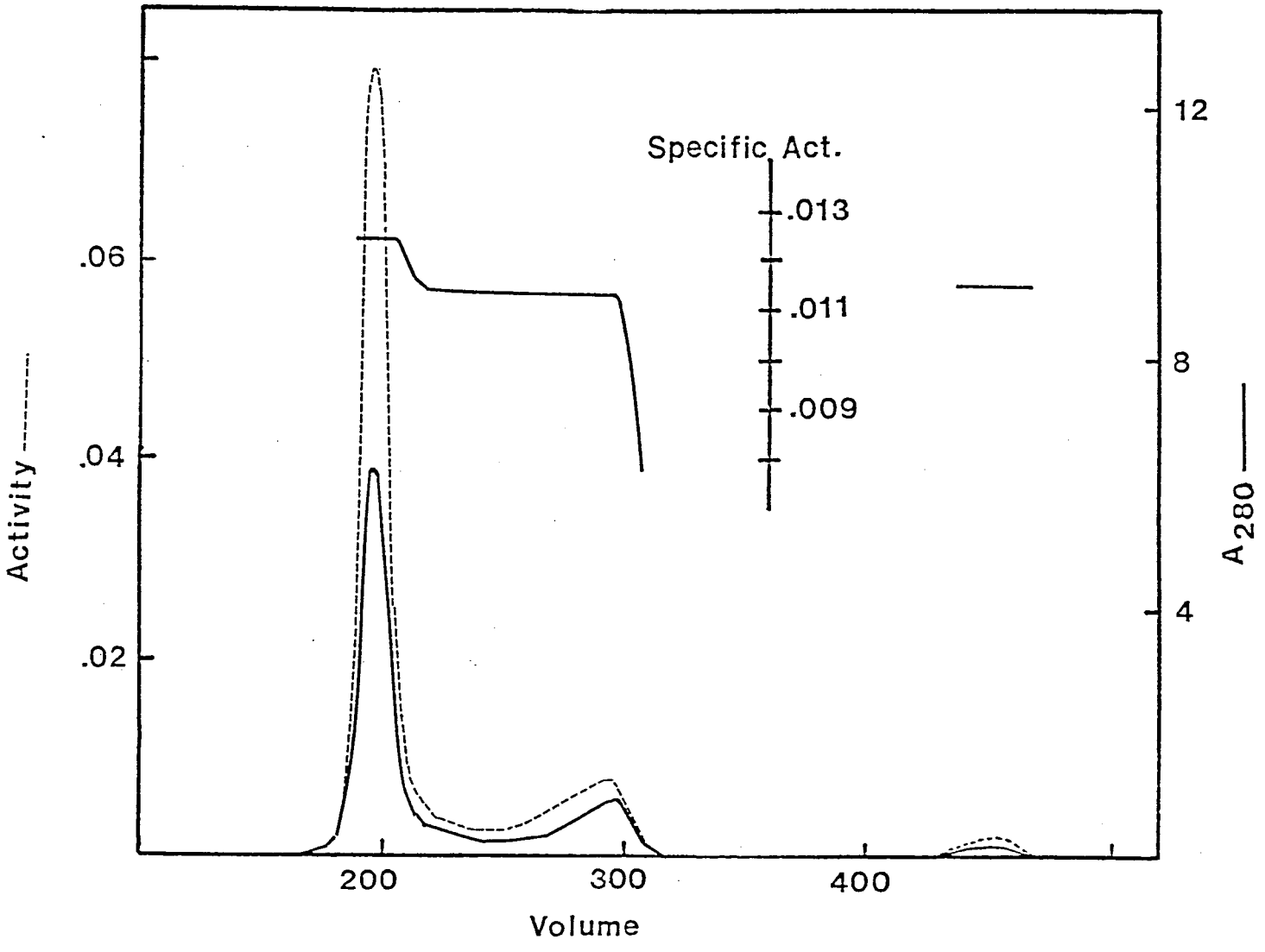

Specific Act.
.013
.011
.009
.06
.04
.02
Activity
12
8
4
A_{280}
200
300
400
Volume

Table II

Effect of Storage on Activity of Alpha$_2$-Macroblogulin

Storage Condition	Percent Loss of Trypsin Binding Activity After Storage[a]
Freezing (0°C)	53
Freezing (0°C) in 10% glycerol	11
Freezing (0°C) in 20% glycerol	10
Lyophilyzation	90

[a]. = Value are average of two determinations.

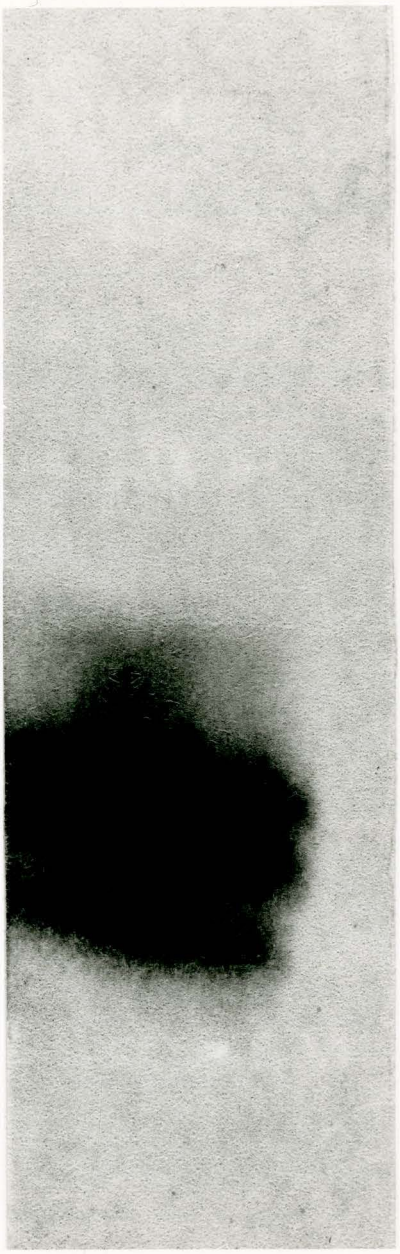
B. Isoelectric Focusing of Alpha$_2$-Macroglobulin

IEF was performed on the alpha$_2$-Macroglobulin purified through the CM-cellulose chromatography step. Two types of support media, Sephadex and polyacrylamide, were utilized. All attempts at flat bed polyacrylamide IEF were unsuccessful. The IEF was performed under various operating voltages with numerous methods of sample application. The alpha$_2$-Macroglobulin failed to penetrate commercial pre-cast gels. Instead, the protein seemed to migrate in the surface water film rather then through the gel matrix. Alternatively, polyacrylamide, ampholytes, and alpha$_2$-Macroglobulin were premixed prior to polymerization of the gels, but alpha$_2$-Macroglobulin inhibited polymerization. Flat bed IEF was then performed on alpha$_2$-macroglobulin from the cation exchanger using Sephadex G-75 SuperFine as the support. The focusing revealed the presence of 5 bands with pI's ranging from 4.8 to 5.2 Isoelectric focusing of the eluate from the cation exchanger indicated that the first peak was composed of three species (Figure 4), while the second peak consisted of two species. All five species were active in the trypsin binding assay for alpha$_2$-macroglobulin.

C. Immunological Analysis of Purified Alpha$_2$-Macroglobulin

Purified alpha$_2$-macroglobulin was subjected to immunodiffusion and immunoelectrophoresis. Both procedures produced identical results. No precipitation lines were detected between purified alpha$_2$-Macroglobulin and anti IgM or anti-human fibrinogen. One precipitation line appeared between purified alpha$_2$-Macroglobulin and anti-human alpha$_2$-Macroglobulin and between purified alpha$_2$-Macroglobulin and anti-human whole serum.

FIGURE 4 Isoelectric focusing of alpha$_2$-Macroglobulin obtained from the cation exchanger. Twenty micrograms of alpha$_2$-Macoglobulin from peak 1 of the CM-Cellulose column was applied to a Sephadex G-75 gel layer which was prefocused for 4 hrs. at 200 v. Following application of the protein to the gel, the voltage was increased to 500 v and maintained there for 4 hours. Experiment was repeated ten times.



D. Phospholipid Determination

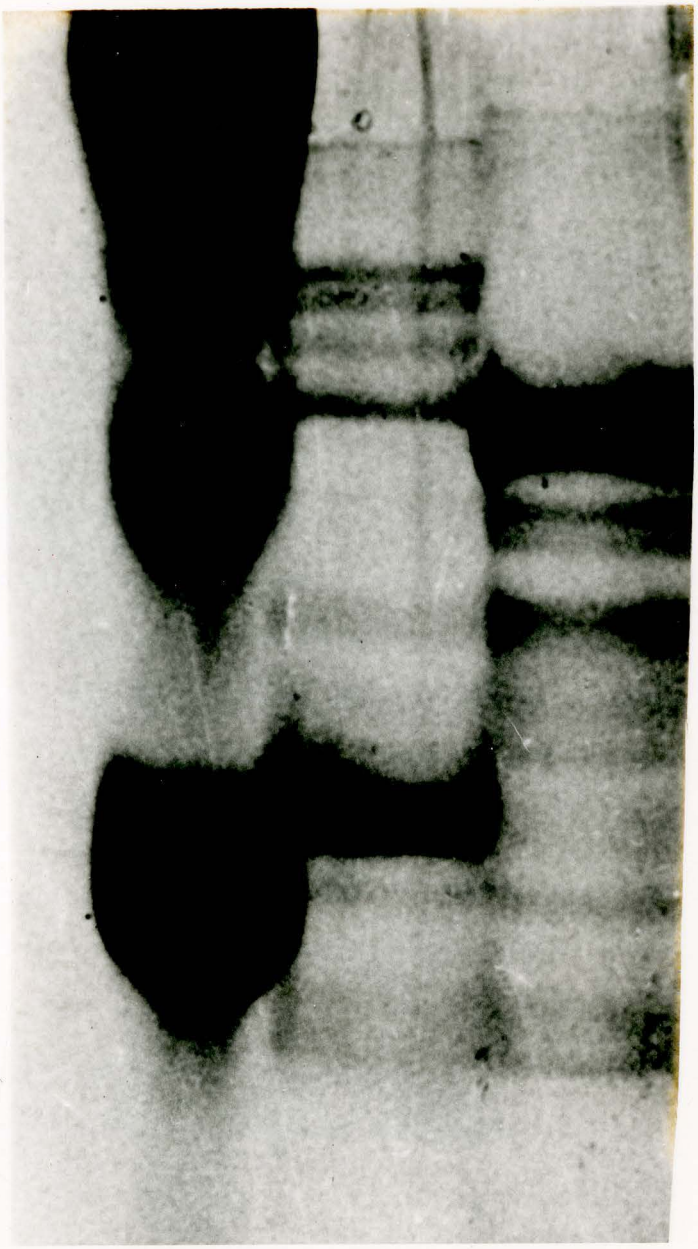
The assay for phospholipid was performed on native alpha$_2$-macroglobulin and on a chloroform:ethanol (1:1) extract of alpha$_2$-Macroglobulin. Both samples were subjected to Bartlett's procedure which measures inorganic phosphate formed by the hydrolysis of the phospholipids. In both cases no inorganic phosphate could be detected.

E. Electrophoresis

Electrophoresis was used to determine the purity and subunit structure of alpha$_2$-Macroglobulin. Increasing amounts of purified alpha$_2$-macroglobulin were subjected to discontinuous PAGE under non denaturing conditions. Following electrophoresis, the gels were either stained for protein and carbohydrate or were cut into 1.5 mm segments. Only a single protein band was observed, which contained all the alpha$_2$-macroglobulin activity present in the gel.

Alpha$_2$-Macroglobulin was then subjected to SDS-polyacrylamide gel electrophoresis in the presence or in the absence of reductant. With only SDS present, a single protein band corresponding to a molecular weight of 360,000 was observed after staining. When mercaptoethanol was added to the sample of native alpha$_2$-Macroglobulin in SDS prior to electrophoresis, the stained gel indicated the presence of a major band at 180,000 with numerous minor bands (Figure 5). Electrophoresis of alpha$_2$-Macroglobulin trypsin complex under identical conditions resulted in the conversion of the 180,000 molecular weight band to a band corresponding to an apparent molecular weight of 85,000 daltons. A number of minor bands were also evident. In order to rule out the possibility that the minor bands resulted

FIGURE 5 Native $alpha_2$-Macroglobulin (Lane 3), $alpha_2$-Macroglobulin trypsin complex (Lane 2) and cross-linked hemocyanin molecular weight standards (Lane 1) were subjected to SDS PAGE under reducing conditions. The standards exhibit molecular weights of 70,000 daltons, 140,000 daltons, 210,000 daltons and 280,000 daltons. This photo is one representative sample from ten runs.



from trypsin digestion of the complex during denaturation with SDS, the sample was adjusted to pH 3.0 prior to SDS-polyacrylamide gel electrophoresis. This treatment had no effect on the electrophoretic pattern, suggesting that the complexity of the pattern may be due to limited proteolysis of alpha$_2$-Macroglobulin which had occurred prior to the acidification and denaturation steps.

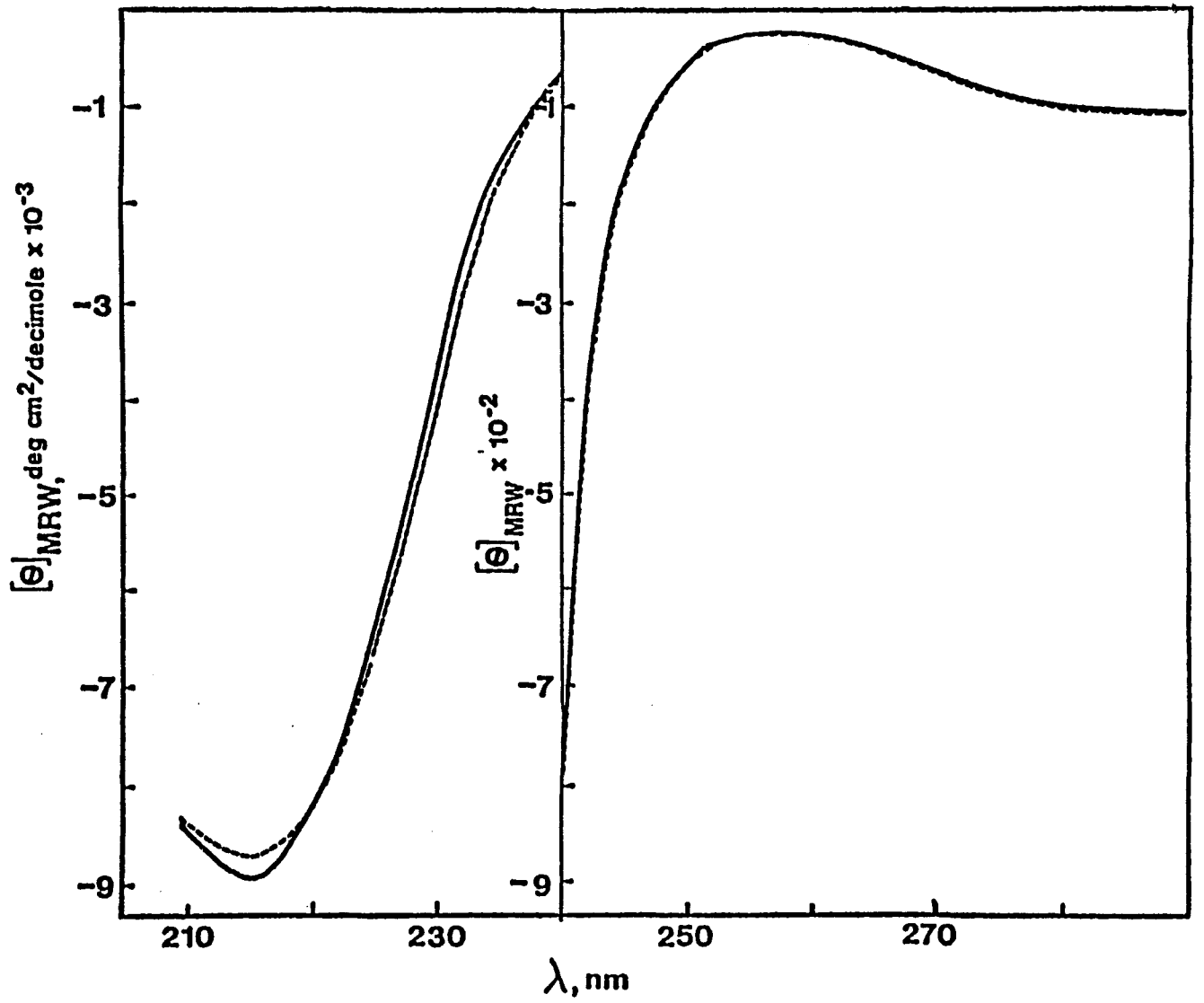
F. Circular Dichroism Spectroscopy

It has been suggested that alpha$_2$-Macroglobulin undergoes a conformational change upon protease binding, which results in the entrappment of the bound protease (Barrett, A.J., 1973). Circular dichroism was performed in an attempt to verify this theory. A Jasco CD/ORD spectropolarimeter was used to obtain CD spectra of native and complexed forms of alpha$_2$-macroglobulin between 209 nm and 290 nm. Spectra for alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex are shown in Figure 6. The results indicated that the native and complexed forms of alpha$_2$-macroglobulin yielded virtually identical spectra. However, the ratio of signal to noise in the spectra declined significantly below 245 nm. As a consequence, small changes in the circular dichroism spectra below 245 nm would have been difficult to detect. At wavelengths greater than 250nm, changes in the circular dichroism spectra were not detected possibly due to insufficient protein being present in the cuvette.

G. Fluorescent Spectroscopy

The circular dichroism data in Figure 6 seemed to indicate that alpha$_2$-macroglobulin did not undergo major conformational changes upon complex formation. In light of this, a more subtle technique, fluorescence spectroscopy, was chosen to investigate changes in the

FIGURE 6 Far u.v. and near u.v. circular dichroism spectra of alpha$_2$-Macroglobulin alone (———) and alpha$_2$-Macroglobulin allowed to react with affinity purified trypsin (- - - - -). Measurements were done with 1 cm cells with alpha$_2$-Macroglobulin and trypsin at 0.52 uM and 0.47 uM, respectively. Each spectra is a representative sample from eight runs.


$[\theta]_{MRW}$, deg cm^2/decimole x 10^{-3}
$[\theta]_{MRW}$ x 10^{-2}
-1
-3
-5
-7
-9
210
230
250
270
λ, nm

microenvirnoment of tyrosine and tryptophan residues in the native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complexes. Fluorescent emission spectra were obtained between 300 nm and 375 nm at an excitation wavelength of 288 nm. Originally, fluorescence measurements were performed with an Aminco-Bowman Spectrophotofluorometer. The spectra, which are shown in Figure 7, indicated that complex formation resulted in an increase in the fluorescent intensity of the protein, as well as a shift in the maxima from 331.3 nm to 330.0 nm. When corrected spectra of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex were obtained with the Perkin Elmer MPF 44B spectrophotofluorometer, the increase in fluorescence upon complex formation was again observed, but the blue shift in the maxima was not seen. Apparently, the small blue shift seen earlier was an artifact of the Aminco-Bowman Spectrophotofluorometer.

H. Titration of Alpha$_2$-Macroglobulin with Proteases

The increase in fluorescence of alpha$_2$-macroglobulin upon binding proteases was used to determine the stoichiometry of binding. Increasing amounts of affinity purified trypsin solution of a known normality, (determined by previous titration with p-nitrophenyl guanidinobenzoate), were added to a fluorometer cell containing alpha$_2$-Macroglobulin at a final concentration of 0.24 uM together with an appropriate volume of 0.02 M NaPhosphate buffer, pH 7.40, to maintain a final volume of 3.0 ml. The fluorescent intensity was then measured at excitation and emission wavelengths of 288 nm and 331 nm respectively. The results are shown in Figure 8. It is evident that the fluorescence of the sample increases with increasing amounts of

FIGURE 7 Fluorescence emission spectra of alpha$_2$-Macroglobulin alone (———), and alpha$_2$-Macroglobulin reacted with affinity purified trypsin (----). Entrance and exit slits of 1 mm were used to measure the fluorescence of alpha$_2$-Macroglobulin and trypsin at 0.37 u$\underline{M}$ and 0.48 u$\underline{M}$, respectively. The protein solutions, which were excited at 288 nm were placed in a 0.05 $\underline{M}$ Tris·HCl, pH 7.40 buffer. Each spectra is a representative sample from ten determinations.

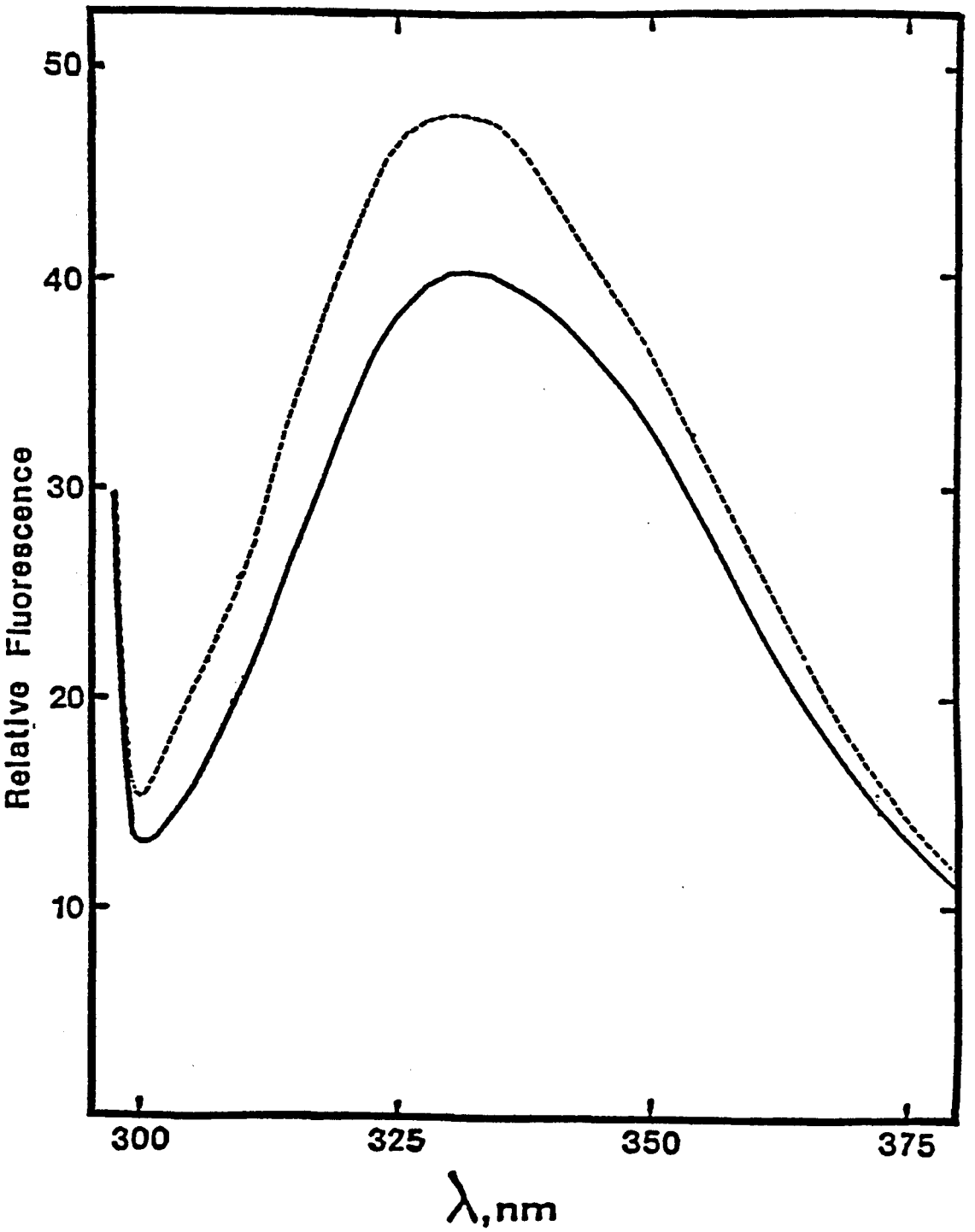
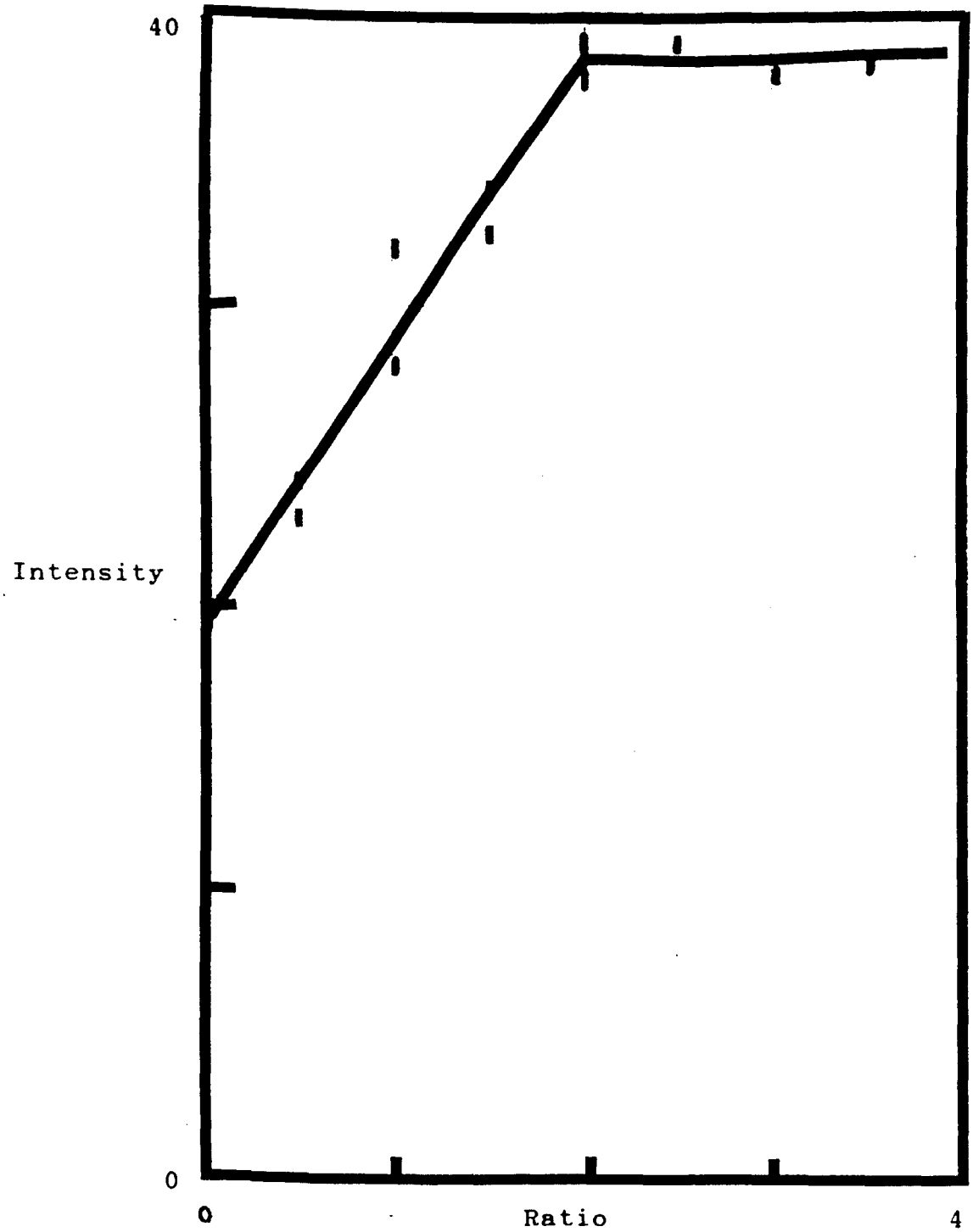

50
40
30
20
10
Relative Fluorescence
300
325
350
375
λ,nm

FIGURE 8 The relative fluorescence intensity of alpha$_2$-Macroglobulin trypsin complex versus the molar ratio of trypsin to native alpha$_2$-Macroglobulin. Increasing amounts of trypsin were reacted with native alpha$_2$-Macroglobulin (0.29 uM). The complex was excited at 288 nm while the emission was measured at 330 nm.


40
Intensity
0
0
Ratio
4

trypsin until a plateau is reached corresponding to a molar ratio of trypsin to alpha$_2$-macroglobulin of 2 to 1. Using identical conditions affinity purified chymotrypsin was reacted with 0.24 uM alpha$_2$-Macroglobulin. The change in fluorescence indicated that chymotrypsin reacts with alpha$_2$-Macroglobulin with a 2 to 1 stoichiometry.

I. Gel Filtration of Native Alpha$_2$-Macroglobulin and Alpha$_2$-Macroglobulin Protease Complex

Native alpha$_2$-Macroglobulin and the alpha$_2$-Macroglobulin trypsin complex were subjected to gel chromatography on Sepharose CL-6B in order to determine whether the formation of complex is accompanied by a change in the frictional coefficient of alpha$_2$-Macroglobulin. Calibration of the column was performed as described in Methods. Seive coefficients were calculated for all the proteins and the results are shown in Table III. The seive coefficient for alpha$_2$-macroglobulin-trypsin complex, is smaller than the sieve coefficient for the native protein, indicating that the complex appears to compact upon complex formation.

Table III Gel Filtration of Proteins on Sepharose CL-6B

Marker Protein	Molecular Weight of Marker Proteins	Marker Seive Coefficient
$(Albumin)_2$	134,000	.685
Xanthine Oxidase	275,000	.482
Thyroglobulin	670,000	.322

Sample Protein	Sample Seive Coefficient (T)	Average	Apparent Molecular Weight
Native $alpha_2$-Macroglobulin	.284		
	.298	.295 $\pm$ 0.10	732,875
	.303		
$Alpha_2$-Macroglobulin Trypsin Complex	.335		
	.314	.328 $\pm$ 0.12	625,014
	.334		

J. Ultraviolet Difference Spectra

Ultraviolet difference spectroscopy was also employed to investigate changes which occurred upon binding proteases to alpha$_2$-macroglobulin. Figure 9 shows difference spectra obtained using native alpha$_2$-Macroglobulin, trypsin, and papain. The difference spectra obtained on binding of trypsin and papain to alpha$_2$-Macroglobulin were similar; each spectra was characterized by two negative extremum. One extrema had a maximum at 200 nm for both alpha$_2$-Macroglobulin-trypsin and alpha$_2$-Macroglobulin-papain complex. Additionally, the magnitude of this extremum was identical for both protease complexes. The second extremum, which appeared to have a maximum at 233 nm for alpha$_2$-Macroglobulin-trypsin complex and 232 nm for alpha$_2$-Macroglobulin-papain, complex differed in magnitude for the two proteases, being 25% more negative for the alpha$_2$-Macroglobulin trypsin complex.

K. Titration of Native and Complexed forms of Alpha$_2$-Macroglobulin with DTNB

The sulfhydryl titer of alpha$_2$-macroglobulin and alpha$_2$-macroglobulin-trypsin complex was compared and the results tabulated in Tables IV and V. First order plots of this data demonstrate that the addition of trypsin caused the appearance of new thiol groups, which could be detected with DTNB (Ellman, 1959). At a trypsin to alpha$_2$-Macroglobulin molar ratio of 2:1, .pa four new sulfhydryl groups are generated per mole of alpha$_2$-macroglobulin.

FIGURE 9 U.V. absorption difference spectra measured between alpha$_2$-Macroglobulin treated with trypsin (·····) or papain (··▲··▲··) and native alpha$_2$-Macroglobulin alone. The spectra were performed in tandem cells at 25°C with native alpha$_2$-Macroglobulin, trypsin, and papain present at concentrations of 0.27 u$\underline{M}$, 0.57 u$\underline{M}$, and 0.57 u$\underline{M}$, respectively. The spectra are the average results from two analyses.

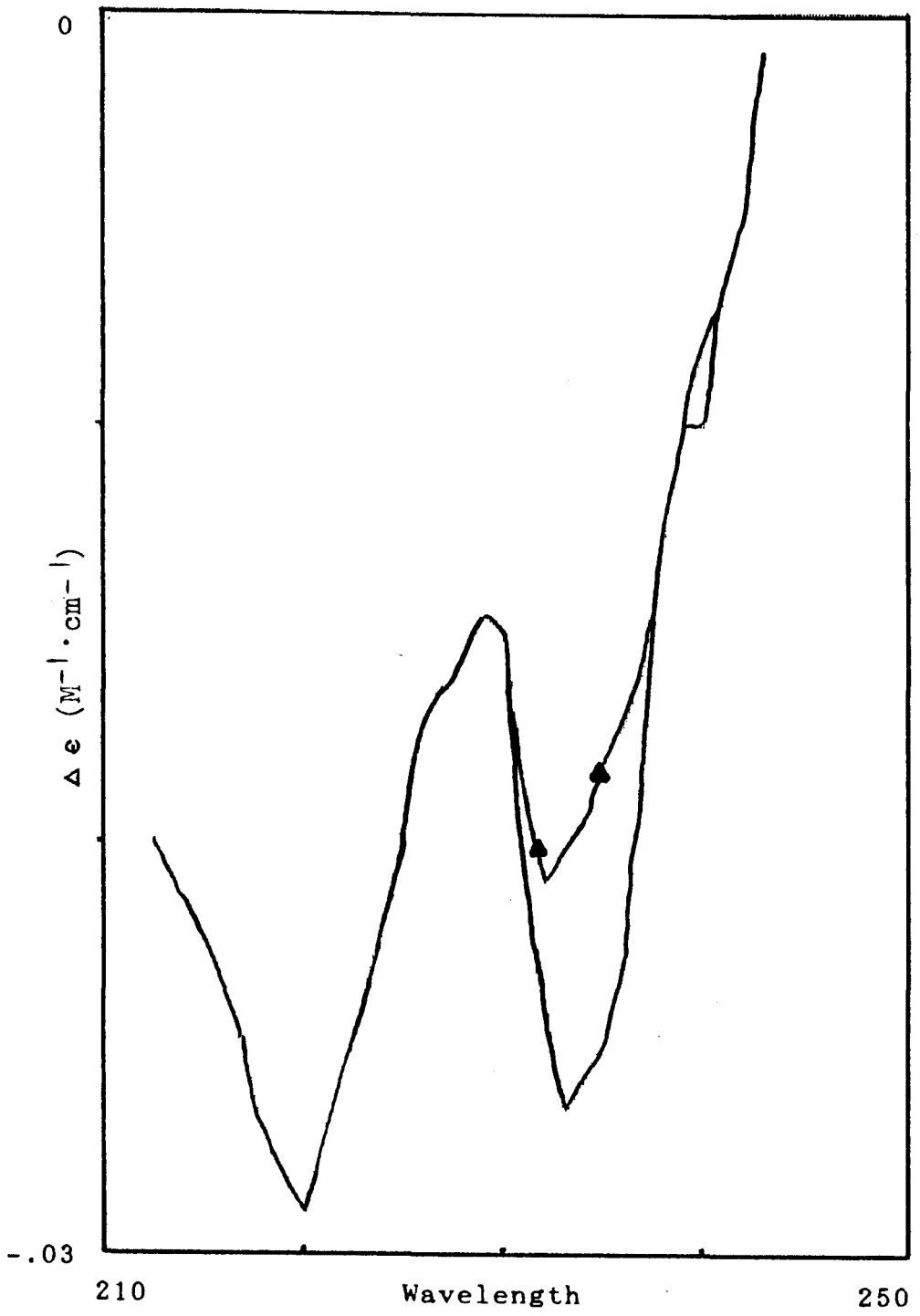

0
-.03
Δ e (M⁻¹·cm⁻¹)
210
Wavelength
250

Table IV DTNB Titration of Native alpha$_2$-Macroglobulin

Time (min)	(A_2-A_t)	In (A_x-A_t)	Analysis of 1st Order Plot
			Linear Regression
0	.0158	-4.148	
.33	.0147	-4.220	Y-intercept = -4.099
.83	.0131	-4.335	
1.33	.0116	-4.457	4.099 = .0166
1.83	.0103	-4.576	
2.33	.0091	-4.700	Slope(k_{obs})=.275 min^{-1}
2.83	.0078	-4.854	
3.33	.0066	-5.021	
3.83	.0058	-5.150	Correlation coefficient=.998
4.33	.0050	-5.298	
4.83	.0043	-5.449	Molar Ratio of [SH]/[a_2-M]
5.33	.0040	-5.521	[SH] = .0166 = 1.22 uM
5.83	.0036	-5.627	1.36×10^4
6.33	.0029	-5.843	
6.83	.0024	-6.032	[SH] = 1.22 uM = 12.1
7.33	.0021	-6.166	[a_2-M] .10 uM

Table V DTNB Titration of $alpha_2$-Macroglobulin Trypsin

Time(min)	$(A_p - A_t)$	In $(A_p - A_t)$	Analysis of 1st Order Plot
			Linear Regression
0	.0216	-3.835	
.67	.0163	-4.117	Y-intercept = -3.808
1.17	.0130	-4.343	
1.67	.0111	-4.501	$e^{-3.808} = .0222$
2.17	.0086	-4.756	
2.67	.0069	-4.976	
3.17	.0057	-5.167	Slope(k_{obs}) = .443 min^{-1}
3.67	.0045	-5.404	
4.17	.0034	-5.684	
4.67	.0026	-5.952	Correlation Coefficient=.998
5.17	.0022	-6.119	
5.67	.0020	-6.215	
6.17	.0013	-6.645	Molar Ratio of $[SH]/[a_2\text{-M}]$
6.67	.0011	-6.812	
7.17	.0010	-6.908	

$$[SH] = \frac{.0222}{1.36 \times 10^4} = 1.638 \text{ uM}$$

$$\frac{[SH] = 1.63 \text{ uM}}{1.0 \text{ uM}} = 16.3$$

L. Sialic Acid Removal

The effect of complex formation on the accessibility of $alpha_2$-macroglobulin's sialic acid residues to enzymatic hydrolysis by Vibrio Cholerae (sialidase) was examined. The results are presented in Table

VI. The incubation of the Vibrio Cholerae enzyme with native alpha$_2$-macroglobulin or alpha$_2$-macroglobulin-trypsin complex resulted in the removal of similar quanitities of sialic acid as determined by the Papermaster assay. In addition, the rates of cleavage of sialic acid from both forms of alpha$_2$-Macroglobulin were similar (Figure 10 and Figure 11). The sialidase cleaved off 16.10 ug of sialic acid per mg of native alpha$_2$-Macroglobulin and 16.44 ug of sialic acid per mg of alpha$_2$-Macroglobulin-trypsin complex. This corresponds to a molar ratio of sialic acid to alpha$_2$-macroglobulin of 38 to 1. In contrast, the chemical cleavage of sialic acid appeared to be more effective than the enzymatic removal. Thus, treatment with 0.1 N sulfuric acid resulted in the removal of 36.9 $\pm$ 2.7 ug of sialic acid per mg of native alpha$_2$-Macroglobulin and 30.5 $\pm$ 2.9 ug of sialic acid per mg of alpha$_2$-macroglobulin-trypsin complex. The sulfuric acid treatment leaved similar amounts of sialic acid from native and complexed alpha$_2$-Macroglobulin.

Table VI Sialidase Treatment of Native and Complexed Alpha$_2$-Macroglobulin

Test Tube Number	Incubation Time hrs	[Sialic Acid] (ug/ml)
Native Alpha$_2$-Macroglobulin		
1	1.05	4.50
2	1.05	4.74
3	3.30	7.14
4	3.30	7.50
5	5.50	9.9
6	5.50	10.5
7	24.5	12.0
8	24.5	12.8
Alpha $_2$-Macroglobulin-Trypsin Complex		
9	1.00	3.10
10	1.00	3.52
11	3.00	8.48
12	3.00	9.60
13	5.00	10.80
14	5.00	11.30
15	26.00	11.90
16	26.00	12.20

FIGURE 10 Plot of sialic acid cleaved from native alpha$_2$-Macroglobulin versus time. Sialic acid was cleaved from native alpha$_2$-Macroglobulin (1.1 u$\underline{M}$) with sialidase (7.5 units/ml) in a 0.05 $\underline{M}$ Na Acetate 0.15 $\underline{M}$ NaCl 8.0 mM $ClCl_2$, pH 5.20 buffer at 37^{o}C for 26 hour. At various times aliquots were removed and assayed for free sialic acid.

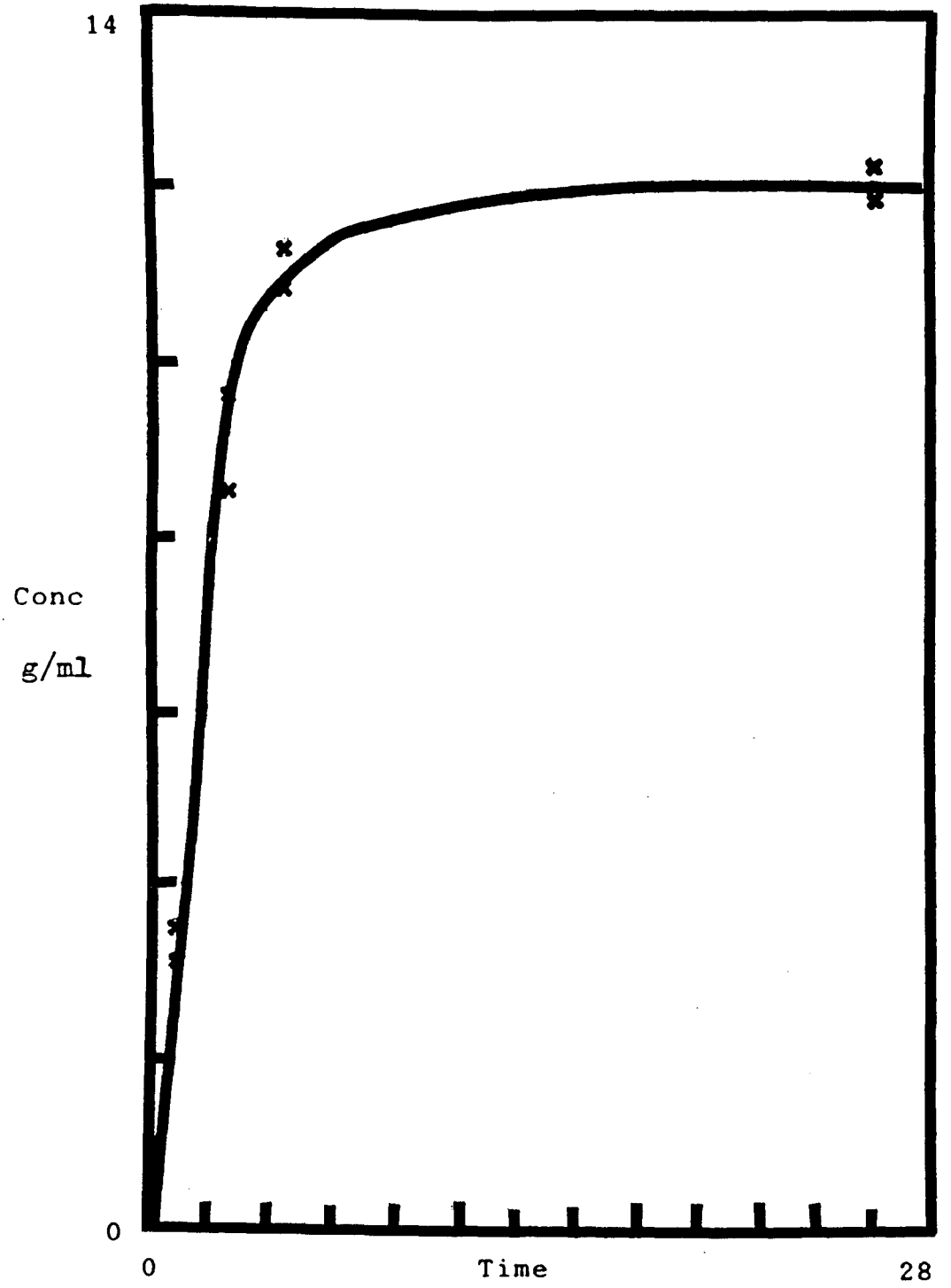
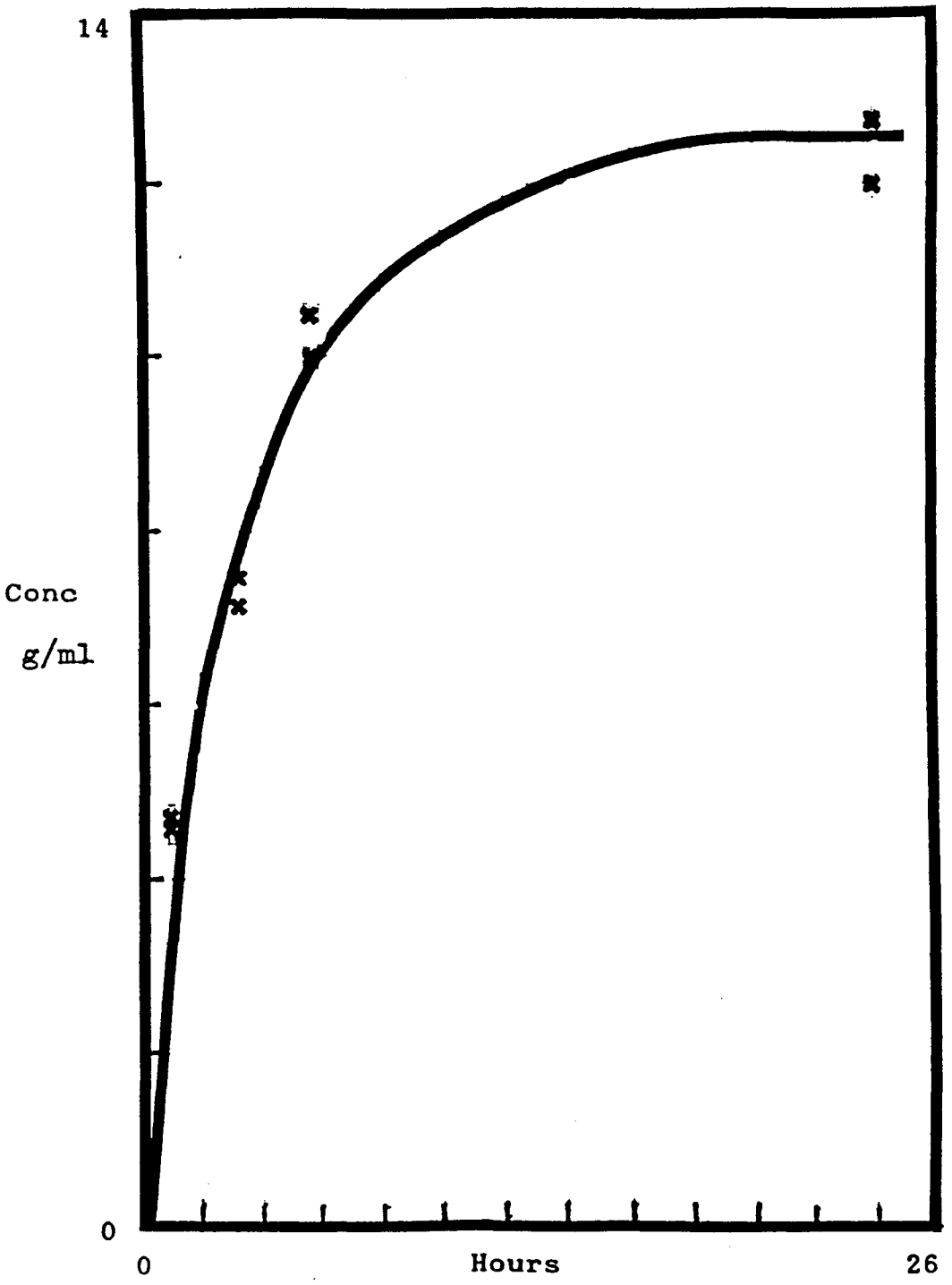

14
Conc
g/ml
0
0
Time
28

FIGURE 11 Plot of sialic acid cleaved from alpha$_2$-Macroglobulin trypsin complex versus time. Native alpha$_2$-Macroglobulin (1.1 u$\underline{M}$) was reacted with trypsin (2.1 u$\underline{M}$) and subjected to sialidase (7.5 units/ml) at 37^{o}C for 26 hours in a 0.05 $\underline{M}$ Na Acetate 0.15 $\underline{M}$ NaCl 8.0 mM $CaCl_2$, pH 5.20 buffer. At various times, aliquots were removed and assayed for free sialic acid.


14
Conc
g/ml
0
0
Hours
26

M. Effect of Metal Cations on Alpha$_2$-Macroglobulin's Trypsin Binding Activity

The observation that metal ions altered alpha$_2$-macroglobulin's trypsin binding activity led to the following series of experiments. Alpha$_2$-macroglobulin was assayed with metals in the presence and the absence of trypsin. As expected, in the absence of trypsin no enzymatic activity was observed. The presence of Mg^{++} and Zn^{++} altered the trypsin binding activity as shown in Figures 12 and Figures 13. Increasing the concentration of zinc chloride or magnesium chloride produced a progressive decrease in the trypsin binding activity below that of alpha$_2$-Macroglobulin assayed without any cations. Magnesium chloride or zinc chloride alone had no effect on trypsin. The addition of the metal ions to the assay did not alter the pH nor result in the precipitation of the alpha$_2$-macroglobulin. In addition, chloride ions had no effect on trypsin binding activity.

N. Analysis of Iodinated Alpha$_2$-Macroglobulin

Alpha$_2$-Macroglobulin was iodinated by four different methods. The primary means of evaluating the four methods of iodination was the recovery of trypsin binding activity. The results are summarized in Table VII. The enzymobead procedure of iodination appeared to have no noticeable effects on the trypsin binding activity, whereas Cuatrecacus' and Marchaloni's procedures completely abolished the ability of the protein to bind trypsin Hunter and Greenwood's procedure resulted in a minor reduction in the trypsin binding activity. Some iodinated protein samples, despite removal of free iodine by either dialysis or gel filtration yielded a significant fraction of label which was not precipitable with TCA. Consequently,

FIGURE 12 Semilog plot of trypsin binding activity of alpha$_2$-Macroglobulin incubated with Mg^{++} versus the concentration of Mg^{++}. The concentration of Mg^{++} was varied from 10^{-7} M to 10^{-3} M. The trypsin binding activity of alpha$_2$-Macroglobulin in the absence of Mg^{++} was 0.0014 units/ml. Each sample was run in duplicate.

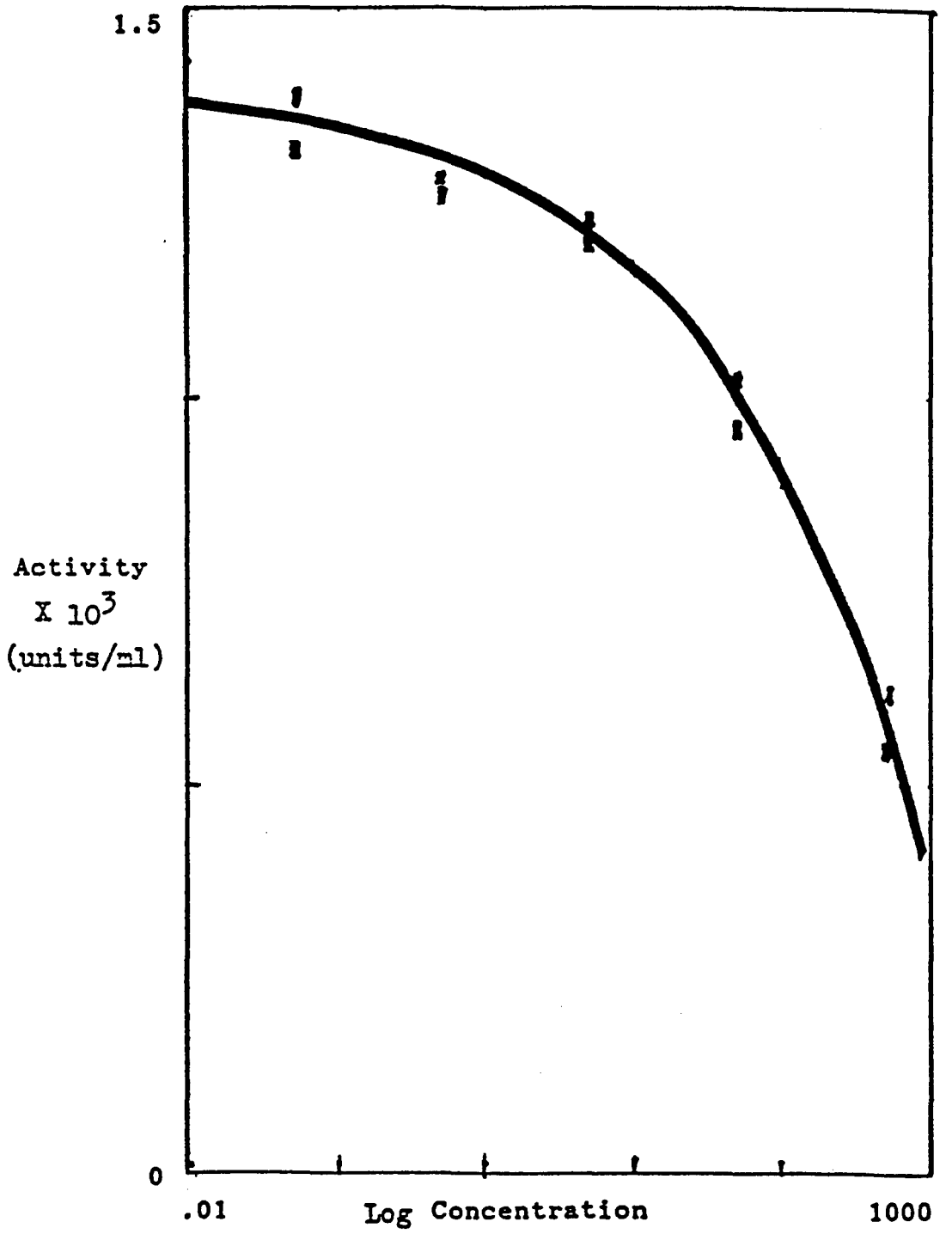

1.5
Activity
X 10^3
(units/ml)
0
.01
Log Concentration
1000

FIGURE 13 Semilog plot of trypsin binding activity of alpha$_2$-Macrglobulin incubated with Zn^{++} versus concentration of Zn^{++}. The concentration of Zn^{++} was varied from 10^{-7} M to 10^{-4}M. The tryspin binding activity of alpha$_2$-Macoglobulin in the absence of Zn^{++} was 0.0014 units/ml. Each sample was run in duplicate. Zinc acetate and Zinc chloride produced identical results.

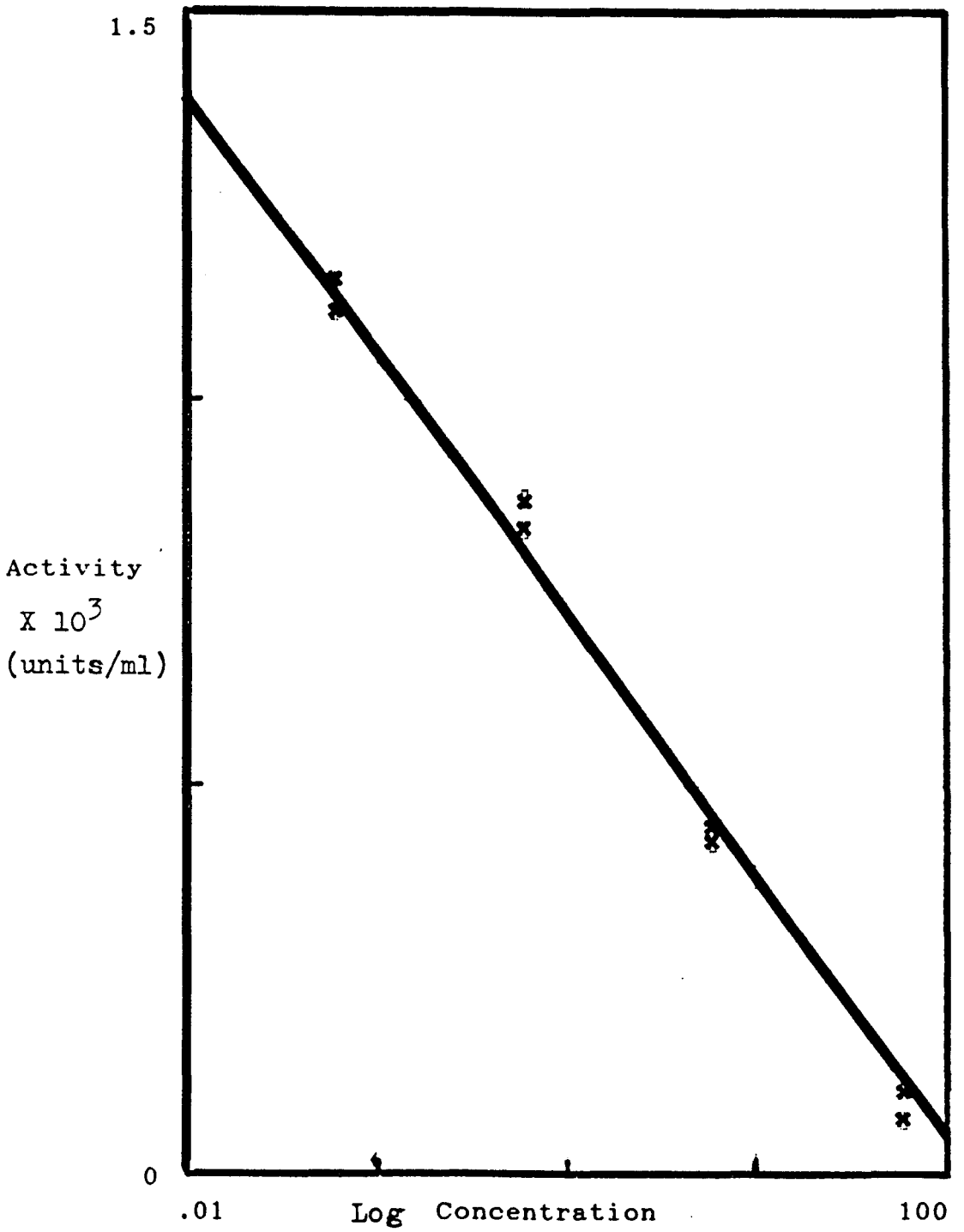

1.5
Activity
X 10^3
(units/ml)
0
.01
Log Concentration
100

TABLE 7

EFFECT OF VARIOUS IODINATION PROCEDURES OF ALPHA$_2$-MACROGLOBULIN'S TRYPSIN BINDING ACTIVITY

Procedure	% Activity Remaining Following Iodination[a]
Cuatrecacus	0
Marchaloni's	0
Hunter	85
Enzymobeads	100

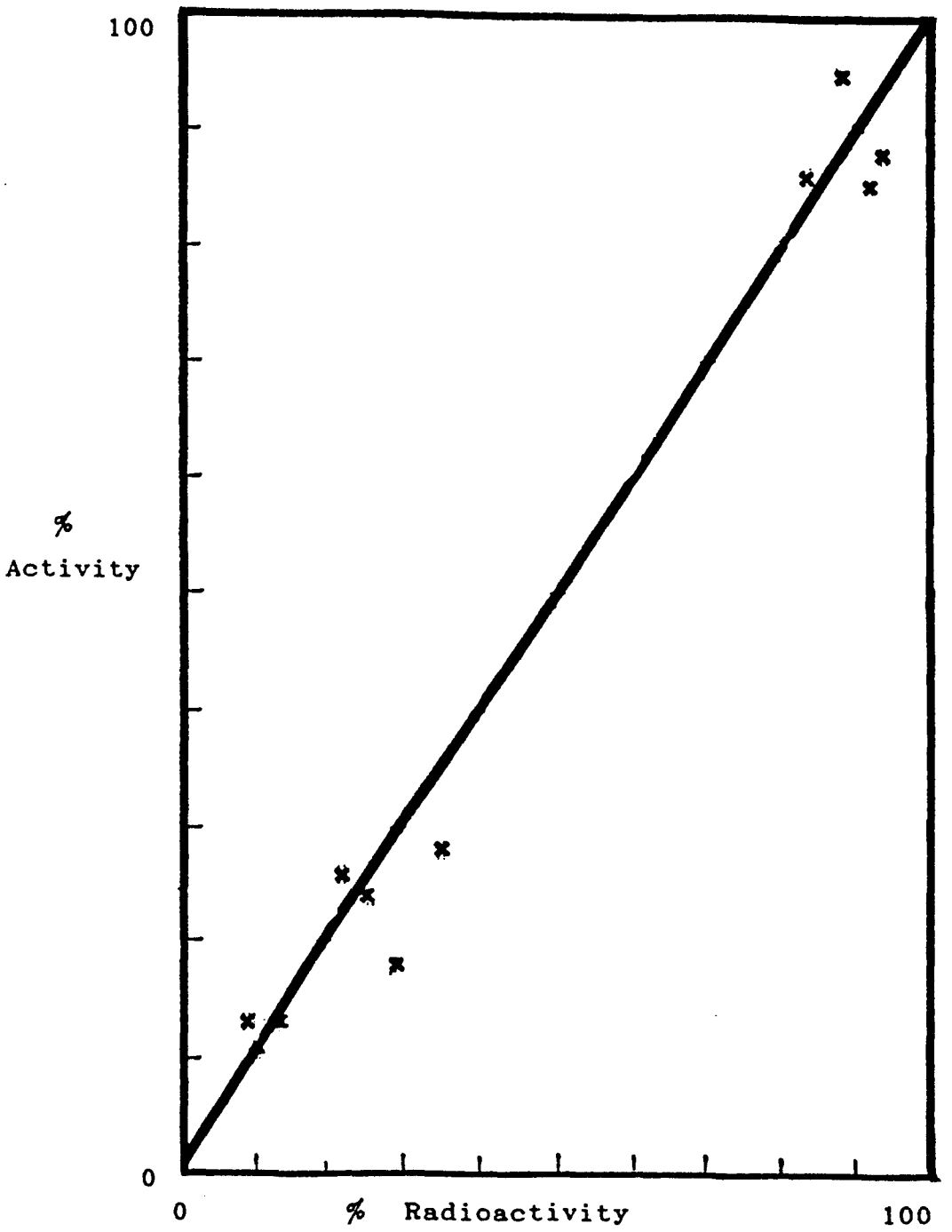
a. Values are an average of two determinations.

the TCA precipitability of the (Enzymobead) labeled $alpha_2$-Macroglobulin was routinely ascertained. ConA precipitation of native and iodinated $alpha_2$-Macroglobulin was also performed in order to verify that both proteins behaved similarly. ConA precipitation of $alpha_2$-Macroglobulin was performed by adding increasing amounts of ConA to plastic test tubes containing I^{125}-labeled and unlabeled $alpha_2$-Macroglobulin in 0.2 M NaPhosphate 10 uM $CaCl_2$ 1.0 uM $MaCl_2$, pH 7.40 at room temperature. Following incubation and centrifugation, aliquots of the supernatant were assayed for trypsin binding activity and counted in the gamma counter. The results in Figure 14 demonstrated that iodinated $alpha_2$-Macroglobulin was precipitated in an identical manner as unlabeled $alpha_2$-Macroglobulin. The I^{125} $alpha_2$-Macroglobulin utilized for experimentation had a TCA precipitability of at least 92%. The specific activity of the I^{125} $alpha_2$-Macroglobulin ranged between 0.1uCi/ug and 3.8 uCi/ug.

O. Lectin Binding Studies

The interaction of $alpha_2$-Macroglobulin with Concanavalin A and wheat germ lectin was performed with lectins free in solution or immobilized on Sepharose 4B. Initially, Concanavalin A was coupled to cyanogen bromide activated Sepharose at several different pH values and temperatures. Resins 2 and 3 (Table VIII) were both coupled at pH 9.0. The temperature used for coupling resin 2 was room temperature, while resin 3 was coupled at $4^{o}C$. Resins 4 and 5 were both coupled at pH 8.0 and at room temperature, but the coupling buffer for resin 5 contained only Concanavalin A whereas the coupling buffer for resin 4 contained equimolar quantities of Concanavalin A and alpha-methyl mannoside. Table VIII illustrates the conditions used for coupling as

FIGURE 14 Precipitation of I^{125} alpha$_2$-Macroglobulin and unlabeled alpha$_2$-Macroglobulin by various concentrations of Concanavalin A. Native alpha$_2$-Macroglobulin (0.19 uM) was incubated with increasing concentrations of Con A (0 to 18 uM). The percent of trypsin binding activity remaining in solution following precipitation by Con A was plotted versus percent of radioactivity remaining in solution following precipitation by Con A. Linea regression of the data indicates that the line has a slope of 1.02 with a correlation coefficient of 0.992.


100
%
Activity
0
0
% Radioactivity
100

well as the binding capacity of each resin. The coupling conditions used for resin 5 resulted in the greatest binding capacity e.g. 1.1 micromoles of alpha$_2$-Macroglobulin per millileter of gel. Consequently, this resin was employed in further binding studies. Native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex were each applied to a column containing resin 5 and subjected to a variety of eluting buffers.

TABLE VIII Coupling Condiitons and Binding Capacity for Concanavalin A Affinity Resins

Resin	pH	Temperature (^{o}C)	Micromoles Bound
1*	11	Unknown	0
2	9	Room Temperature	0.02
3	9	4^{o}	0.03
4xx	8	Room Temperature	0.60
5	8	Room Temperature	1.10

* Commercial resin - Miles

xx Alpha-methylmannoside was bound to Concanavalin A prior to coupling.

Table IX demonstrates that native alpha$_2$-Macroglobulin could be separated from alpha$_2$-Macroglobulin trypsin by first eluting the resin bound protein with 1.0 M alpha-methylmannoside in the sodium barbital buffer, while the alpha$_2$-Macroglobulin trypsin complex could only be eluted by decreasing the pH to 5.50 with sodium citrate buffer. Another Concanavalin A affinity resin (6) was synthesized using the identical coupling conditions as resin 5. Both native and complexed forms of alpha$_2$-Macroglobulin were each applied to a column containing resin 6 and elution was attempted. The results obtained on Resin 5 could not be reproduced. The binding and elution conditions for Resin 6 are illustrated in Table X. Both forms of alpha$_2$-Macroglobulin appeared to bind and elute from resin 6 in a similar manner as compared to resin 5 where differential elution had been obtained previously. Another affinity resin (7) was synthesized using the identical coupling conditions as resin 5, except following the coupling of Concanavalin A, any residual cyanogen bromide activated sites in the resin were blocked with methylamine. Native alpha$_2$-Macroglobulin was applied to resin 7 before the usual wash buffers. No alpha$_2$-Macroglobulin could be detected in any of the elution buffers. The anthrone sulfuric assay was used to confirm the presence of alpha-methylmannoside in the elution buffers. Repeated attempts to elute the alpha$_2$-Macroglobulin from the resin failed. Subsequently, it was discovered that methylamine could abolish the typsin binding activity of native alpha$_2$-M. Consequently, no further studies were performed with resin 7.

Because of lack of reproducibility, the Concanavalin A affinity resins were abandoned, and further lectin binding studies were

TABLE IX BINDING AND ELUTION OF NATIVE AND COMPLEXED FORMS OF ALPHA$_2$-MACROGLOBULIN TO RESIN 5

Protein Applied	Amount of Protein Applied (Micromole)	Equilibration Buffer[2]	Eluting Buffer[1]	[alpha$_2$-M] in Eluting Buffer (M)	Amount of Protein Eluted (Micromole)
Native alpha$_2$-M	1.0	Barbital	Barbital	0	0,0,0
Native alpha$_2$-M	1.0	Barbital	Barbital	1.0	.88,.87,.86
Alpha$_2$-M-Tryspin Complex	1.0	Barbital	Barbital	0	0,0,0
Alpha$_2$-M-Tryspin Complex	1.0	Barbital	Barbital	1.0	0,0,0
Alpha$_2$-M-Tryspin Complex	1.0	Barbital	Borate	1.0	0,0,0
Alpha$_2$-M-Tryspin Complex	1.0	Barbital	Citrate	1.0	.88,.88,.87

Equilibration Buffer + Eluting Buffer[1]

Barbital = 0.0125 M Na Barbital 1.0 uM $MnCl_2$ 1.0 M $CaCl_2$, pH 7.40

Borate = 0.1 M Na Borate 1.0 uM $MnCl_2$ 1.0 uM $CaCl_2$, pH 6.0

Citrate = 0.1 M Na Citrate 1.0 uM $MnCl_2$ 1.0 uM $CaCl_2$, pH 5.5.

TABLE X BINDING AND ELUTION OF NATIVE AND COMPLEXED FORMS OF ALPHA$_2$-MACROGLOBULIN TO RESIN 6

Protein Applied	Amount of Protein Applied (Micromole)	Equilibration Buffer[2]	Eluting Buffer[1]	[alpha$_2$-M] in Eluting Buffer (M)	Amount of Protein Eluted (Micromole)
Native alpha$_2$-M	1.0	Barbital	Barbital	0	0,0
Native alpha$_2$-M	1.0	Barbital	Barbital	1.0	.87, .80
Alpha$_2$-M-Trypsin Complex	1.0	Barbital	Barbital	0	0,0
Alpha$_2$-M-Trypsin Complex	1.0	Barbital	Barbital	1.0	.58, .60
Native alpha$_2$-M	1.0	Citrate	Citrate	0	.81, .87
Alpha$_2$-M Trypsin Complex	1.0	Citrate	Citrate	0	.89, .94

Equilibration Buffer + Eluting Buffer[1]

Barbital = 0.0125 M Na Barbital 1.0 uM $MnCl_2$ 1.0 uM $CaCl_2$, pH 7.40

Citrate = 0.1 M Na Citrate 1.0 uM $MnCl_2$ 1.0 uM $CaCl_2$, pH 5.5.

performed with Concanavalin A free in solution. Initially, the effect of time and temperature on the ability of Concanavalin A to precipitate alpha$_2$-Macroglobulin was examined. As Figure 15 indicates, this precipitation is completed in 5 minutes. All subsequent studies were then performed with an incubation period of thirty minutes. The effect of temperature on the precipitation of I^{125}-labeled and unlabeled alpha$_2$-Macroglobulin was investigated. Following incubation of the alpha$_2$-Macroglobulin and Concanavalin A, the test tube's contents were centrifuged and an aliquot of the supernatant removed for gamma counting. The precipitation of alpha$_2$-Macroglobulin by Concanavalin A at 4^{o}C and at 25^{o}C were similar as indicated by Table XI. Subsequent precipitations were conducted at 25^{o}C. The interaction of Concanavalin A with native alpha$_2$-Macroglobulin, alpha$_2$-Macroglobulin protease complex, methylamine pretreated alpha$_2$-Macroglobulin, and H_2O_2 pretreated alpha$_2$-Macroglobulin was then examined. Figure 16 demonstrates that native alpha$_2$-Macroglobulin was precipitable by Concanavalin A whereas alpha$_2$-Macroglobulin trypsin complex was not. Iodinated alpha$_2$-Macroglobulin and non-iodinated alpha$_2$-Macroglobulin were allowed to react with a chymotrypsin, followed by the addition of Concanavalin A. As Table XII indicates, the Concanavalin A was also unable to precipitate alpha$_2$-Macroglobulin chymotrypsin complex to any great extent. Similar results were also obtained with alpha$_2$-Macroglobulin papain in place of alpha$_2$-Macroglobulin chymotrypsin. Iodinated alpha$_2$-Macroglobulin was pretreated with either methylamine or H_2O_2 as described in the Methods section, and subjected to a Concanavalin A precipitation. Tables XIII and XIV demonstrated that Concanavalin A

FIGURE 15 Plot of precipitation of alpha$_2$-Macroglobulin by Con A versus time. Alpha$_2$-Macroglobulin (0.68 u$\underline{M}$) was incubated with Con A (16.3 u$\underline{M}$) for varying amounts of timefrom 0 minutes to 63 minutes. For each point, aliquots of the supernatant were removed after centrifugation and assayed for alpha$_2$-Macroglobulin's trypsin binding activity. The percent of trypsin binding activity remaining in the supernatant was plotted versus time.

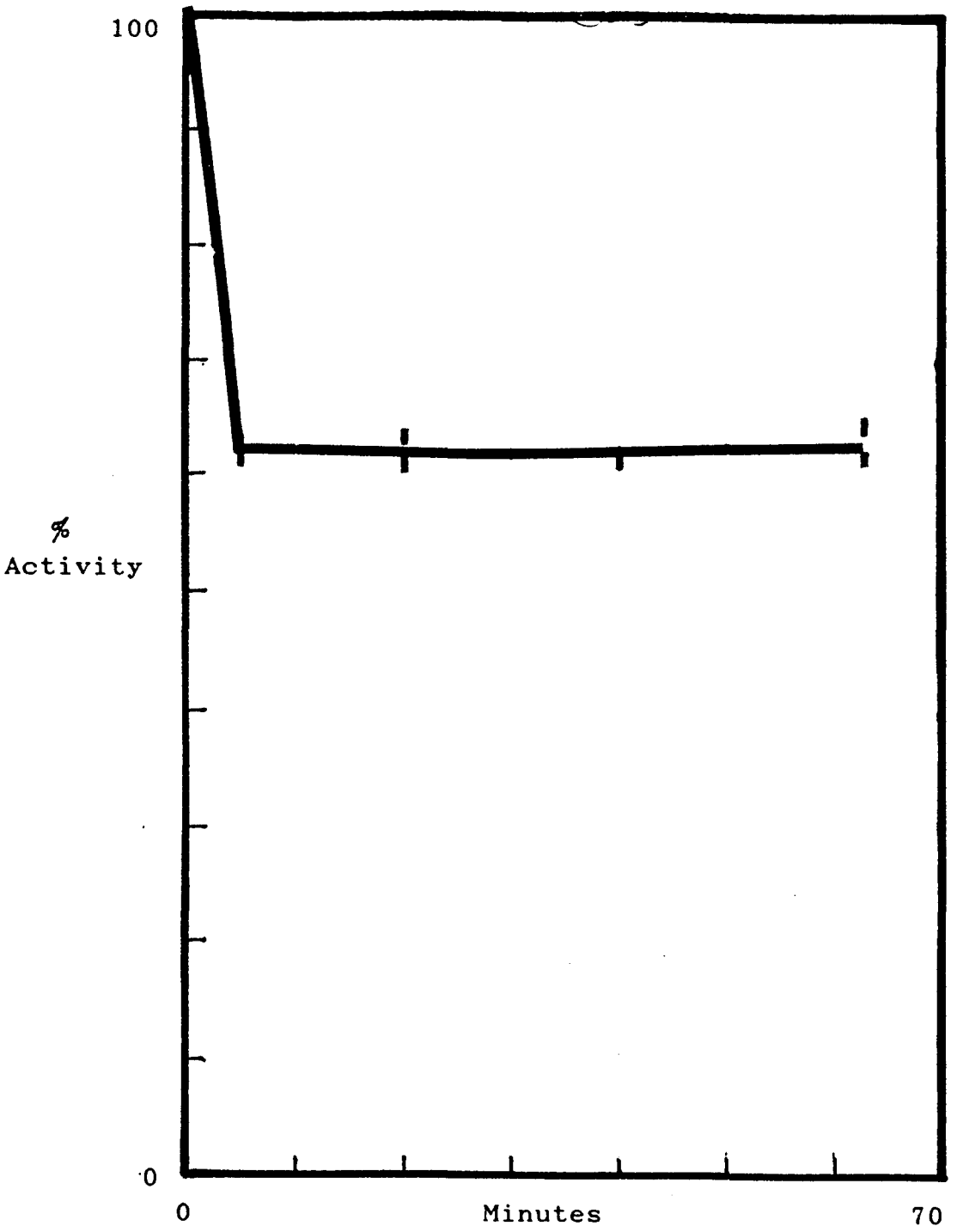

100
%
Activity
0
0
Minutes
70

TABLE XI CON A PRECIPITATION OF I^{125}-ALPHA$_2$-MACROGLOBULIN AS A FUNCTION OF TEMPERATURE

Tube No.	Temperature of Incubation (^{o}C)	Radioactivity in Supernatant (cpm)	Mean
1	4	27,716	
2	4	28,122	29,191 $\pm$ 2212
3	4	31,734	
4	25	31,955	
5	25	28,179	31,333 $\pm$ 2893
6	25	33,864	

FIGURE 16 Percent of trypsin binding activity remaining in supernatant following precipitation of native alpha$_2$-Macroglobulin (X) and alpha$_2$-Macroglobulin trypsin complex (▮) by Concanavalin A versus the molar ratio of Con A to alpha$_2$-Macroglobulin. The precipitation was conducted at room temperature and at pH 7.4. These values are a representative sample of six runs. Alpha$_2$-Macroglobulin protease complex was formed using trypsin, chymotrypsin and papain. Con A was incapable of precipitating any of these complexes.

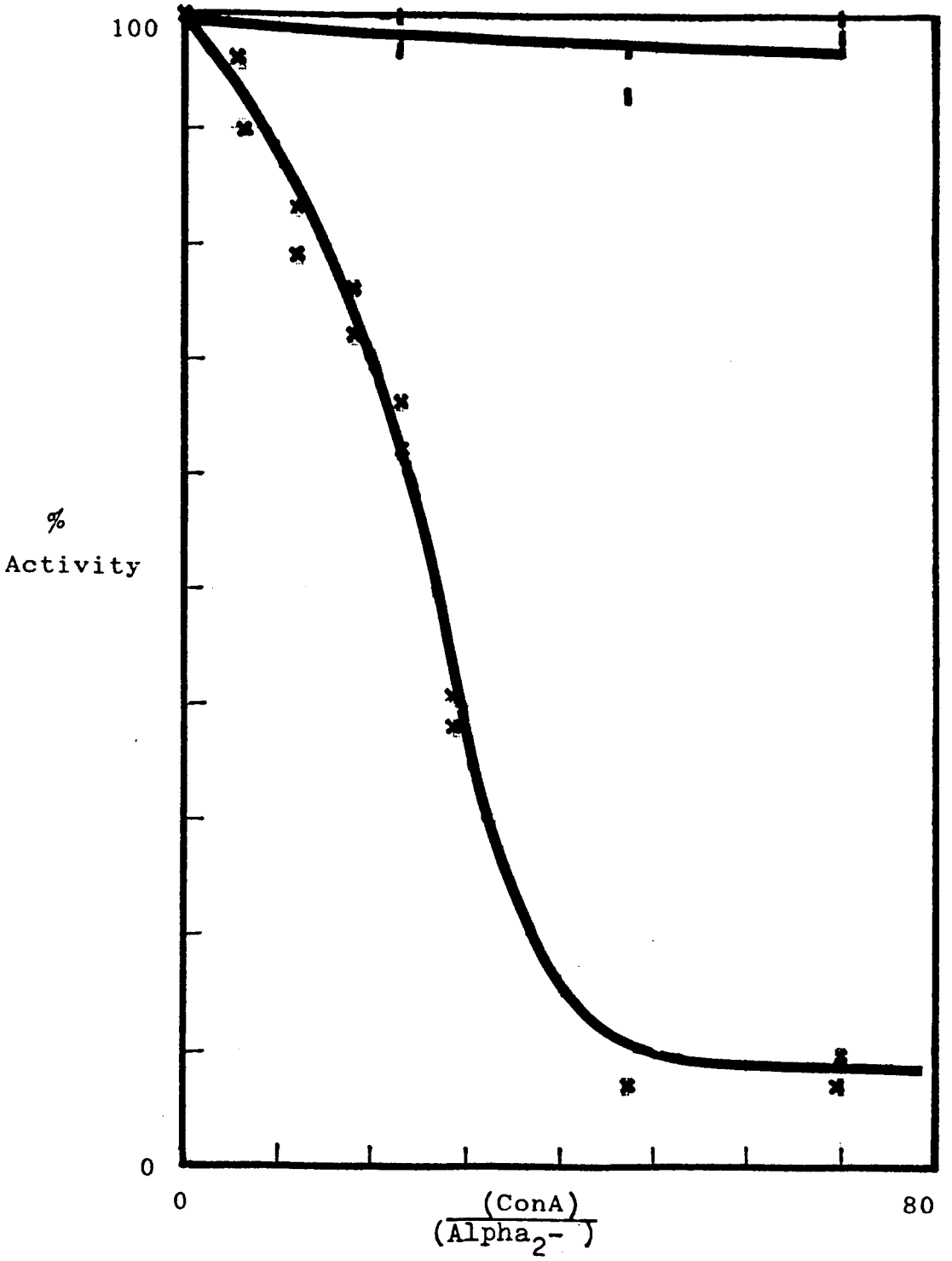

100
0
% Activity
0
80
(ConA) / (Alpha2-)

TABLE XII CONCANAVALIN A PRECIPITATION OF ALPHA$_2$-MACROGLOBULIN CHYMOTRYPSIN COMPLEX

Tube No.	[alpha$_2$-M] (u.M)	[Chymotrypsin] (u.M)	[Con A] (u.M)	Radioactivity Present in Test tube (cpm)	Radioactivity Present in Precipitate (cpm)
1	0.42	0.86	19.0	74,600	2664
2	0.42	0.86	19.0	74,600	3092
3	0.42	0.86	19.0	74,600	7266

TABLE XIII CONCANAVALIN A PRECIPITATION OF METHYLAMINE-ALPHA$_2$-MACROGLOBULIN

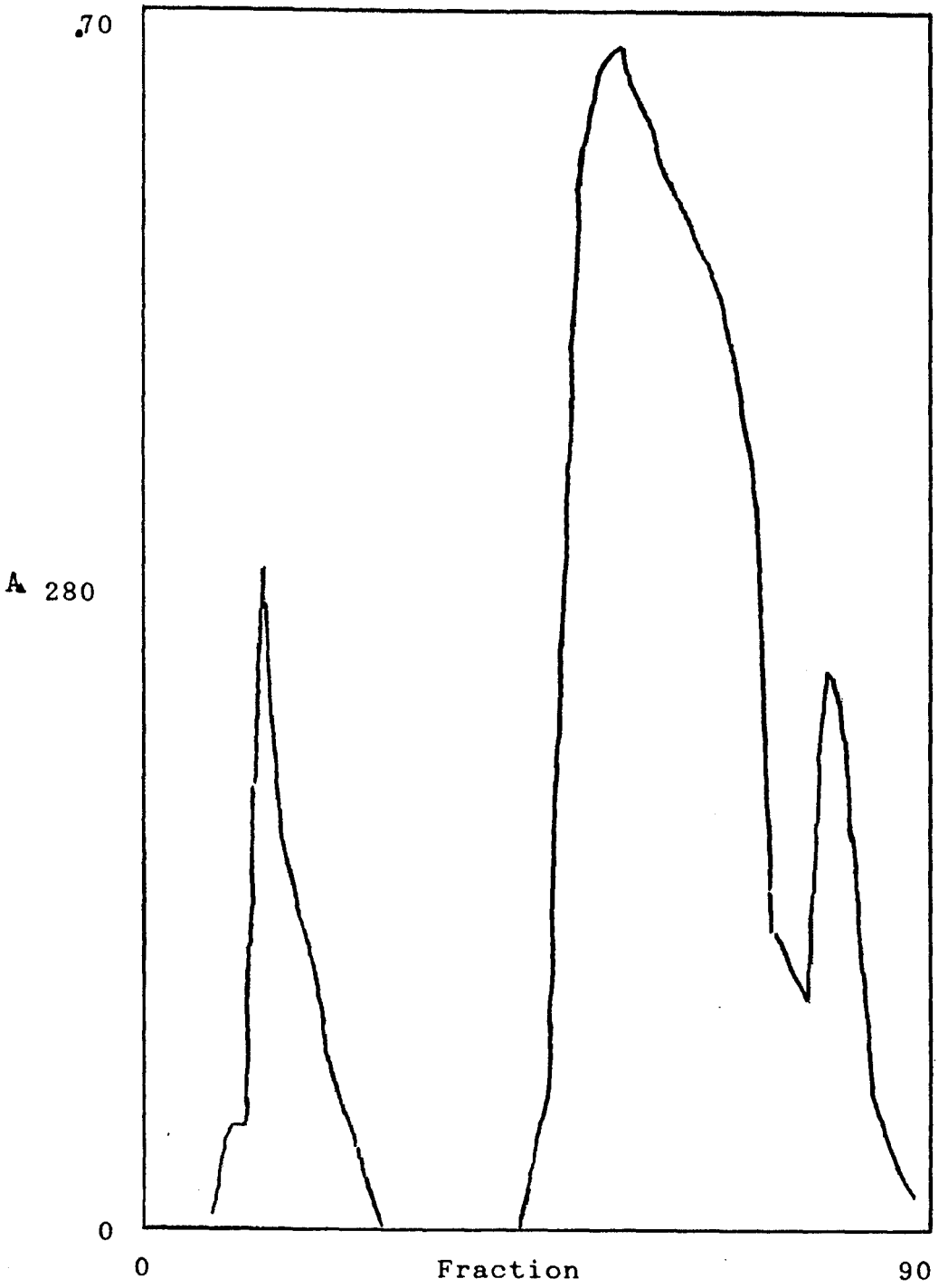
Tube No.	Methylamine [alpha$_2$-M] (u.M)	[Con A] (u.M)	Radioactivity Present in Test tube (cpm)	Radioactivity Present in Precipitate (cpm)	Percent of Level in Precipitate (%)
1	0.16	18.0	135,500	6233	4.6
2	0.16	18.0	135,500	6098	4.5
3	0.16	18.0	135,500	6369	4.7

TABLE XIV CON A PRECIPITATION OF H_2O_2 TREATED ALPHA$_2$-MACROGLOBULIN

Tube No.	[alpha$_2$-M] (u.M)	[Con A] (u.M)	Radioactivity Present in Test tube (cpm)	Radioactivity Present in Precipitate (cpm)	Percent of Level in Precipitate (%)
1	0.12	17.0	44,500	1400	3.2
2	0.12	17.0	46,000	1425	3.1
3	0.12	17.0	42,000	1075	2.6

was also largely unable to precipitate methylamine pretreated I^{125} labeled alpha$_2$-Macroglobulin and H_2O_2 pretreated I^{125} labeled alpha$_2$-Macroglobulin. These initial results seemed to indicate that Concanavalin A could selectively precipitate native alpha$_2$-Macroglobulin but not alpha$_2$-Macroglobulin protease complex or methylamine and H_2O_2 treated alpha$_2$-M. These experiments were repeated using alpha$_2$-Macroglobulin from several different purifications with each batch of alpha$_2$-Macroglobulin yielding identical results. The Concanavalin A used in these studies was purchased from two sources, Sigma and Pharmacia. All bottles of Concanavalin A produced results identical to those presented above, except for the last bottle of Concanavalin A purchased from Pharmacia. Previously, the Concanavalin A precipitated native alpha$_2$-Macroglobulin but not alpha$_2$-Macroglobulin protease complex. This new bottle of Concanavalin A precipitated both native and complexed forms of alpha$_2$-Macroglobulin. Since the conditions used for the precipitation were identical to the previous studies, these new results were rather puzzling. Consequently, a new batch of alpha$_2$-Macroglobulin was purified and subjected to the Concanavalin A precipitation. Once again the Concanavalin A precipitated both native and complexed forms of alpha$_2$-Macroglobulin. The new bottle of Concanavalin A was then subjected to an affinity purification on a column of Affigel-ovalbumin (See Methods). The elution profile obtained from the Affigel Ovalbumin column is illustrated in Figure 17. The first peak represents material which did not bind to the resin. The second and third peaks correspond to Concanavalin A which bound and could be eluted with alpha-methylmannoside. These peaks

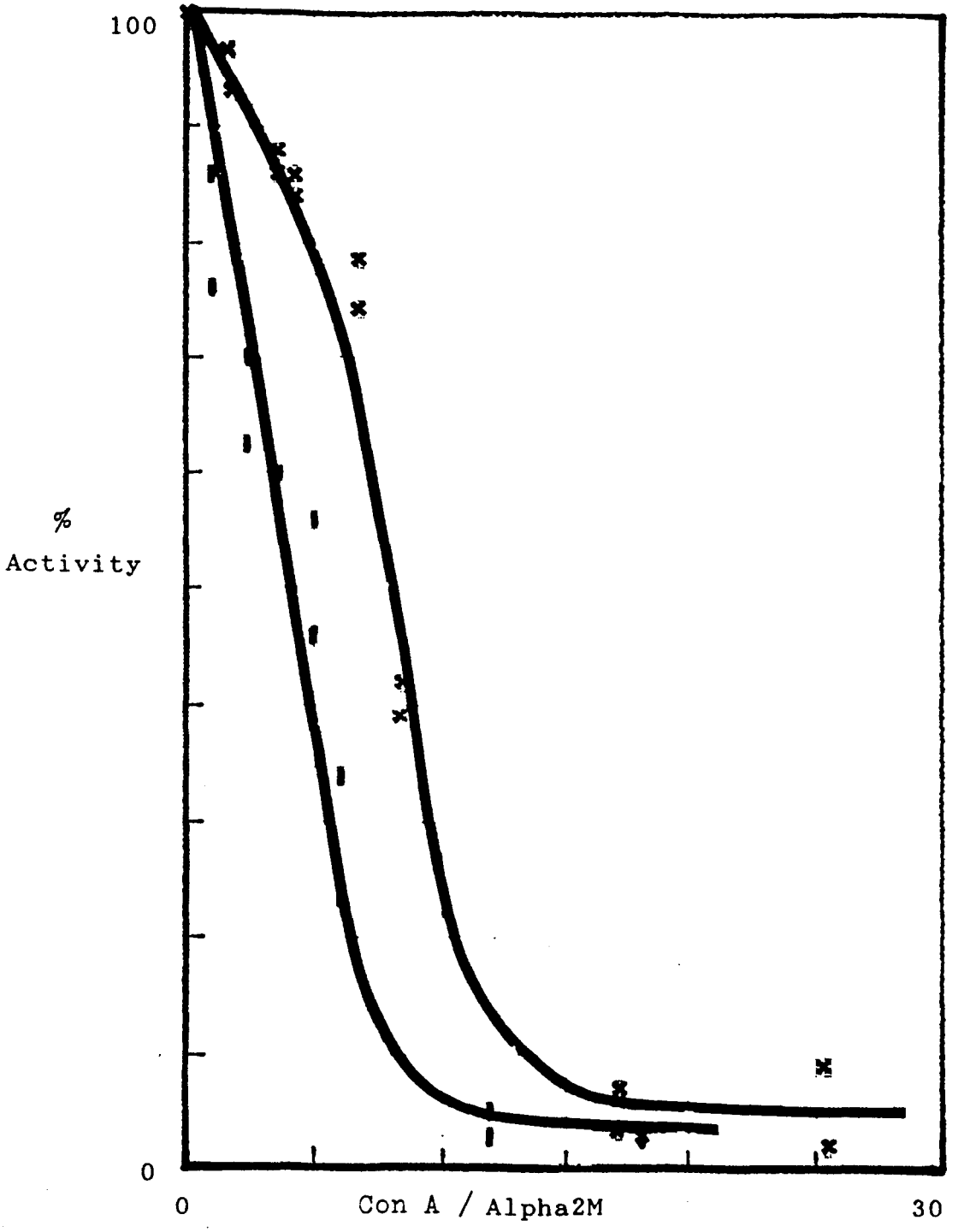
FIGURE 17 Chromatography of Concanavalin A on Affigel Ovalbumin. An Affigel ovalbumin column was equilibrated with 0.02 M Hepes 1.0 uM M_2Cl_2 1.0 uM $CaCl_2$, pH 7.40 at a flow rate of 10 ml/hr. Following sample application the non-binding proteins were allowed to elute from the column. When the A_{280} remained zero for one entire bed volume, Con A was eluted from the resin with 0.5 M alpha-methylmannoside.


.70
A 280
0
0
Fraction
90

containing purified Concanavalin A were combined, concentrated, and exhaustively dialyzed against the 0.0125 M Sodium Barbital 10 uM Calcium $Chloride_2$ 10 uM $MnChloride_2$, pH 7.0 buffer. This purified Concanavalin A was used to repeat the precipitation of native and complexed forms of $alpha_2$-Macroglobulin at pH 7.40. These results (Figure 18) corroborated the findings obtained with the unpurified Concanavalin A e.g. both native and $alpha_2$-Macroglobulin tryspin complex were precipitated by the Concanavalin A. The only major difference between the experiments using purified and unpurified Concanavalin A was that a much smaller quantity by weight of the purified Concanavalin A was required to precipitate the same amount of $alpha_2$-Macroglobulin. The precipitation of native and complexed forms of $alpha_2$-Macroglobulin with purified Concanavalin A was repeated at pH 5.29 using a 0.05 M Sodium Acetate 10 uM Calcium Chloride 10 uM MnChloride buffer. The results obtained at pH 5.29 were similar to the resulted obtained at 7.40 e.g. both native and complexed forms of $alpha_2$-Macroglobulin were precipitated by affinity purified Concanavalin A.

Wheat germ lectin was then utilized for affinity resins and lectin binding studies in place of Concanavalin A. The wheat germ affinity resins were made according to the procedure and conditions utilized for Concanavalin A resin 5. Native $alpha_2$-Macroglobulin and $alpha_2$-Macroglobulin trypsin complex were applied to affinity columns. Native $alpha_2$-Macroglobulin failed to bind to the affinity resin at both pH 8.45 and 7.40, while 40% and 23% of the applied $alpha_2$-Macroglobulin trypsin complex bound to the resins at pH 8.45 and 7.40 respectively. Wheat germ lectin $alpha_2$-Macroglobulin studies were

FIGURE 18 Percent of trypsin binding activity remaining in supernatant following precipitation of native alpha$_2$-Macroglobulin (■) and alpha$_2$-Macroglobulin trypsin complex (X) by affinity purified Concanavalin A versus the molar ratio of Con A to alpha$_2$-Macroglobulin. The precipitation was conducted at room temperature and at pH 7.4.


100
%
Activity
0
0
Con A / Alpha2M
30

also performed utilizing the wheat germ lectin free in solution. Initially, the interaction of alpha$_2$-Macroglobulin with wheat germ lectin was examined as a function of time. The results indicated that an incubation period of one hour would be sufficient for completion of the precipitation reaction. Additional binding studies were conducted with affinity purified and unpurified wheat germ lectin at pH 7.40 and pH 5.20. As Figures 19, 20 and 21 illustrate, the alpha$_2$-Macroglobulin trypsin complex tends to be slightly more precipitable than native alpha$_2$-Macroglobulin.

P. Hydrophobic Interaction Chromatography

Native and complexed forms of alpha$_2$-Macroglobulin were chromatographed on ethyl, butyl, and octyl sepharose to determine whether complexation resulted in changed in the exposure of hydrophobic sites on the surface of the protein. The results (Figure 22) seem to indicate that native and complexed forms of alpha$_2$-Macroglobulin interacted with the ethyl agarose in a similar manner, with native alpha$_2$-Macroglobulin being slightly retarded. Application of native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex to butyl agarose resulted in a greater retardation of the native alpha$_2$-Macroglobulin as compared to the protease complexed alpha$_2$-Macroglobulin (Figure 23).

In constrast, the octyl Sepharose was capable of completely binding both the native and the complexed forms of alpha$_2$-Macroglobulin (Table XV). The equilibration of the octyl sepharose with a buffer containing chaotropic ions and/or ethylene glycol did not attenuate the binding of either native or complexed alpha$_2$-Macroglobulin, thereby resulting in an inability to elute them from the

FIGURE 19 Percent of tryspin binding activity remaining in supernatant following precipitation of native alpha$_2$-Macroglobulin (■) and alpha$_2$-Macroglobulin tryspin complex (X) by wheat germ lectin versus the molar ratio of wheat germ lectin to alpha$_2$-Macroglobulin. The precipitation was conducted at room temperature and at pH 7.40.

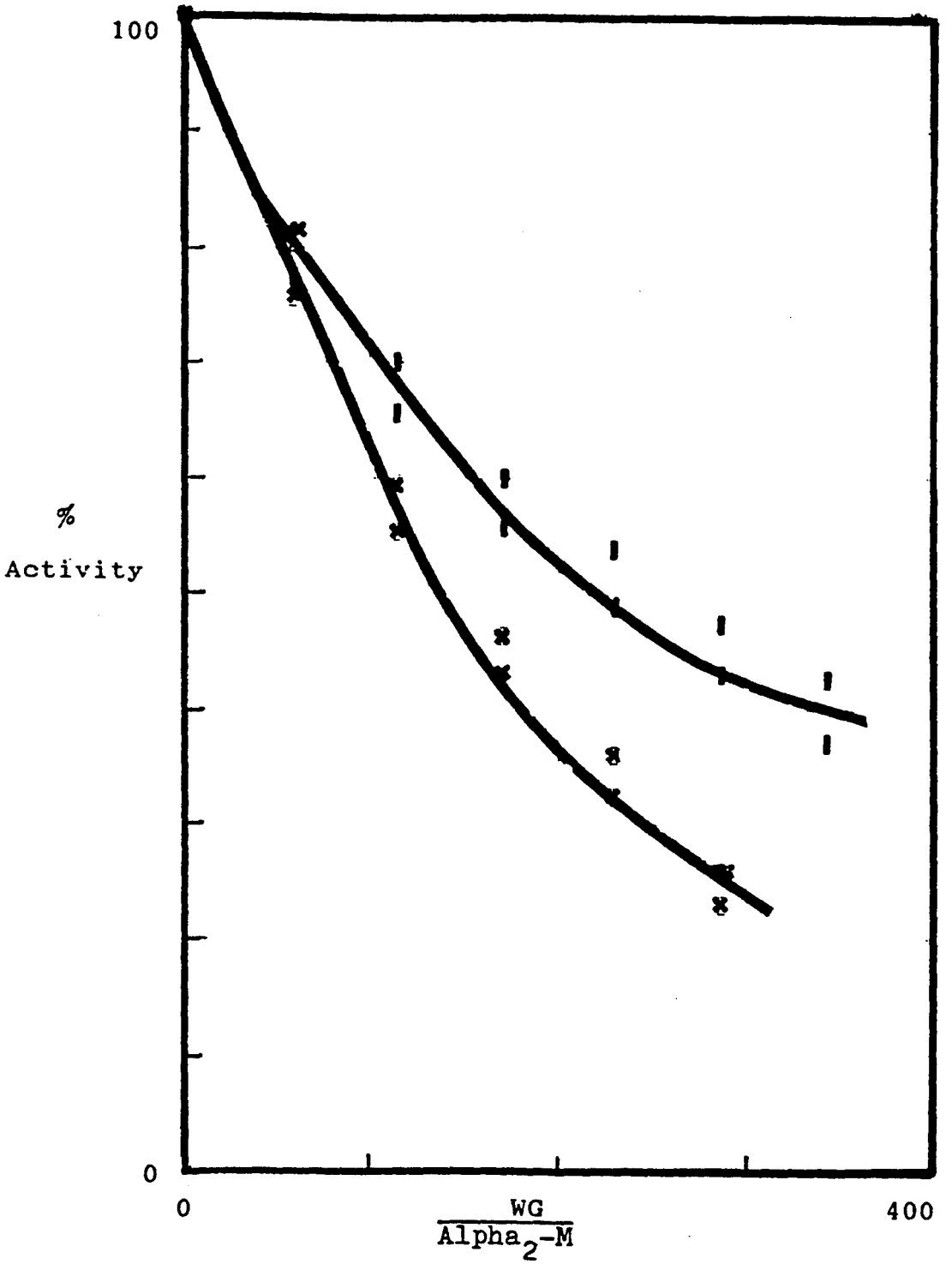

100
%
Activity
0
0
WG
Alpha2-M
400

FIGURE 20 Percent of trypsin binding activity remaining in supernatant following precipitation of native alpha$_2$-Macroglobulin (■) and alpha$_2$-Macroglobulin trypsin complex (X) by affinity purified wheat germ lectin versus the molar ratio of affinity purified wheat germ lectin to alpha$_2$-Macroglobulin. The precipitations were conducted at room temperature at pH 7.40.

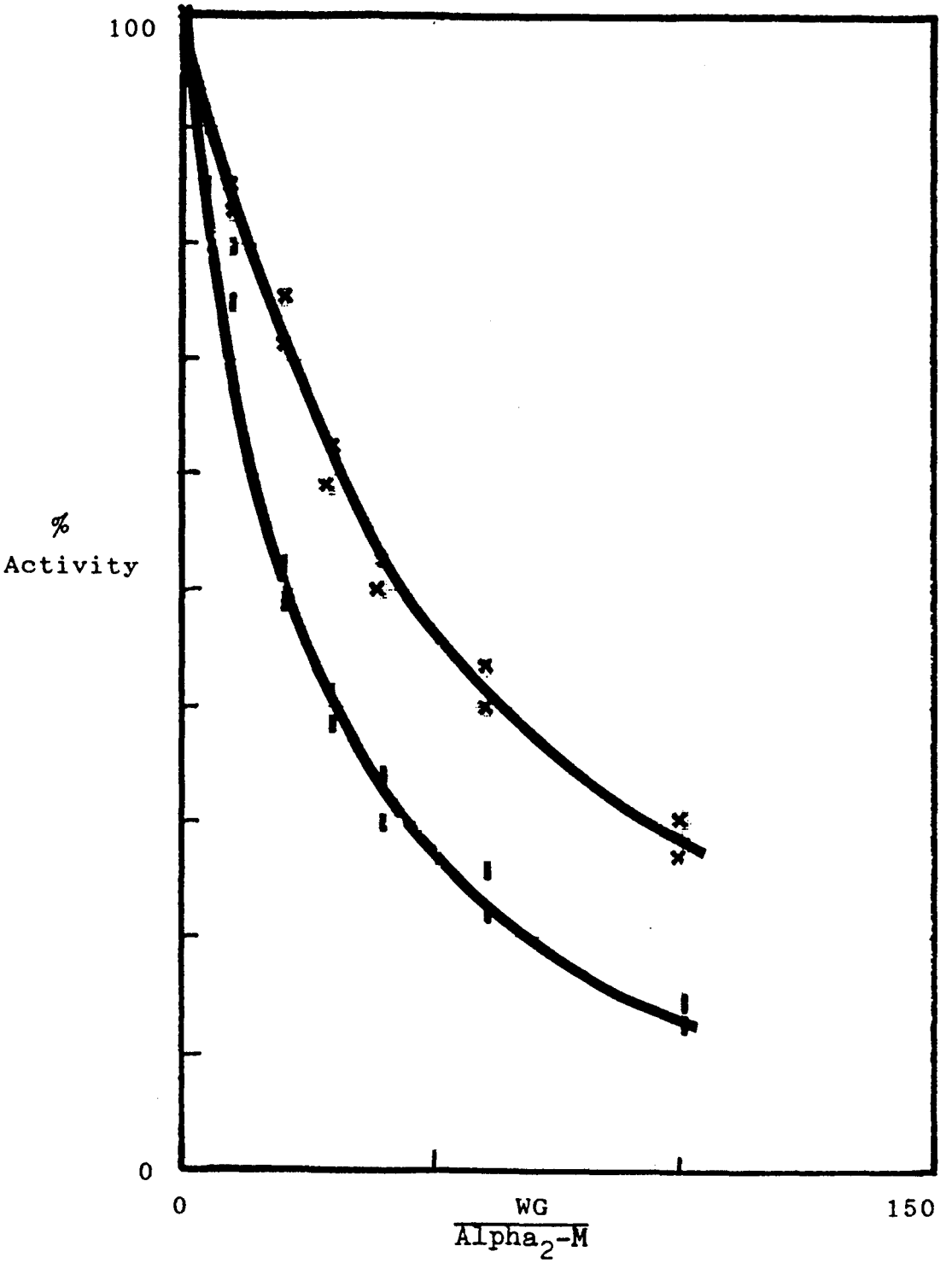

100
0
% Activity
0
WG / Alpha2-M
150

FIGURE 21 Percent of trypsin binding activity remaining in supernatant following precipitation of native $alpha_2$-Macroglobulin (X) and $alpha_2$-Macroglobulin trypsin complex (■) by affinity purified wheat germ lectin. The precipitation was conducted at room temperature at pH 5.20.

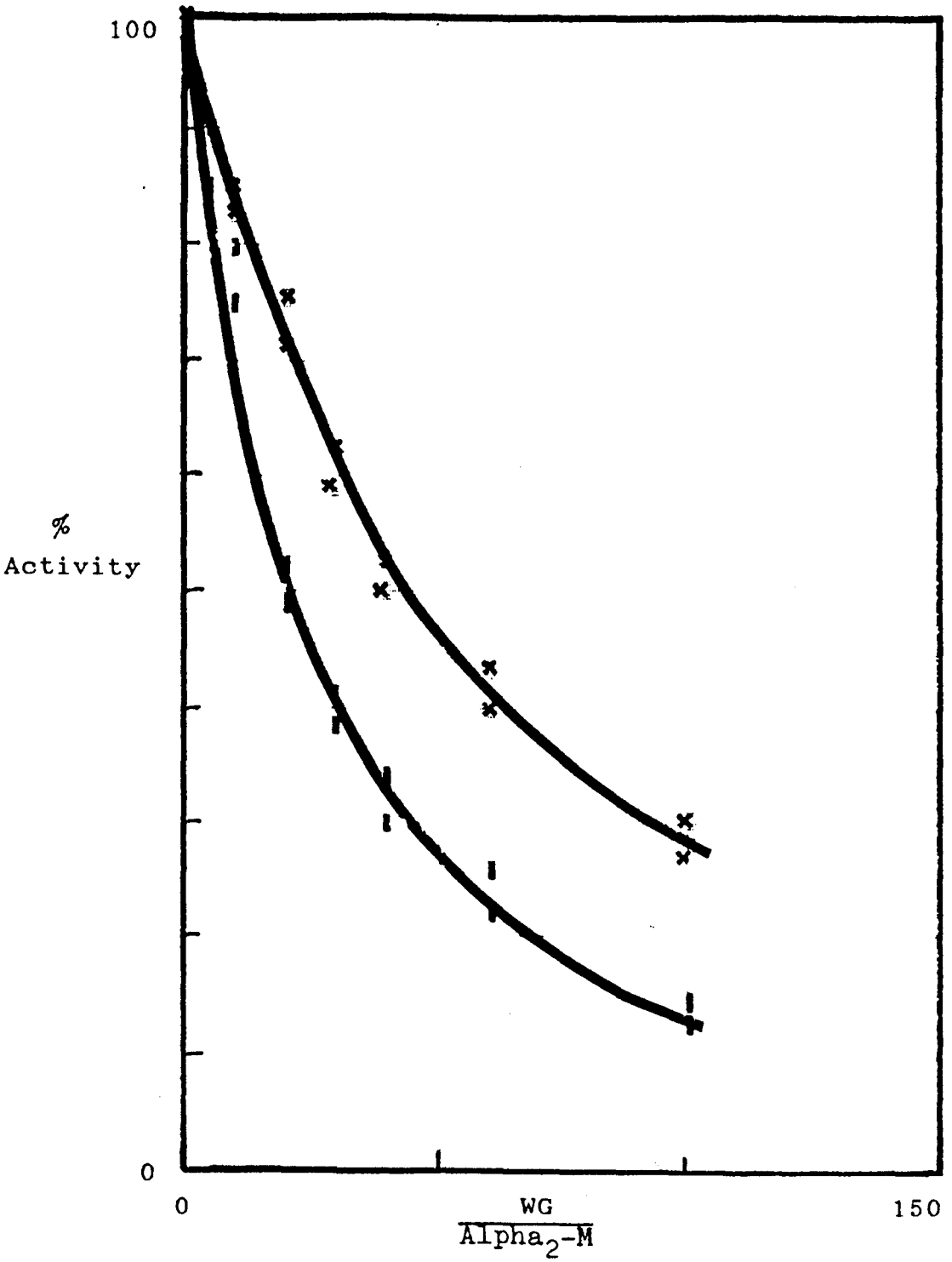

100
%
Activity
0
0
WG
Alpha2-M
150

FIGURE 22 Chromatography of native alpha$_2$-Macroglobulin (■) and alpha$_2$-Macroglobulin trypsin complex (▲) on ethyl agarose. The resin was equilibrated with 0.05 M Na Phosphate 0.1 M NaCl, pH 7.40 at a flow rate of 10 ml/hr. Fractions (0.3 ml) were collected and assayed for alpha$_2$-Macroglobulin's trypsin binding activity. This elution profile is a representative sample of three column runs.

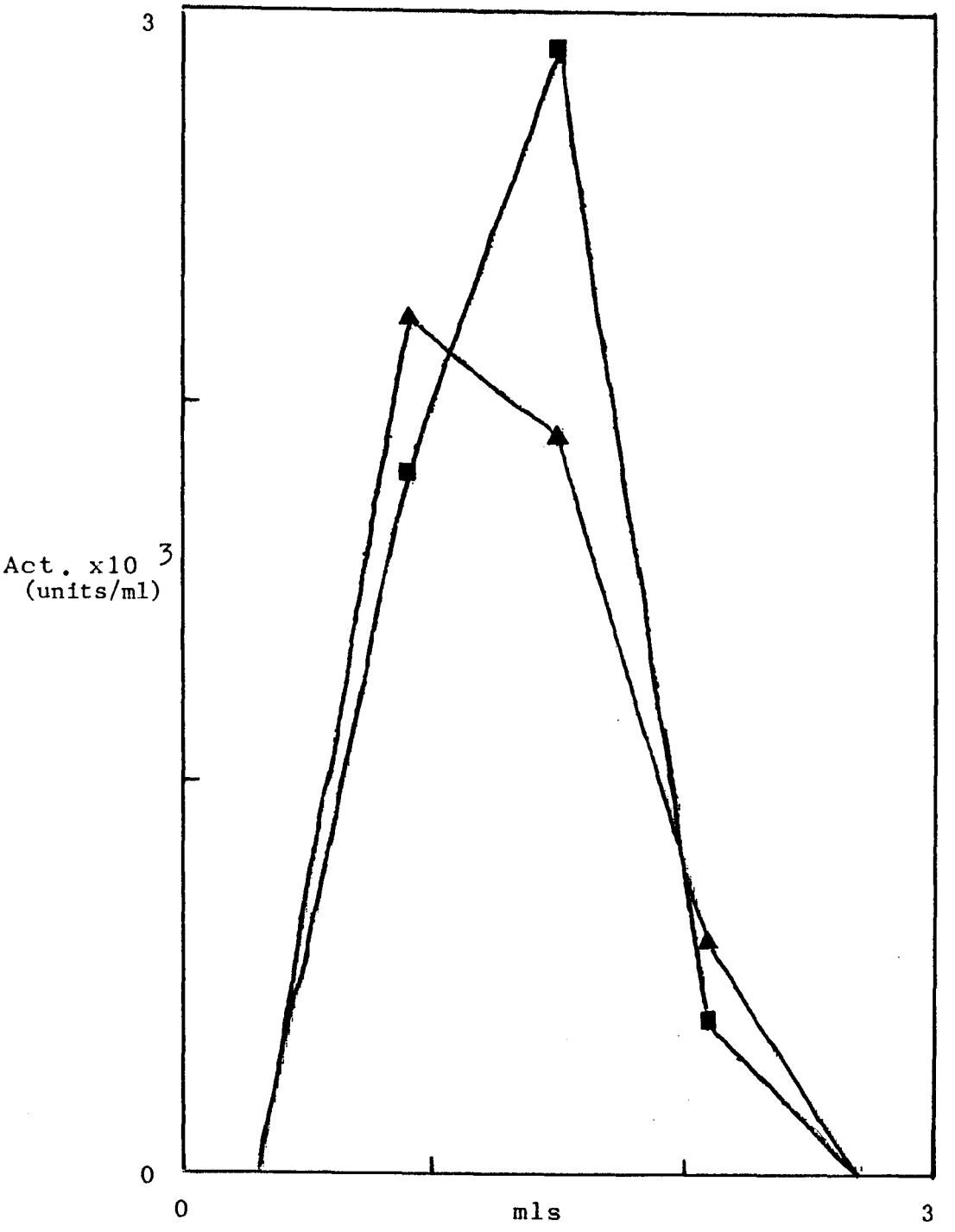

3
Act. x10 3
(units/ml)
0
0
mls
3

FIGURE 23 Chromatography of native alpha$_2$-Macroglobulin (■) and alpha$_2$-Macroglobulin trypsin complex (▲) on butyl agarose. The resin was equilibrated with 0.05 M Na Phosphate 0.1 M NaCl, pH 7.40 at a flow rate of 10 ml/hr. Fractions (0.3 ml) were collected and assayed for alpha$_2$-Macroglobulin's tryspin binding activity. This elution profile is a representative sample of three column runs.

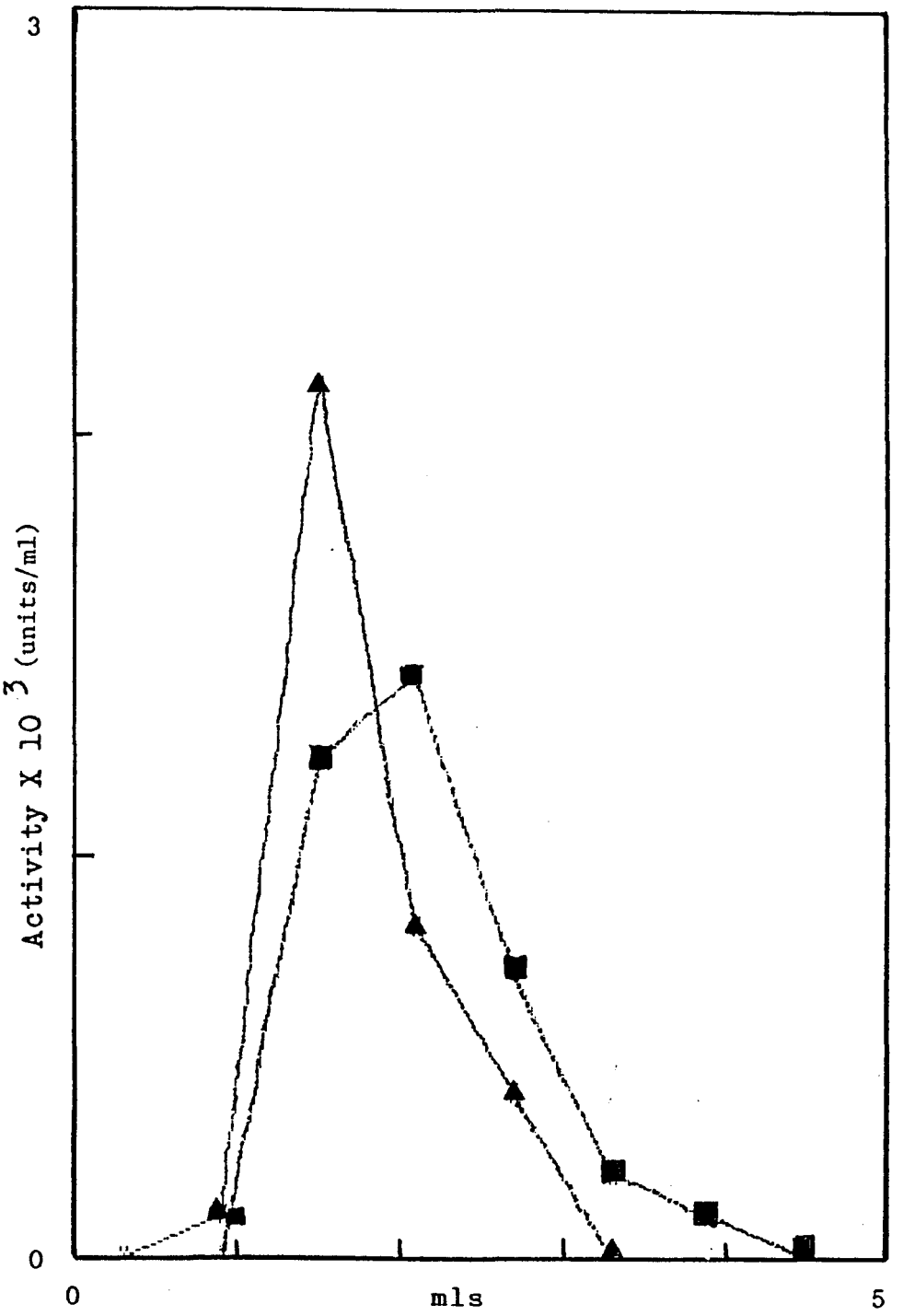

3
0
Activity X 10 3 (units/ml)
0
mls
5

TABLE XV

HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF NATIVE AND COMPLEXED ALPHA $_2$-MACROGLOBULIN

Sample[a] Applied	Resin	Buffer	% Bound
Native & Complex	Octyl	0.02 M Na Acetate, pH 5.20	100
Native & Complex	Octyl	0.1 M Na Citrate - 10% ethylene glycol	100
Native & Complex	Octyl	0.05 M NaPhosphate 0.1 M NaCl, pH 7.4	100
Native & Complex	Octyl	0.02 M NaPhosphate 10% glycerol, pH 7.4	100
Native & Complex	Octyl	0.02 M NaPhosphate 50% glycerol, pH 7.4	100
Native & Complex	Octyl	0.05 M Tris·HCl, pH 8.10	100
Native & Complex	Octyl	0.05 M Tris· 0.3 M KCNS 0.3 M $BaCl_2$, pH 8.10	100
Native & Complex	Octyl	0.05 M Tris·HCl 0.3 M KCNS 0.3 M $BaCl_2$ 50% ethylene glycol, pH 8.10	100
Native & Complex	Octyl	0.02 M NaPhosphate 50% glycerol, pH 9.25	100

a = 1 micromole of native or alpha$_2$-Macroglobulin trypsin complex was applied to the column

chromatography column. Equilibration of the octyl agarose with buffer containing chaotropic ions and/or ethylene glycol prior to sample application failed to facilitate the elution of native or complexed alpha$_2$-Macroglobulin. Therefore, octyl agarose irreversibly binds both forms of alpha$_2$-Macroglobulin.

Q. Receptor Mediated Uptake by Cells

The ability of alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin tryspin complex to be internalized by receptor mediated endocytosis was examined using cultured human fibroblasts. I^{125}-labeled alpha$_2$-Macroglobulin was preincubated in serum free media at 37^{o}C for sixty minutes prior to initiation of the experiment. The prewarmed media containing I^{125}-labeld alpha$_2$-Macroglobulin was then added to a monolayer of cultured human fibroblasts which had been incubated for 75 minutes in serum free media. The accumulation of radioactivity in the monolayer with time was then measured. The experiment was terminated by removing the media and washing the cells 5 times with cold PBS. The cellular radioactivity was removed with formic acid, after which aliquots were taken and counted in the gamma counter. From Figure 22, it can be seen that the amount of I^{125}-labeled alpha$_2$-Macroglobulin accumulated in the fibroblasts is proporational to time. Furthermore, the accumulation appears to be biphasic, with the initial rapid phase being complete by 20 minutes. Next, increasing amounts of labeled alpha$_2$-Macroglobulin were incubated for 30 minutes with the cells in an attempt to saturate the receptors (Figure 25). As can be seen, the accumulation of the inhibitor was essentially linear in alpha$_2$-Macroglobulin. To determine if the binding was receptor mediated, an excess of unlabeled cold alpha$_2$-Macroglobulin was

FIGURE 24 Plot of uptake of I^{125}-alpha$_2$-Macroglobulin versus time. Labeled alpha$_2$-Macroglobulin was incubated with human fibroblasts in serum free media for varying amounts of time (0 to 48 min.). At each time point, the media was removed and cells were washed with cold phosphate buffered saline. Following removal of the cells by formic acid, aliquots from the acid solubilized cells were counted in the gamma counter. Each value represents a single plate.

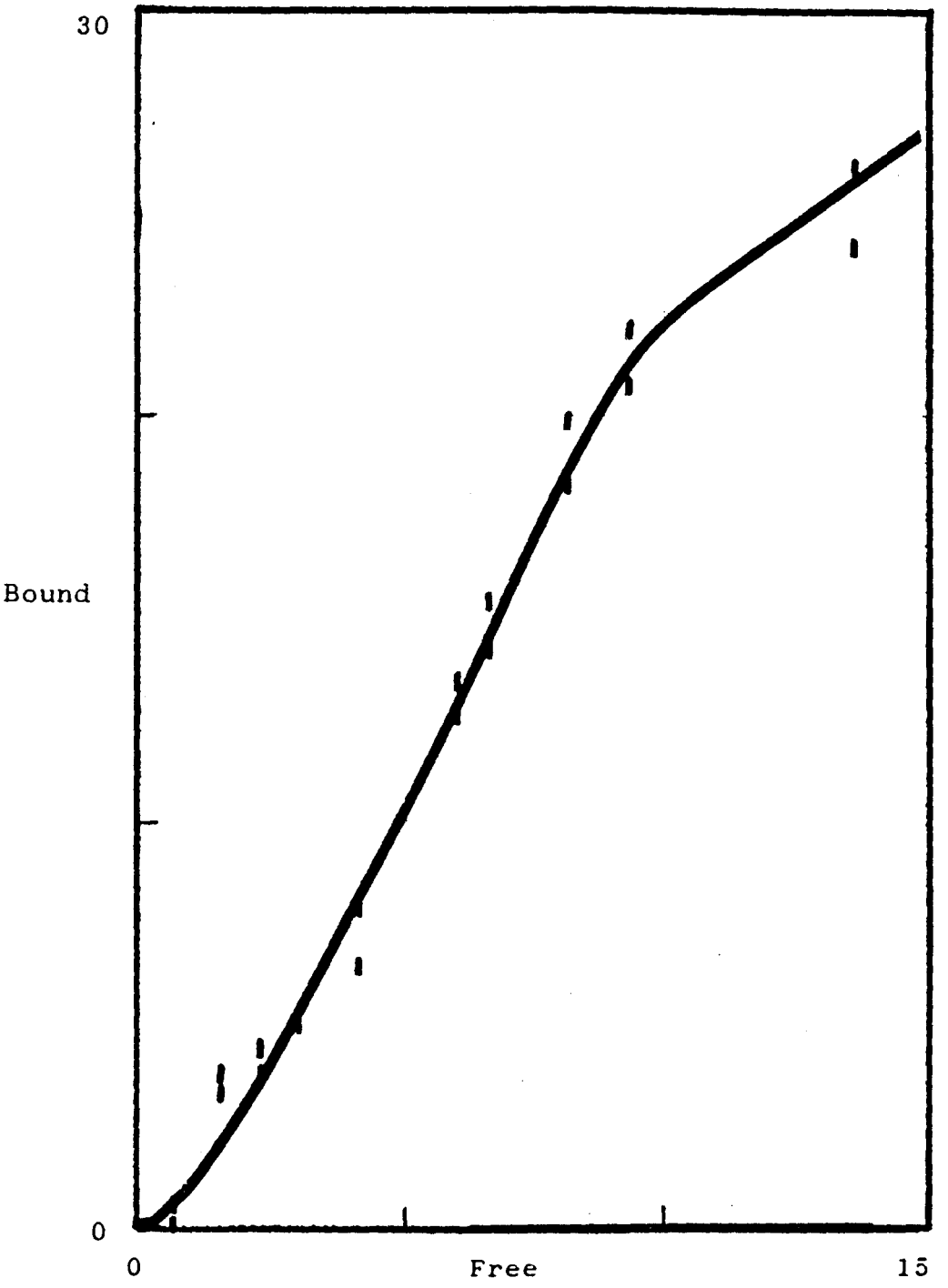
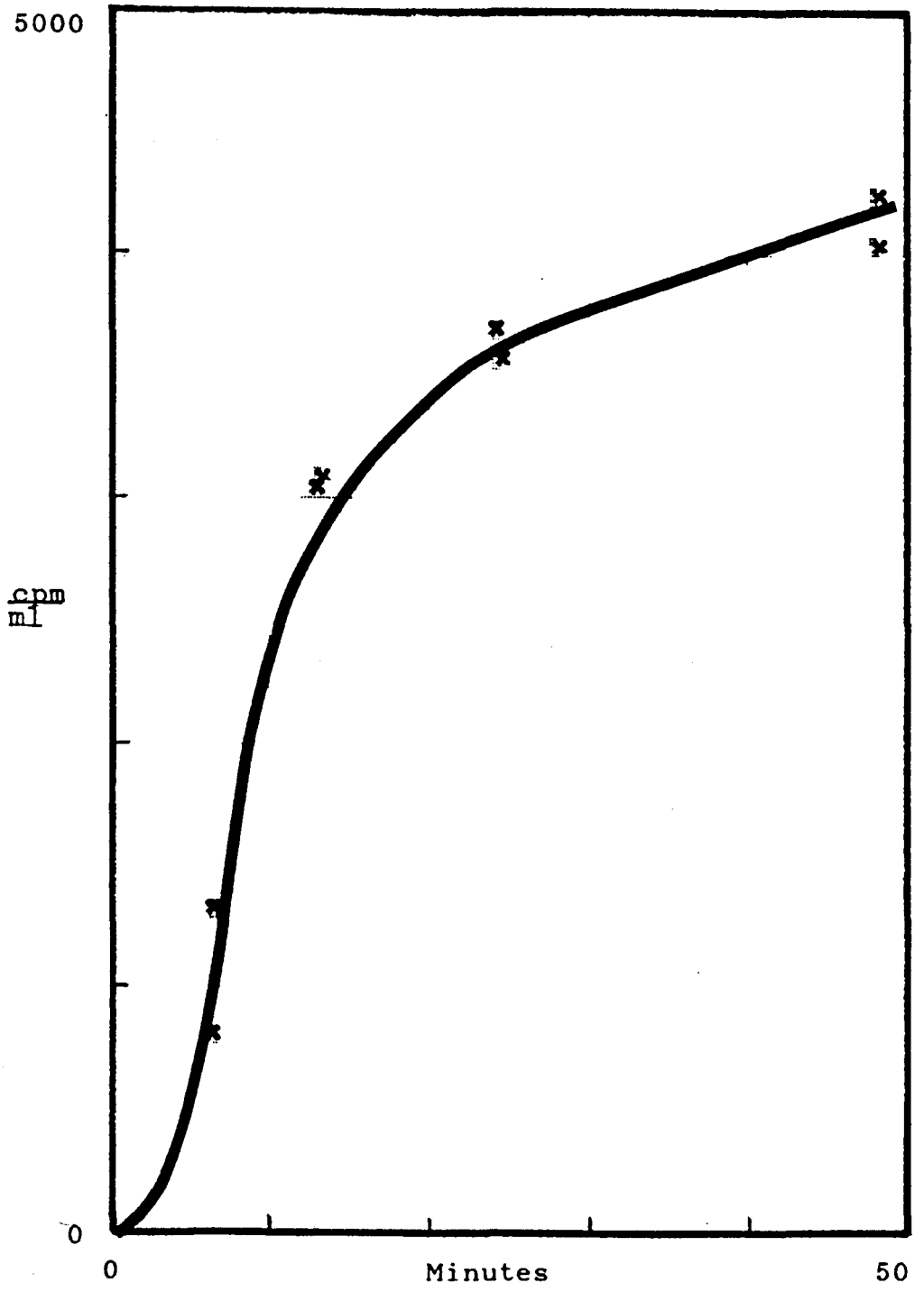

30
Bound
0
0
Free
15

FIGURE 25 Plot of I^{125}-alpha$_2$-Macroglobulin incorporated by human fibroblasts versus concentration of alpha$_2$-Macroglobulin free in the incubation media. Increasing amounts of I^{125}-alpha$_2$-Macroglobulin were incubated with cells for 30 minutes, after which media was removed and cells were washed with cold phosphate buffer saline. Following removal of the cells by formic acid, aliquots from the acid solubilized cells and media were counted in the gamma counter.


5000
cpm
ml
0
0
Minutes
50

incubated with increasing amounts of labeled alpha$_2$-Macroglobulin. The results in Table XVI demonstrate the ability of the unlabled alpha$_2$-Macroglobulin to compete with the labeled alpha$_2$-Macroglobulin, suggesting that the uptake of labeled alpha$_2$-Macroglobulin is mediated by receptors. Thus, the results suggest that fibroblasts contain receptors for native alpha$_2$-Macroglobulin.

TABLE XVI Displacement of I^{125}-Alpha$_2$-Macroglobulin with Unlabeled Alpha$_2$-Macroglobulin

I^{125}-Alpha$_2$-Macroglobulin in Media	Alpha$_2$-Macroglobulin Bound	Alpha$_2$-Macrogolbulin bound with Cold Alpha$_2$-Macroglobulin Present
(cpm/ml)	(cpm/ml)	(cpm/ml)
2×10^6	2452	2000
4×10^6	7352	3967
6×10^6	9294	4165
8×10^6	12285	6304

Con. cold alpha$_2$-Macroglobulin = 0.73 mg/ml

CHAPTER IV

DISCUSSION

A. Purification of Alpha$_2$-Macroglobulin

A three step purification scheme was developed for human alpha$_2$-Macroglobulin. The bentonite precipitation, the gel chromatography, and ion exchange chromatography are all mild procedures. During the two year developmental period, numerous changes were made to perfect this procedure. Replacement of bentonite with Mg-bentonite enhanced the removal of fibrinogen and lipoproteins. In addition, numerous chromatographic resins and various sized glass columns were used before the best one was finally selected. For molecular exclusion chromatography, Sepharose 4B, Sepharose CL6B, and Sephacry 300 were tested for their ability to fractionate alpha$_2$-Macroglobulin from the bulk of the plasma proteins. DEAE-Sephadex, Sulphopropyl-Sephadex, DEAE-Cellulose, CM-Cellulose, hydroxyl apatite, Affigel Blue, and metal chelate chromatography were evaluated for the capability of removing trace impurities and resolving alpha$_2$-Macroglobulin into its isoenzymic forms. Removal of the bound inhibitor from ion exchangers was effected by batch and gradient elution. A Gilson mixograd gradient maker permitted creation of various types of salt and pH gradients. A batch elution was found to be the method of choice. Following elution from the ion exchanger, the protein was concentrated, dialized, and stored in the refrigerator at 4^{o}C.

Several different procedures were tested for their ability to store alpha$_2$-Macroglobulin without incurring a loss of trypsin binding activity. The best method for storing alpha$_2$-Macroglobulin appeared

to be at 4°C in 0.05 M Na Phosphate, pH 7.4 buffer, after sterilizing the alpha$_2$-Macroglobulin solution by filtration. Lyophilization resulted in a 90% loss of activity, while freezing led to a similar substantial loss (50%). Other laboratories appear to freeze or lyophilize their purified alpha$_2$-Macroglobulin preparations with out severe losses in activity. The reason for this different remains unclear.

Polyacrylamide gel electrophoresis, immunodiffusion, and immunoelectrophoresis were utilized to detect impurities. Discontinuous polyacrylamide gel electrophoresis under non-denaturing conditions failed to detect significant levels of impurities even for gels which had been overloaded with protein. When SDS-PAGE were conducted under reducing conditions, we were unable to detect the 85,000 dalton fragment indicative of prior exposure to proteases. Thus, our purification scheme did not lead to formation of detectable amounts of alpha$_2$-Macroglobulin protease complex. The purity of the alpha$_2$-Macroglobulin preparation was also assessed by the techniques of immunodiffusion and immunoelectrophoresis. In both cases, the commercially purchased antibodies only detected the presence of alpha$_2$-Macroglobulin. Isoelectric focusing was used to further confirm the purity of the alpha$_2$-Macroglobulin. With this technique, I was able to resolve alpha$_2$-Macroglobulin into five distinct isoenzyme forms having isolelectric points which ranged from 4.8 to 5.2. Each band exhibited trypsin binding activity. No band corresponding to inactive protein was detected. Ion exchange chromatography was also able to resolve alpha$_2$-Macroglobulin into several species. Thus, chromatography on CM-cellulose yielded three

peaks of trypsin binding activity in the proportions of 75:20:5. Isolelectric focusing revealed that peak 1 contains three isoenzymic forms of alpha$_2$-Macroglobulin, while peak 2 contains two isoenzymic forms of alpha$_2$-Macroglobulin. The low concentration of peak 3 prevented us from performing isoelectric focusing on it.

Our method of purifying alpha$_2$-Macroglobulin attempts to improve upon the current procedures now in use. The less desirable features of other purifications include the use of harsh techniques, failure to minimize activation of endogenous proteases and poor yields. Another drawback to these procedures is their inability to fractionate all the alpha$_2$-Macroglobulin in the plasma from the remaining plasma proteins. Silanized glassware, plastic labware, and protease inhibitors (Benzamidine, Sodium Heparin and SBI) are utilized in our purification to minimize activation of endogenous proteases. All other purification procedures fail to incorporate any of these precautions into their methods for purifying alpha$_2$-Macroglobulin. Our purification scheme's recovery of 75% exceeds the yields of the other procedures, which range between 20 and 50%. All my purifications were performed using citrated plasma from one individual at a time. Pooled citrated plasma would have been a better source of starting material to eliminate any individual differences in alpha$_2$-Macroglobulin.

B. Physical Characterization of Alpha$_2$-Macroglobulin

One of the fundamental properties of alpha$_2$-Macroglobulin is its capacity to interact with virtually all endopeptidases. The "trap hypothesis" (Barrett, 1973) postulated that when a protease cleaves the bait region in alpha$_2$-Macroglobulin, a conformational change occurs which results in entrapment of the protease. Investigation of

this conformational change can provide data for elucidation of the mechanism for complex formation between alpha$_2$-Macroglobulin and proteases. In this dissertation a number of spectroscopic techniques were used to analyze the changes in alpha$_2$-Macroglobulin on forming complexes with proteases. For all these spectroscopic measurements, the trypsin was first purified by affinity chromatography and quantitated by active site titration with p-nitrophenyl-p'-guanidine benzoate. Following purification of the trypsin on a turkey ovomucoid resin, the purity of the trypsin rose from 63% to at least 90%. Affinity purified chymotrypsin, which was a gift from Dr. Susan Buktenica, revealed a purity of 91%.

In an attempt to verify Barrett's trap hypothesis, circular dichroism was performed on native and complexed alpha$_2$-Macroglobulin. The circular dichroic spectra for native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex were measured and compared in Figure 4. In the far U.V. region between 230 nm and 250 nm, both native and complexed alpha$_2$-Macroglobulin exhibit negative ellipticity with a minimum at 216 nm. Complexation appears to result in a very small increase in ellipticity in the far U.V. region. At wavelengths greater than 250 nm, changes in circular dichroism were not detected. These results were unexpected, because Barrett had postulated that a large conformational change accompanied entrapment. My work was the first to demonstrate that alpha$_2$-Macroglobulin appears to undergo only a small change in secondary structure upon enzyme binding (Buktenica, Frankfater, 1978). This data can be explained in an alternative manner. The minor change in ellipticity in the far U.V. region may reflect the cancellation of positive and negative bands in this region

instead of a small change in secondary structure. For example, the presumably positive tyrosine bands (Woody, 1978) could be cancelled by the negative bands of the B tunrs (Brahms, et al., 1977) in the region of 200 nm - 230 nm. The contributions of aromatic chromophores to the far U.V. circular dichroism spectra below 250 nm is poorly understood for proteins. Usually this region is apparently dominated by the secondary structures of the polypeptide chain, but it cannot automatically be assumed that the effects of aromatic groups are negligible. At wavelengths greater than 250 nm, I failed to observe any changes in ellipticity. This result is probably due to insufficient protein being present in the cuvette.

Other investigators have utilized circular dichroism to investigate the interaction of alpha$_2$-Macroglobulin with proteases [Gonais, (1982), Bjork, (1982)]. Their CD spectra of native alpha$_2$-Macroglobulin are virtually identical to mine except their spectra demonstrate a shoulder near 210 nm, which disappears upon complex formation. This shoulder is not evident in my spectra possibly because of the poor signal to noise ratio obtained with the Jasco CD/ORD spectrometer. These investigators reported a small increase in ellipticity upon complex formation, but the magnitude they observed was slightly larger than mine.

Fluorescence spectroscopy was used to assess possible conformational changes on complex formation which are too small to result in substantial reorganization of the polypeptide backbone of alpha$_2$-Macroglobulin. The fact that proteins absorb radiation in the region of 270 nm to 300 nm had been known for a long time, but Shore and Pardii (1957) were first to report that proteins are fluorescent

when excited at 280 nm. Teale and Weber's (1957) report on the fluorescent properties of free aromatic amino acids elucidated the role of tyrosine and tryptophan in fluorescence. Their work demonstrated that tryptophan and tyrosine have similar fluorescence efficiencies in water, but the former contributes more to fluorescence because of its higher extinction coefficient. The fluorescence contribution of phenylalanine is extemely low. They reported that the excitation maxima in neutral water for tryptophan, tyrosine, and phenylalanine were 287 nm, 275 nm, and 260 nm, respectively, while the fluorescence emission maxima of tryptophan, tyrosine, and phenylalanine were 348 nm, 303 nm, and 282 nm respectively. Teale (1957) and Velick (1958) have reported a failure to detect tyrosine fluorescence in tryptophan containing proteins. Additionally, Velick (1958) and Konev (1957) agree that there is considerable enhancement of the 350 nm fluorescence in intact proteins as compared to the component amino acids. Furthermore, the 350 nm fluorescence of lactic dehydrogenase was found to be 5 times as intense as glyceraldehyde-3-phosphate dehydrogenase per units weight of protein, whereas the tryptophan content was only twice as high. Clearly, these data indicate a transfer of energy between aromatic amino acid residues within the protein molecule.

Our fluorescent work was the first documented evidence that fluorescence could be used to investigate the interaction between $alpha_2$-Macroglobulin and proteinases (Buktenica and Frankfater, 1978). Fluorometric studies on $alpha_2$-Macroglobulin were conducted by running excitation and emission spectra for $alpha_2$-Macroglobulin. The excitation spectra indicated that the excitation maxima for native

alpha$_2$-Macroglobulin is 288 nm, while the emission spectra revealed that the emission maxima was 331.3 nm. Results from the Aminco-Bowman spectrofluorimeter suggested that complex formation between alpha$_2$-Macroglobulin and trypsin resulted in an increase in fluorescence intensity and a shift in the maxima from 331.3 nm to 330 nm (Figure 5). Various alpha$_2$-Macroglobulin protease complexes were formed using trypsin, chymotrypsin, and papain. Their subsequent fluorescence emission spectra revealed that all the complexes produced an increase in fluorescence intensity with the corresponding blue shift of the maxima. Corrected spectra obtained with the Perkin Elmer MPF 44B spectrofluorometer also showed the increase in fluorescence intensity at 330 nm upon complex formation, but the small blue shift was no longer evident.

Additional fluorescent studies were performed to further clarify our results. Attempts were made to determine whether the increase in fluorescence was characteristic of complex formation. To confirm this, the molar ratio of protease to alpha$_2$-Macroglobulin was varied from 0.5:1 to 3:1 (enzyme: alpha$_2$-Macroglobulin). For trypsin and chymotrypsin, the fluorescence intensity increased until a molar ratio of 2:1 (enzyme:alpha$_2$-Macroglobulin) was reached. Further increase in enzyme concentration led to no additional increase in fluorescence. These results suggested that the increase in fluorescence was characteristic of complex formation. Additionally, the change in fluorescence could be used to determine the stoichiometry of binding between alpha$_2$-Macroglobulin and proteases. At this time, the literature contained no general consensus on the stoichiometry of binding. This was not surprising since previous investigators had

failed to perform active site titration or affinity purification of their enzymes. Many investigators were conducting experiments using impure preparations of alpha$_2$-Macroglobulin, which also accounts for the variation in the observed stoichiometry.

Interpretation of my spectral data is difficult since several explanations could account for the observed change in fluorescence upon complexation. With the excitation monochromator set at 288 nm, the most probable fluorophores are tyrosine and tryptophan, with excitation maximas at 275 nm and 287 nm respectively. The emission spectra of alpha$_2$-Macroglobulin revealed the absence of a peak at 303 nm, indicating no tyrosine fluorescence. This result agrees with previous investigators who failed to observe tyrosine fluorescence, suggesting that energy was probably transferred from tyrosine to tryptophan. The possibility of tryptophan being the primary fluorescent emitter is enhanced by the fact that tryptophan's extinction coefficient is four times larger than tyrosine's. Additionally, excitation of alpha$_2$-Macroglobulin occurred very near tryptophan's excitation maxima. The increase in fluorescence due to complex formation could mean that the tyrosine or tryptophan fluorescence is less quenched in the complex. Indirect support for this explanation can be found in the literature (Gonias, et al., 1982). His ultraviolet difference spectrum between methylamine treated alpha$_2$-Macroglobulin and native alpha$_2$-Macroglobulin demonstrates the exposure of tyrosine residues to the aqueous media following complex formation. Exposure of tyrosine residues to the solvent could possibly result in a decrease in fluorescent quenching of this amino acid. Alternatively, complex formation may reposition

the tyrosine and tryptophan residues closer to one another. This action would elevate the efficiency of energy transfer between the two amino acids and thereby cause an increase in fluorescence upon complex formation.

Intrinsic and extrinsic fluorescence was utilized by other investigators to analyze the interaction of alpha$_2$-Macroglobulin with proteinases (Bjork, 1982; Straight, 1982; Steiner, 1985). Bjork utilized an Aminco SPF500 spectrofluorimeter with proteinases and amines. Their corrected spectra for the complexation of alpha$_2$-Macroglobulin with trypsin resembles ours except their fluorescence maxima occurs at 326 nm and is blue shifted 3 nm upon complex formation. The differences between the results are primarily due to the fact that Bjork used 280 nm as his wavelength for excitation, and we used 288 nm. Nevertheless, complexation produces an increase in fluorescence intensity when excitation occurs at either 280 nm or 289 nm. TNS, [6(-p-toluidino)-2 napthalenesulfonic acid] was used as an extrinsic probe to study the conformational changes that occur in alpha$_2$-Macroglobulin following complexation with amines or proteases. The emission spectra of TNS-alpha$_2$-Macroglobulin is enhanced upon methylamine treatment or complex formation with trypsin or plasmin (Strickland, 1984). Additionally, there is a shift in maximal emission from 430 nm to 412 nm. The TNS fluorescence change induced by melthylamine was compared to the inactivation of alpha$_2$-Macroglobulin measured by the loss of trypsin binding ability. These authors concluded that the conformational change measured by this probe appears to reflect alterations in the functional activity of alpha$_2$-Macroglobulin and correlates with the loss of trypsin binding

activity of the molecule.

Ultraviolet difference spectroscopy is commonly used to compare proteins for possible changes in conformation or the environment of its constituent amino acids. When the spectrum of pepsin at pH 5.7 is compared to the one at pH 7.3, the resultant difference spectrum exhibits difference peaks at 278 nm., 286 nm, and 292 nm. with no significant changes in the spectrum from 225 nm to 245 nm (Glaser, A.N. and Smith, E.L., 1961). These wave lengths indicate absorption of light by tyrosine and tryptophan residues. Other investigators have demonstrated that these three difference peaks represent changes in the environment of tryptophan and tyrosine residues (Donovan, J.W., et al., 1958; Wetlaufer, D.B., 1958). The difference spectrum obtained by comparing pepsin at pH 7.3 to pepsin at pH 1.4 reveals difference peaks at 279 nm., 286 nm., and 230 nm. (Donovan, J.W., et al., 1958; Wetlaufer, D.B., 1958). The decrease in the pH from 7.3 to 1.4 results in a change in the environment of the tyrosine and tryptophan residues as well as a change in the conformation of the protein. Glaser (1961) also utilized difference spectroscopy to detect a change in conformation of poly-L-glutamic acid by comparing the absorption spectrum of poly-L-glutamic acid at pH 4.0 to the spectrum at pH 8.0. The resultant difference spectrum indicates a large change at 225 nm., suggesting that changes in the conformation of the peptide backbone are reflected in this region of the spectrum. Subsequent experimentation illustrated that the rise in pH is accompanied by a conformational change from alpha-helix to random coil.

Ultraviolet difference spectroscopy was used to compare $alpha_2$-

Macroglobulin, alpha$_2$-Macroglobulin trypsin complex and alpha$_2$-Macroglobulin papain complex. The difference spectrum obtain from alpha$_2$-Macroglobulin treated with trypsin and untreated alpha$_2$-Macroglobulin (native) closely resembled the difference spectrum produced from alpha$_2$-Macroglobulin treated with papain and untreated alpha$_2$-Macroglobulin. As shown by Fig. 8 these far U.V. difference spectra are identical except in the region between 230 nm and 238 nm. The difference spectrum measured between native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin papain complex exhibits negative extreme at 232 nm, while the difference spectrum measured between native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin demonstrates an even larger negative extreme at 233 nm. Tyrosine, tryptophan, and thiolesters may contribute to absorption in the region of 230 nm to 240 nm (Wetlaufer, 1962). Spectral differences in the region of 230 nm to 240 nm may reflect changes in the environment of tyrosine and tryptophan residues as well as changes in the conformation. However, changes in the environment of aromatic side chains are not necessarily accompanied by a change in the spectral region from 230 nm to 240 nm. The differences in the magnitude and position of the negative extrema for alpha$_2$-Macroglobulin papain difference spectra may be due to failure to fully activate papain. Based on this data alone, it is not possible to say whether the difference peak is due to a change in the environment of the chromophore, or due to a change in the conformation, or a combination of both of these.

Other investigators have measured difference spectra between alpha$_2$-Macroglobulin treated with amines or proteinases and alpha$_2$-Macroglobulin alone, but all the experiments were performed in the

spectral region from 250 nm. to 310 nm. (Bjork, 1982; Gonais, 1982). These near U.V. difference spectra measured between the $alpha_2$-Macroglobulin protease complex ($alpha_2$-Macroglobulin thrombin, $alpha_2$-Macroglobulin trypsin, and $alpha_2$-Macroglobulin chymotrypsin) and $alpha_2$-Macroglobulin alone are very similar to each other, although their magnitudes differ somewhat. These spectra are similar to, but not identical with spectra produced with amines. Difference spectra from our lab and other labs indicate minor differences do exist between the native and complexed forms of $alpha_2$-Macroglobulin, but these are subtle differences and not a major conformational change as postulated by the 'trap hypothesis'.

$Alpha_2$-Macroglobulin's conformational change was further assessed by molecular exclusion chromatography. If protease binding alters $alpha_2$-Macroglobulin's conformation sufficiently, the chromatographic behavior of native and protease bound $alpha_2$-Macroglobulin will differ. Native $alpha_2$-Macroglobulin and $alpha_2$-Macroglobulin protease complex were chromatographed on Sepharose CL-6B (Table III). The differential elution of native and complexed $alpha_2$-Macroglobulin seems to suggest that protease binding is accompanied by a conformational change which transforms complexed $alpha_2$-Macroglobulin into a more compact conformation than native $alpha_2$-Macroglobulin. These chromatographic results appear to be in concert with Barrett's theory, which postulates that $alpha_2$-Macroglobulin engulfs and entraps the protease. Electron microscopy, sedimentation velocity, rate electrophoresis, and differential scanning calorimetric analysis all support our chromatographic results (Barrett, A.J., 1974; Barrett, A.J., 1979; Gonias, S.L., 1982; Cumming, H.S., et al., 1984).

This diverse group of techniques all demonstrated that alpha$_2$-Macroglobulin appears to compact upon complex formation.

SDS polyacrylamide gel electrophoresis was performed to investigate the subunit structure of alpha$_2$-Macroglobulin and to determine the extent of complex formed during purification. SDS-PAGE of native alpha$_2$-Macroglobulin under nonreducing conditions reveals only a single protein band, while SDS-PAGE under reducing conditions results in the appearance of one major protein band and numerous minor protein bands. The major band corresponds to an apparent molecular weight of 180,000 daltons, while the minor bands exhibit molecular weights above and below the 180,000 dalton band. If these other minor bands were due to impurities, they would have been observed previously by immunoelectrophoresis, isoelectric focusing, or discontinuous PAGE with overloaded samples. Inactivations of the alpha$_2$-Macroglobulin trypsin complex immediately following complex formation but prior to the addition of SDS failed to eliminate the minor bands. Possibly, these minor bands may be the result of alpha$_2$-Macroglobulin being nicked by proteases at sites other than the bait region prior to purification.

Parisi (1970) identified alpha$_2$-Macroglobulin as a major serum binding protein. Roche and Pizzo (1987) have demonstrated the role of histidyl residues in Zn^{2+} binding. Diethylpyrocarabonate, a histidine specific acylating reagent was capable of preventing a zinc induced precipitation of alpha$_2$-Macroglobulin trypsin complex and alpha$_2$-Macroglobulin methylamine complex.

My experiments were conducted to further clarify the role of divalent cations. The effect of divalent cations on trypsin binding

activity was examined. The addition of Zn^{2+} or Mg^{2+} to the assay for alpha$_2$-Macoglobulin led to decreases in the trypsin binding activity. Neither Zn^{2+} or Mg^{2+} alone had any effect on trypsin. These results suggest that the binding of divalent cations may alter the conformation of alpha$_2$-Macroglobulin sufficiently to produce changes in the conformation of the bound protease, thereby altering the catalytic activity of the bound enzyme. Alternatively, the metal cations may act to reduce the protease binding activity of alpha$_2$-Macroglobulin. The physiological significance of this observation is not clear.

C. Chemical Characterization of Alpha$_2$-Macroglobulin

Some chemical characterization of alpha$_2$-Macroglobulin was performed in an attempt to clarify its mechanism and physiological role. More specifically, we were interested in detecting changes in exposure of surface amino acds or oligosaccharide side chains following complex formation and assessing their possible involvement in receptor mediated uptake of alpha$_2$-Macroglobulin by cells. Fluorescence and U.V. spectroscopy indicated that aromatic amino acids may be exposed following reaction with trypsin. Consequently, hydrophobic interaction chromatography was utilized to probe the surface structure of alpha$_2$-Macroglobulin.

Hydrophobic interaction chromatography permits nonpolar regions on the resin to interct with nonpolar regins on the protein. The driving force for the hydrophobic bond is the gain in entropy which results from a decrease in contact between the hydrophobic groups and water, and not from an attractive force between the hydrophobic groups per se. Commonly, hydrophobic interaction chromatography is conducted

at high salt concentrations e.g. 1.0 M NaCl to 4.0 M NaCl to minimize electrostataic interactions. High salt concentrations were found to substantially reduce alpha$_2$-Macroglobulin's trypsin binding activity. Since the possibility existed that high salt concentrations may induce an unusual conformation, the experiments were conducted at low ionic strength (0.05 M NaPhosphate).

Ethyl, butyl and octyl sepharose agarose were allowed to interact with 1.6 uM of native alpha$_2$-Macroglobulin or alpha$_2$-Macroglobulin protease complex. On ethyl agarose, native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin trypsin complex were eluted similarly from the gel, with the native form of alpha$_2$-Macroglobulin being slightly retarded. Application of native and complexed alpha$_2$-Macroglobulin on butyl agarose resulted in an even greater retardation of native alpha$_2$-Macroglobulin. In contrast, octyl sepharose totally bound both forms of alpha$_2$-Macroglobulin. Attempts to elute the bound protein from the octyl agarose resins with buffers containing ethylene glycol or chaotropic agents or both were unsuccessful. Raising or lowering the pH of the elution buffers containing chaotropic ions caused no desorption of protein. The use of lyophilic agents in the equilibration buffer failed to decrease the binding of native or complexed alpha$_2$-Macroglobulin to the octyl agarose, and appeared to have a negligible effect on elution. The inability to elute native or complexed alpha$_2$-Macroglobulin from the octyl resin may be due to the increased length of the ligand coupled to the agarose or an increased concentration of the ligand. In either case, the distance between the resin bound ligands is smaller than the diameter of the protein,

resulting in multipoint attachment. The use of neutral alkyl derivatives of agarose excluded the participation of electrostatic interactions. The low protein load (1.6 uM) in comparison to the ligand concentration (40 uM) maximizes the ability of the resin to detect the exposure of hydrophobic areas on the protein. When the degree of hydrophobicity of the ligand was as low as possible, differences in the surface amino acids between native and complexed alpha$_2$-Macroglobulin were observed. The hydrophobic character of the interaction between alpha$_2$-Macroglobulin and the resins is supported by the fact that an increase in the length of the resin bound ligand led to an increase retardation of the uncomplexed alpha$_2$-Macroglobulin.

Assays were performed on alpha$_2$-Macroglobulin to detect the presence of phospholipids. Initially, native alpha$_2$-Macroglobulin was subjected to a lipid extract. An aliquot of native alpha$_2$-Macroglobulin and the lipid extract were assayed for phospholipid. Bartlett's ANSA reagent failed to detect inorganic phosphate.

During development of the purification scheme and preparation of Concanavalin A affinity columns, some unusual and related observations were made. During synthesis of a Concanavalin A affinity column, the use of methylamine to block cyanogen bromide activated sites led to the discovery that methylamine inactivates alpha$_2$-Macroglobulin. At the time of this observation, the literature made no mention of methylamine's ability to inactivate alpha$_2$-Macroglobulin. Another interesting observation was made while examining the possible utility of phenylchloromercuric Sepharose chromatography column for the purpose of purifying alpha$_2$-Macroglobulin. A DTNB titration of

alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex was undertaken. Surprisingly, the data indicated that complex formation resulted in an increase of the sulfydral titer by four moles/mole of alpha$_2$-Macroglobulin. The importance of these observations were not clear until later, when Sottrup-Jensen et al., (1980) and Howard (1981) examined the reaction of alpha$_2$-Macroglobulin with primary amines. Alpha$_2$-Macroglobulin contains four labile thiol ester bonds (one per monomer), which are formed from the B-sulfhydryl group of cysteine and the - carbonyl group of glutamine. These investigators demonstrated that primary amines cleave the thiol ester bonds, resulting in the appearance of free sulphydral groups.

Carbohydrate studies were initiated to assess changes in the exposure of oligosaccharide chains and examine their involvement in receptor mediated endocytosis of alpha$_2$-Macroglobulin by cultured cells. The presence of carbohydrate moieties in alpha$_2$-Macroglobulin was confirmed through the use of Periodic Schiff reagent. Cleavage of the terminal sialic acid from native and complexed forms of alpha$_2$-Macroglobulin was conducted to ascertain whether exposure of sialic acid changes upon complex formation. The rates of cleavage of sialic acid from native and protease complexed forms of alpha$_2$-Macroglobulin by neuraminidase appeared similar. Chemical cleavage of sialic acid was performed to determine the sialic acid content of native alpha$_2$-Macroglobulin trypsin complex. Complexation appeared to result in a negligible decrease in sialic acid content as compared to the native. Thus, no detectable amounts of sialic acid are released from alpha$_2$-Macroglobulin upon complexation.

The exposure of surface carbohydrates as well as branched

carbohydrate chains was examined with Concanavalin A and wheat germ lectin. Wheat germ lectin, a protein purified from Triticum vulgare binds primarily N-acetyl-B-glucosamine residues. (Debray, 1981). Concanavalin A (ConA), derived from Canavalia ensiformis binds primarily alpha-D-glucopyranosyl and alpha-D-mannopyranosyl residues (Goldstein, 1972). At pH 7 the Con A exists as a tetramer, with four saccharide binding sites (Becker, J.W. et al., 1971). Below pH 7 the dimeric form of the protein begins to predominate. Each monomer contains one calcium and one maganese atom which are essential for carbohydrate binding to the lectin (Wang, J.L., et al., 1971; Lagrawal, B.B. et al., 1968). The precipitation reaction between ConA and branched chain polysaccharides is inhibited by low molecular weight carbohydrates which do not themselves cause precipitation of the lectin (Goldstein, I.J. and So., L.L., 1969).

Our Concanavalin A experiments attempted to examine the exposure of branched carbohydrate chains which contain alpha-D-glucopyranosyl and alpha-D-mannopyranosyl moieties. This differs from the sialidase experiment which examined the exposure of a single carbohydrate residue. Initial studies employed ConA either coupled to a resin or free in solution. Immobilization of the lectin can impose restrictions on the ConA which permit it to detect structural differences that the soluble lectin can not. Molecules of soluble ConA are free to move in relation to each other, and can concentrate at points where the degree of chain branching is greatest. By doing this, differences between polysaccharides may not be observed. Immobilization of ConA prevents this movement, and consequently only carbohydrates with a particular distance between non-reducing ends

would interact with the maximum possible strength. Thus, in the immobilized case, molecules may be fractionated according to differences in the length of branches or the distribution of branch points which are not apparent when complexation is carried out in solution.

The lectin binding experiments were initiated by synthesizing a number of affinity resins. The coupling conditions were altered to obtain a resin with the greatest binding capacity. A decrease in the pH of the coupling buffer resulted in an increased binding capacity. Possibly the change in pH reduced the number of multipoint attachments of the ConA, leading to less distortion of ConA's conformation. Maximizing binding conditions lead to the development of an affinity column (resin 5) with a binding capacity of 1.1 micromoles of alpha$_2$-Macroglobulin per ml of gel. All subsequent columns utilized the same coupling conditions as resin 5.

The interaction of native alpha$_2$-Macroglobulin and complexed alpha$_2$-Macroglobulin with Concanavalin A was conducted using a wide range of buffers and concentrations of alpha-methylmannoside. ConA resin 5 exhibited differential elution of native and complexed forms of alpha$_2$-Macroglobulin. The use of 1.0 M alpha-methylmannoside to elute alpha$_2$-Macroglobulin from the affinity resin appeard to be rather high when compared to other studies which used between 0.02 M and 0.4 M alpha-methylmannoside. Another affinity resin (6) was synthesized using the same conditions as resin 5. However, resin 6 was unable to affect the differential elution of native and complexed alpha$_2$-Macroglobulin. Application of a new batch of purified alpha$_2$-Macroglobulin to resin 6 indicated that this resin was incapable of

separating native alpha$_2$-Macroglobulin from complexed alpha$_2$-Macroglobulin. Due to this lack of reproducibility, the ConA studies were continued in solution. First, the conditions for the precipitation were optimized. The soluble ConA appeared to precipitate native alpha$_2$-Macroglobulin, but not alpha$_2$-Macroglobulin treated with various proteases or methylamine. Oxidation of the carbohydrate residues on native alpha$_2$-Macroglobulin, abolished ConA's ability to precipitate the native form of alpha$_2$-Macroglobulin. Later studies with a new bottle of ConA failed to demonstrate the differential precipitation of native and complexed alpha$_2$-Macroglobulin. A new batch of alpha$_2$-Macroglobulin was purified and the commercially purchased ConA was subjected to a purification. Repetition of the experiment indicated that both the native and complexed forms of alpha$_2$-Macroglobulin were precipitated similary by ConA. The only major difference between the experiments using purified and unpurified ConA was that a much smaller quanatity by weight of purified ConA was required to precipitate the same amound of alpha$_2$-Macroglobulin.

These conflicting results are difficult to explain. Either differences exist in the ConA and/or alpha$_2$-Macroglobulin. Calls to Pharmacia's technical service were made in the hope that the manufacturer could provide some reason(s) for our conflicting data. To the best of their knowledge, the manufacturing process as well as the chemical suppliers for the ConA have not changed. The existence of a hydrophobic binding site in addition to a sugar binding site, has also been postulated for ConA (Poretz and Goldstein, 1971; Yang, et al., 1974). Davey et al., (1976) demonstrated that ethylene glycol in

combination with alpha-methylmannoside considerably increased the recovery of absorbed proteins from ConA-agarose affinity columns. Other investigators (Lotan, et al., 1977) have reported that detergents greatly affect the total binding capacity of ConA affinity resins without changing its ability to recognize specifically mannosyl and/or glycosyl containing glycoproteins. Studies by Ochoa (1979) have shown that ConA is indeed absorbed to alkylagaroses by hydrophobic interaction chromatography. Additionally, the presence of glucose in the ConA sugar binding site results in a 20% increase in the total amount of ConA bound to the hydrophobic column. The occurrence of hydrophobic binding and its modulation by sugars in the sugar binding site could lead to variability in the data. Some investigators have suggested that the Concanavalin A coupled to cyanogen bromide activated Sepharose may be bound in a distorted conformation with exposes the interior of the protein leading to greater hydrophobic binding (Davey et al., 1976). The use of partially purified ConA could have led to conflicting results since partially purified extract contains variable concentrations of ConA depending on the source of the meal and the manner of extraction (Goldstein, et al., 1965). Variability in $alpha_2$-Macroglobulin's carbohydrate composition may have contributed to the conflicting results. Since the $alpha_2$-Macroglobulin used for the experiments was from an individual, slight variations in carbohydrate chain length and distribution of branch points may occur. The apparent variability in $alpha_2$-Macroglobulin's carbohydrate content is suggested by the studies on cystic fibrosis. The issue of carbohydrate alterations in cystic fibrosis is currently a matter of controversy. The literature

contains reports that there exist differences between cystic fibrosis and normal in the sialic acid content of alpha$_2$-Macroglobulin (Ben-Yoseph, 1979), its binding to Coancanavalin A and wheat germ lectin (Ben-Yosehp, 1979; Shapira, E., 1980), and in the ability of desialylated preparations of alpha$_2$-Macroglobulin to serve as sialic acid acceptors for sialytransferases (Ben-Yoseph, 1981). In contrast, cystic fibrosis and normal alpha$_2$-Macroglobulin have been shown to be identical with respect to hexose content (Bridges, 1982), neutral and amino sugar content (Park, C.M., 1985), and gel electrophoretic behavior (Comings, D.E., 1980). The reasons for the conflict results remain unclear. These articles suggest that there may be different population of alpha$_2$-Macroglobulin which differ in their carbohydrate content.

ConA was to be utilized in experiments designed to determine whether trypsin binding shows cooperativity. The successful execution of this study required ConA to bind and precipitate only native alpha$_2$-Macroglobulin. Since the new bottle of ConA bound native and complexed alpha$_2$-Macroglobulin, the cooperativity experiments could not be conducted.

Additional lectin studies were performed with wheat germ lectin. Initial experiments utilized unpurified wheat germ lectin. Following purification of the wheat germ lectin, the binding studies were repeated. Both sets of experiments indicated the native and complex forms of alpha$_2$-Macroglobulin are precipitated similarly, suggesting that the exposure of N-acetyl glucosamine residue is not dramatically altered upon complex formation.

D. Interaction of Alpha$_2$-Macroglobulin with Cells

The literature contained reports that both native and complexed alpha$_2$-Macroglobulin bound cellular receptors. The possibility existed that the receptor bound only complexed alpha$_2$-Macroglobulin, but the preparations of native alpha$_2$-Macroglobulin used for the studies may have been contaminated with complexed alpha$_2$-Macroglobulin. Our efforts were focused towards examining this possibility. Originally we planned to use ConA to separate native alpha$_2$-Macroglobulin from complexed alpha$_2$-Macroglobulin. When the new bottle of ConA failed to discriminate between the native and complexed forms of alpha$_2$-Macroglobulin, an alternative approach was needed.

The ability of iodination to alter alpha$_2$-Macroglobulin was investigated next. The iodination procedures could have possibly altered the inhibitor in such a manner that the receptor recognition sites would become exposed upon iodination. All the investigators performing cellular binding studies failed to ascertain whether iodination has any effect on alpha$_2$-Macroglobulin's trypsin binding activity. Consequently, experiments were conducted to determine if iodination of alpha$_2$-Macroglobulin alters its trypsin binding activity or its binding to Concanavalin A. Alpha$_2$-Macroglobulin was iodinated with chloramine T, soluble lactoperoxidase, and enzymobeads. The method of iodination that effected trypsin binding activity the least was chosen as the best procedure to use. Assaying the iodinated alpha$_2$-Macroglobulin from each procedure indicated that the enzymobead method led to no loss of trypsin binding activity. Unlabelled alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin iodinated by enzymobeads were subjected to a Concanavalin A precipitation. The results suggested

that I^{125}-alpha$_2$-Macroglobulin was precipitated in an identical manner as unlabelled alpha$_2$-Macroglobulin.

Cellular binding studies showed that native alpha$_2$-Macroglobulin was bound by fibroblasts, and the amount bound increased with time. Additionally, the receptor bound I^{125}-alpha$_2$-Macroglobulin was capable of being displaced by unlabelled inhibitor. Recent studies using monoclonal antibodies have demonstrated that the receptor recognition site on alpha$_2$-Macroglobulin is exposed after complex formation e.g. cleavage of the thiolester bond. One possible explanation for the binding of native alpha$_2$-Macroglobulin to cells may be the preincubation of alpha$_2$-Macroglobulin of alpha$_2$-Macroglobulin at 37^{o}C for fifty minutes results in conversion of native alpha$_2$-Macroglobulin into the complexed form of alpha$_2$-Macroglobulin. Hall and Roberts (1978) have reported that incubation of native alpha$_2$-Macroglobulin for 5 minutes at 55^{o}C results in loss of trypsin binding activity. Similarly, freezing of alpha$_2$-Macroglobulin was shown to inactivate native alpha$_2$-Macroglobulin. These results tend to support the suggestion that alpha$_2$-Macroglobulin is converted to a complexed form on incubation at 37^{o}C.

In summary, this thesis produced a new purification scheme for alpha$_2$-Macroglobulin. Native alpha$_2$-Macroglobulin and alpha$_2$-Macroglobulin protease complex were subjected to a variety of physical and chemical characterization procedures. Circular dichroism, fluorescent spectroscopy, U.V. difference spectroscopy, gel filtration, and hydrophobic interaction chromatography were some of the physical techniques used to assess alpha$_2$-Macroglobulin, while DTNB, sialidase, and lectins were utilized for chemical analysis of

native and complexed alpha$_2$-Macroglobulin. This work attempted to assess the magnitude and nature of the conformational changes which occurred upon reaction of alpha$_2$-Macroglobulin with proteases. The results demonstrated that only small changes in the secondary and tertiary structure of alpha$_2$-Macroglobulin appeared to occur on enzyme binding. Additionally, the sialidase and lectin studies indicated only minor changes in the exposure of the oligosaccharide chains. In contrast, the DTNB experiments demonstrated a large increase in the sulfhydryl titer occurred upon enzyme binding. Hydrophobic interaction chromatography of native and protease bound alpha$_2$-Macroglobulin on butyl-sepharose demonstrated that a change in the surface amino acids was observed upon enzyme binding.

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Biochemistry.

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