DENSITY COMPARISONS AND STRUCTURAL STUDIES OF MURINE MEMBRANE AND SECRETED IMMUNOGLOBULIN HEAVY CHAINS

By

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Dedication

To my wife, Wanda, my mother, Chloe, and in loving memory of my grandparents, Floyd and Ardale Beebe.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BSA	-	Bovine serum albumin
C	-	Constant
°C	-	Degrees centigrade
cm	-	Centimeter
CNBr	-	Cyanogen bromide
DMSO	-	Dimethyl sulfoxide
EACA	-	ε-amino caproic acid
EGTA	-	Ethyleneglycol N,N'-tetraacetic acid
FCS	-	Fetal calf serum
g	-	Gravity
gm	-	Gram
H	-	Heavy
Ig	-	Immunoglobulin(s)
KIU	-	Kallikrein inhibitor units
L	-	Light
M	-	Molar
mA	-	Milliampere
mCi	-	Millicurie
mg	-	Milligram
ml	-	Milliliter
mM	-	Millimolar
mm	-	Millimeter

Nonidet P40	-	Nonionic detergent
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PMSF	-	Phenyl methyl sulfonyl fluoride
rpm	-	Revolutions per minute
S	-	Sedimentation coefficient
SDS	-	Sodium dodecyl sulfate
TIU	-	Trypsin inhibitor unit
TPCK	-	L-1-tosyl-amide-2-phenyl ethyl chloromethyl ketone
Tris	-	2-amino-2-(hydroxymethyl)-1,3-propanediol
µCi	-	Microcurie
µg	-	Microgram
µl	-	Microliter
V	-	Variable
$\bar{v}$	-	Partial specific volume

CHAPTER I

INTRODUCTION

It has been recognized for many years that immune responses are of two general types, broadly termed cell-mediated immunity- (delayed type hypersensitivity responses, transplantation immunity and immunity to some viral, bacterial and parasitic infections) and humoral immunity- (the production of circulating antibody molecules). In all types of immune responses, lymphocytes play a central role in both the initiation and development of the response (Gowans and McGregor, 1965). Lymphocytes are comprised of two discrete cellular populations. A class of bone marrow derived lymphocytes migrates to the thymus where these cells develop the ability to respond to antigen. These thymus-processed lymphocytes, generally referred to as T cells, are responsible for the various phenomena of cell-mediated immunity. The second lymphocyte population also arises in the bone marrow and settles ultimately in the peripheral lymphoid tissues where these cells give rise to the precursors of antibody secreting cells (Davies, 1969). This review is concerned primarily with immunoglobulins as antigen receptors located on the surface of precursor cells to antibody secreting cells and their involvement in the activation of the humoral immune response.

The idea of an immunoglobulin as an antigen receptor on the surface of lymphocytes is not a new one; in fact, it was first clearly enunciated by Ehrlich in 1900 (Ehrlich, 1900). The concept of specific

preformed receptors for antigen was not followed up in the ensuing decades, since the instructional theory of antibody formation was popular (Pauling, 1940). It was thought that the antigen itself in some manner directed the formation of complementary structures using the protein synthesizing system of the cell. In the 1950's, the selection theory of immunity (Burnet, 1959) proposed the concept of precommitment of immunocompetent cells for antigen. It was considered that precursor cells exist bearing receptors for antigen on their surfaces. Each precursor cell carries antibody receptors with only one specificity, which is identical to the antibody whose synthesis is induced when the cell is triggered to divide and differentiate by interaction with a specific antigen. Antigen receptors therefore form the central hub of the selection theory and are operationally defined as membrane bound molecules with the following properties: 1) a binding site with stereochemical complementarity for the corresponding ligand, and 2) the capacity to initiate the transmission of a signal, resulting from the binding of the ligand, to the interior of the cell. The nature of the signals involved in lymphocyte activation has been the subject of much speculation. Ions, cyclic nucleotides and other hormones have been implicated (Wedner and Parker, 1976).

Existence of Membrane Receptors

The existence of membrane immunoglobulin (Ig) has been established by many methods. Membrane Ig was first demonstrated by Raff (1970) who mixed fluorescein or radioactive iodine tagged anti-Ig with intact cells. Bound anti-Ig was detected by fluorescence microscopy or autoradiography. Radioactive membrane Ig has been demonstrated by immunoprecipitation

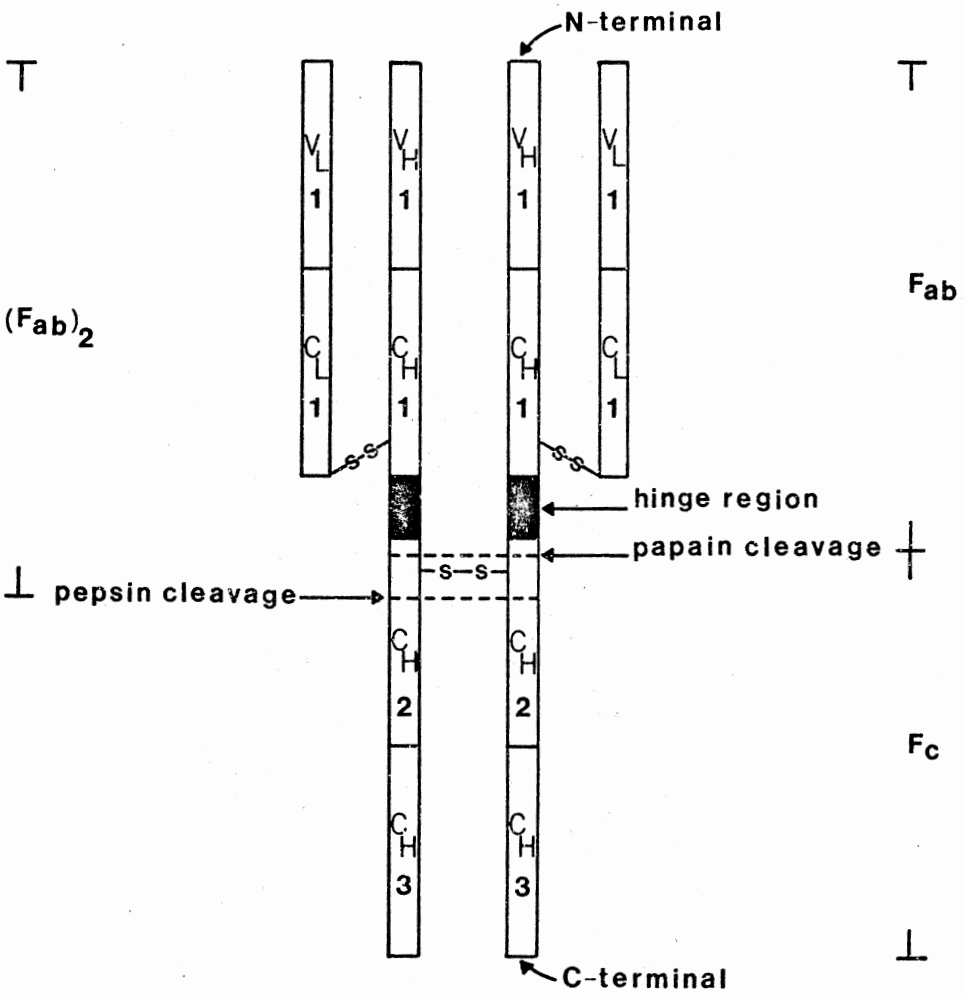
with anti-Ig from lysates of cells whose surfaces had been labeled with ^{125}I (Vitetta et al., 1971; Marchalonis et al., 1972) by the techniques of Phillips and Morrison (1970). Other methods, such as inhibition of anti-Ig-Ig precipitation by whole cells (Rabellino et al., 1971); binding of anti-Ig to cells as detected by various techniques including peroxidase coupling (Gonatas et al., 1972); ferritin coupling (dePetris and Raff, 1972) and rosette formation with red blood cells (Paraskevas et al., 1971) have also been used.

General Ig Structure

A basic feature of all immunoglobulins is a four chain structure: two heavy (H) and two light (L) chains, see Figure 1. The heavy and light chains are usually covalently linked by a single disulfide bond while one or more inter-heavy disulfide bond(s) are present. Reduction of the interchain disulfide bonds yields H and L chains.

Each H and L chain consists of distinct stretches of amino acids called domains. A domain is approximately 110 amino acid residues in length. The domains are termed variable (V) or constant (C), an implicit definition denoting the variability of amino acid sequences within the H and L chains. The H chain has one variable and three to four constant domains while the L chain has one variable and one constant domain. The variable domains of the H and L chains contribute amino acid residues which constitute the antigen binding site. The high degree of antibody diversity, that is the ability to recognize and bind a multitude of antigens, is a function of amino acid changes within the H and L chain variable domains. The three dimensional structure of immunoglobulins obtained by the folding of V and C domains has been

Figure 1. General Structural Features of the Immunoglobulin Molecule


N-terminal
$(F_{ab})_2$
F_{ab}
F_c
hinge region
papain cleavage
pepsin cleavage
s—s
C-terminal

reviewed (Davies et al., 1975).

Structural features of serum Igs were first characterized by fragments obtained upon treatment with proteolytic enzymes. The structural features of serum Igs also apply to membrane Igs. Porter (1959) reported that cleavage of IgG by papain gave rise to large fragments with distinctive properties. The products of this cleavage are also shown in Figure 1. Papain selectively attacks each heavy chain at a site just N-terminal to the inter-heavy chain disulfide bond(s). This liberates three fragments. Two are called Fab fragments (for antigen binding) and the third is called the Fc fragment (c for crystallizable). Each Fab fragment contains a complete L chain and the N-terminal half of the H chain covalently linked by a disulfide bond. The third fragment, Fc, consists of the C-terminal half of the two H chains also covalently linked by disulfide bond(s).

Pepsin attacks on the C-terminal side, rather than the N-terminal side of the inter-heavy chain disulfide bond(s). Cleavage yields a large bivalent fragment $(Fab)_2$ and a partially degraded Fc fragment.

Because of the susceptibility of a particular area in the middle of the H chain to attack by papain, pepsin and other enzymes, it is generally thought that this region must be loosely folded. This region has been designated as the hinge and is found between the first and second constant domains of the H chain.

Membrane Immunoglobulins

Membrane IgM has been identified as the major class of immunoglobulin on the surfaces of B lymphocytes (Vitetta et al., 1971; Marchalonis et al., 1972). Membrane IgD has been identified as the

second major class of Ig on the human lymphocytes (Knapp et al., 1973; Kubo et al., 1974; Fu et al., 1974; Finkelman et al., 1976), rat lymphocytes (Ruddick and Leslie, 1977) and rabbit lymphocytes (Pernis et al., 1975; Wilder et al., 1979). The presence of an additional class of Ig molecule, in addition to IgM in mice, was first reported by Melcher et al. (1974) and subsequently confirmed by others (Abney and Parkhouse, 1974; Haustein and Goding, 1975).

In the mouse, cell surface IgM and IgD can be precipitated from cellular lysates of externally labeled B lymphocytes with anti-IgM and anti-light chain sera respectively. Cell surface IgD is not precipitated by reaction with anti-μ, γ, or α sera at normal concentrations (Abney and Parkhouse, 1974; Melcher et al., 1974). However, it has been reported that the lack of precipitability of cell surface IgD by anti-μ sera can be overcome when sufficient amounts of purified anti-μ sera are used (Lisowska-Bernstein and Vassalli, 1975; Pernis et al., 1975).

Tissue Distribution

Using external labeling procedures, a marked difference is observed in the relative recovery of surface IgM and IgD from various lymphocyte populations. IgM and IgD were observed to be in approximately equal amounts in spleen, but in lymph nodes and Peyer's patches IgD accounts for 70-90% of the total cell surface Ig (Abney and Parkhouse, 1974; Melcher et al., 1974; Vitetta et al., 1975).

Ontogeny of Membrane Immunoglobulins

Lymphocytes bearing IgM, IgD or both IgM and IgD have been

demonstrated in a variety of species. Early studies in humans suggested that IgD was the primordial cell surface receptor on B lymphocytes (Rowe et al., 1973). In the mouse however, IgM was readily detectable on the surface of splenocytes from 4-day old mice, whereas IgD first became detectable at 10-15 days after birth (Vitetta et al., 1975). By three months of age, IgD is the preponderant cell surface immunoglobulin on peripheral mouse lymphoid tissues. That IgM, and not IgD, was detectable on bone marrow cells suggests that IgM rather than IgD was first in ontogeny, since bone marrow cells are precursors of peripheral cells.

Demonstration that IgM and IgD are present on the same cell supports the hypothesis that a differentiation step, in which the cells switch from IgM to IgD production takes place (Goodman et al., 1975). B lymphocytes from athymic mice and mice harbored in a germ free environment also bear both IgM and IgD (Vitetta et al., 1975). It appears that the class switch is independent of T cell influence and may also be independent of antigenic stimulation.

Fluorescent staining techniques have been used to demonstrate the independent movement of IgM and IgD on the surface of lymphocytes. The binding of surface immunoglobulin by polyvalent antigen or divalent anti-Ig induces the surface Ig to redistribute to initially form patches and finally a polar cap on the cell. Experiments have been performed in which surface IgM was first capped with rhodamine-labeled anti-IgM after which a diffuse staining pattern was observed when the cells were reacted with fluorescein-labeled anti-light chain (Ligler et al., 1977). The capping process is energy and temperature dependent (Knapp et al., 1973) and is followed either by shedding of the membrane Ig-ligand complex

or by endocytosis of the complex. If surface IgD and IgM are capped and internalized simultaneously, their rates of reappearance on the cell surface are approximately the same (Ligler et al., 1977; Rowe et al., 1973). Fourteen to twenty hours after internalization newly synthesized IgM and IgD have reappeared on the cell surface.

Turnover

Several studies have recently been reported on the turnover of cell surface immunoglobulin. Studies so far (Vitetta and Uhr, 1972; Andersson et al., 1974) suggest that immunoglobulins on B lymphocytes have a half life between two to eight hours. This half life is of the same order of magnitude as turnover of other cell surface proteins.

Biosynthesis of Membrane Immunoglobulins

Secreted immunoglobulins are synthesized by ribosomes bound to the endoplasmic reticulum of plasma cells and some B lymphocytes (Bevan et al., 1972; Uhr, 1970). The immunoglobulins are modified during passage through the smooth endoplasmic reticulum and the elements of the Golgi apparatus and are finally released from the cell by exocytosis. During IgM biosynthesis in mouse cells, the heavy and light chains are assembled early into monomer IgM (H_2L_2). Assembly of the monomers into a pentamer takes place at or near the time of secretion (Parkhouse and Askonas, 1969) and may involve the rearrangement of disulfide bonds and the addition of J chain (Della Corte and Parkhouse, 1973).

Glycosylation appears to have a role in the intracellular transport and secretion of immunoglobulins. Hickman et al. (1977) have

studied the secretion of Ig in the presence of tunicamycin, a compound that selectively prevents the glycosylation of newly synthesized proteins. Inhibition of Ig secretion, in the presence of tunicamycin, increases as the carbohydrate content of the Ig increases. At or near the time of secretion, the Ig acquires the terminal sugar residues on its carbohydrate side chains (Melchers, 1973).

The biosynthesis of membrane Igs has not been well studied. It has been hypothesized that Ig destined for the cell surface is synthesized and initially transported along a pathway similar to that of Ig to be secreted (Vitetta and Uhr, 1974). In the Golgi complex the precursor to membrane Ig becomes attached to the Golgi vesicle and the secreted Ig does not. After exocytosis of the Golgi vesicle, attached Ig becomes membrane Ig and released Ig becomes secretory Ig. This hypothesis has received some experimental support from pulse-chase studies (Sherret al., 1971; Vitetta and Uhr, 1974). Ig synthesized on membrane-bound polyribosomes is confined to the microsomal compartment; in addition, a period of 1-2 hours is required after the addition of label before membrane Ig can be detected on the outer surface of the cell. This period is similar to that required for the secretion of newly-synthesized IgM and is probably occupied by intracellular transit.

Physical Properties of Immunoglobulins

Table I summarizes pertinent information concerning physical characteristics of membrane and secreted Igs. This topic has been extensively reviewed (Metzger, 1970; Nisonoff et al., 1975). Information concerning IgG, IgA and IgE has been included in the table to provide a comparative view of the five Ig classes.

TABLE I

PHYSICAL PROPERTIES OF MEMBRANE AND SECRETED IMMUNOGLOBULINS

Ig	H chain designation	chain structure	M.W. intact molecule[b]	No. domain H chain	M.W. H chain[b]	% CHO H chain	serum conc.[c]	references[d]
$sIgG^{a}_{1-4}$	γ_{1-4}	H_2L_2	146	4	51	3-4.5	9	1
sIgM	μ	$(H_2L_2)_5 + J$	956-970	5	72-73	13.3	1.5	2
$sIgA_{1-2}$	α_{1-2}	$(H_2L_2)_{1 \text{ or } 2}$ + S.P.[f]	160-390	4	52-58	7-11	0.5	1
sIgE	ε	(H_2L_2)	188-196	5	72-76	12	3×10^{-4}	1
sIgD	δ	(H_2L_2)	172-184	4	63	15	3×10^{-5}	3
mIgM[e]	μ	(H_2L_2)	192	5	74+	unk	0	4
mIgD	δ	(H_2L_2)	178	4	66	unk	0	5,6

[a] s = secreted; [b] molecular wt x 10^{-3}; [c] mg/ml; [e] m = membrane; [f] S.P. = secretory piece.

[d] references: 1-Nisonoff et al., 1975; 2-Robinson et al., 1973; 3-Spiegelberg, 1977; 4-Melcher and Uhr, 1976; 5-Melcher et al., 1974; 6-Abney and Parkhouse, 1974.

Secreted IgM (19S) is a pentamer consisting of ten heavy chains and ten light chains covalently linked by disulfide bridges. In the secreted molecule there is at least one additional polypeptide chain, the J chain, disulfide bonded to the C-terminal region of the H chain (Mestecky and Schrohenloher, 1974). The J chain functions in the maintenance of the pentameric structure. The molecular weight of human secreted IgM(Ou) calculated from the known amino acid sequence and estimated carbohydrate content is 956,000 (Putnam et al., 1973). The molecular weight of mouse myeloma secreted (MOPC 104E) IgM H chain has been reported at 73,000 (Robinson et al., 1973). This molecular weight supports an H chain structure containing five domains (i.e. one variable and four constant domains).

Serum IgD always exists as a monomer whose sedimentation rate is 6-7S (Spiegelberg, 1972). Employing the equilibrium sedimentation method, molecular weight values of 172,000 (Spiegelberg, 1972) and 184,000 (Rowe et al., 1969) have been obtained. The discrepancy in the molecular weight determined by these two groups was a result of different values employed for the partial specific volume ($\bar{v}$) in the molecular weight calculation. The average molecular weight of the serum δ chain was determined by three independent methods to be 63,000 including carbohydrate (Spiegelberg et al., 1970; Goyert et al., 1977). The intact secreted IgD thus has a molecular weight of 172,000 assuming a molecular weight of 23,000 for the light chain. These data suggest that the δ chain has one variable and three constant domains and might have a relatively extended hinge region. Recent studies by Lin and Putnam (1979) have proven this to be the case. Myeloma IgD (WAS) has an extended carbohydrate-rich hinge region of at least 40 amino acid

residues.

In contrast to secreted IgM, cell surface IgM is a monomer (7S) consisting of two heavy and two light chains covalently linked by disulfide bonds. This has been demonstrated by SDS-PAGE and sedimentation in SDS-sucrose solutions (Vitetta et al., 1971; Kennel and Lerner, 1973; Melcher and Uhr, 1976). Under certain conditions it is possible to find two chain molecules (HL) (Abney and Parkhouse, 1974). These half molecules probably arise through breakdown of membrane IgM during the experimental procedure. The molecular weights of human, rat and mouse membrane IgM H chains have been reported as 73,000, 73,000 and 74,300 respectively (Ruddick and Leslie, 1977; Melcher and Uhr, 1976). Comparison of membrane and secreted IgM H chain molecular weight yields a reproducible 1700 dalton difference, the membrane species being the larger. The additional size of membrane H chain has been implicated in cell surface attachment and will be discussed in a subsequent section.

Like serum IgD, membrane IgD exists as a monomer covalently linked by disulfide bonds. The molecular weight of mouse membrane δ chain has been estimated at 65,800 (Melcher and Uhr, 1976). Although the mouse membrane δ chain as usually identified is smaller than the human membrane δ chain (Warr and Marchalonis, 1976), there is evidence that this δ chain may be a proteolytic fragment of a 'native' δ chain with a size equal to that of mouse membrane μ chain (Sitia et al., 1977). Sitia et al. detected a mouse membrane δ chain with a mobility comparable to human membrane δ chain of molecular weight 73,000. Further degradation products were observed, one of which corresponds to the normally isolated mouse membrane δ chain of molecular weight 65,800. The actual

size of mouse membrane δ chain remains for the most part unclear.

Integral Nature of Membrane Immunoglobulins

Membrane proteins have been classified as integral or peripheral based upon their solubilities (Singer and Nicolson, 1972). Proteins that can be extracted with moderate salt concentration, changes in pH, EDTA and other mild reagents are peripheral proteins. While integral membrane proteins require organic solvents or the presence of detergent for extraction and solubility, membrane immunoglobulins are extractable with nonionic detergent and 8M urea (Vitetta et al., 1971), but are not extractable with a variety of salts including potassium chloride, sodium sulfate and sodium trichloroacetate (Kennel and Lerner, 1973).

Evidence to support the integral nature of cell surface Ig has come from the examination of their solubilities in the presence and absence of detergent. Melcher et al. (1975) have reported mouse membrane Igs require the presence of detergent for solubility. In contrast, mouse secreted Igs do not require detergent for solubility.

Proteolytic Fragmentation

Like most Igs, membrane IgM and IgD can be cleaved by treatment with proteolytic enzymes. The proteolysis of mouse membrane IgM and IgD, with the enzyme papain, has been investigated (Vitetta and Uhr, 1976; Hough et al., 1977). Cell surface IgD was considerably more susceptible than cell surface IgM to papain cleavage when the cleavage was performed with the molecules attached to the cell surface. However in cellular detergent lysates treated with papain, no difference in

susceptibility to enzyme cleavage was observed. The major site of enzyme attack was in the hinge region of the H chain. These observations suggest that attachment to the plasma membrane affects the accessibility of IgM to papain.

The susceptibilities of detergent released mouse membrane IgM and IgD to trypsin cleavage have been compared with their human counterparts (Bourgois et al., 1977; Spiegelberg et al., 1970). The fragments released in both species had similar molecular weights; however the conditions required to cleave the IgM and IgD were quite different. Eighty percent of the IgD molecules could be effectively cleaved by trypsin in 10 minutes at $0^{o}C$, and a complete cleavage was obtained at $25^{o}C$. To cleave the IgM, digestion for several hours at $37^{o}C$ was necessary. The differences reported in the susceptibility of detergent released mouse membrane IgM to papain and trypsin have not been explained but may have been due to the extent of denaturation of the IgM in solution.

The increased susceptibility of the IgD molecule to enzymatic proteolysis has prompted investigation of the structural basis for this phenomenon. The Stokes radius and the sedimentation coefficient for the intact serum IgD and its proteolytic fragments have been determined (Griffiths and Gleich, 1972). Their results suggest that intact IgD is less compact than either IgG, IgE or IgM. Further, the IgD Fc fragment is less compact than that of IgG. The latter observation could be a result of the additional carbohydrate present on the Fc portion of the IgD. An apparent difference in conformation was proposed to account, in part, for its marked susceptibility to enzymatic cleavage.

The amino acid sequence of IgA_1 has been determined (Liu et

al., 1976). In the IgA_1, the hinge region is comprised of 30 amino acid residues, 12 of which are proline. Five O-linked carbohydrate moieties are also located in the hinge region (Baenziger and Kornfeld, 1974). The hinge region polypeptide structure of IgD is very different from that of IgA_1. The IgD hinge region is 40 amino acid residues in length and is also rich in carbohydrate (Lin and Putnam, 1979). However unlike the IgA_1 hinge region, the IgD hinge has a relatively low proline content (three for 40) and a high concentration of glutamic acid, lysine, arginine and no hydrophobic residues (Jefferis and Matthews, 1977; Lin and Putnam, 1979). The length of the IgD hinge and its chemical characteristics suggest an open structure readily accessible to cleavage, especially by trypsin and papain.

Spontaneous Degradation of IgD

Among the different classes of Igs, IgD is unique in that it is highly susceptible to degradation during conventional isolation procedures and storage (Fahey et al., 1968; Goyert et al., 1977). This degradation has been termed 'spontaneous' because the enzyme(s) responsible for it are unknown. It has been suggested that serum enzymes with trypsin-like activity could be responsible for the fragmentation of IgD (Spiegelberg, 1972). This proteolytic degradation can be inhibited by the addition of ε-amino caproic acid (EACA), an inhibitor of plasminogen-plasmin activation (Alkjaersig et al., 1959). Thus it has been suggested that plasmin may be responsible for the so-called 'spontaneous' fragmentation. There is some experimental evidence to support the role of plasmin in the proteolytic process. Panero et al. (1978) studied the effect of maternal serum on the cell surface IgD of

neonatal human lymphocytes in the presence of protease inhibitors. Contact with maternal serum caused a significant reduction in the percentage of neonatal IgD-positive cells as determined by the binding of fluorescein conjugated anti-IgD serum. The addition of either EACA or aprotinin, a trypsin inhibitor, significantly reduced the action of maternal serum. These data suggest that serum enzymes, like plasmin, could be responsible for the proteolysis.

The sites of 'spontaneous' cleavages of serum IgD have been investigated and found to occur first in the hinge region and second near the carboxy-terminal of the IgD H chain (Goyert et al., 1977). In the former instance, 'spontaneous' fragmentation parallels enzymatic action by trypsin and papain, in that proteolysis at the hinge yields Fab and Fc fragments. A similar susceptibility to 'spontaneous' fragmentation has been reported for human IgE (Bennich and Johansson, 1971).

Spontaneous fragmentation of cell surface IgD has yet to be investigated in the mouse. The possibility of fragmentation is real since mouse membrane δ chain as usually identified is smaller than human membrane δ chain (Warr and Marchalonis, 1976). The report of a mouse membrane δ chain larger than normally isolated (Sitia et al., 1977) adds support to the possibility of a proteolytic event. One might speculate that degradation at the C-terminal of the mouse membrane δ chain might remove peptide sequences important in the attachment process. The answer to this question awaits further experimentation, however.

Membrane Immunoglobulin Carbohydrate

Immunoglobulins are glycoproteins and typically the complex oligo-

saccharides attached to immunoglobulins are asparagine-linked branched structures comprised of N-acetylglucosamine and mannose core sugars. The branched structures are completed by the addition of the terminal sugars (galactose, fucose and sialic acid) to the core sugars. In addition, IgD contains O-linked (i.e., serine and threonine) oligosaccharides and the carbohydrate N-acetylgalactosamine (Clamp and Johnson, 1972; Grey et al., 1971). The biosynthesis of these complex oligosaccharide units has been reviewed by Kornfeld and Kornfeld (1976) and the oligosaccharide structures defined for a number of the immunoglobulins (Baenziger and Kornfeld, 1974).

Information concerning the carbohydrate attached to cell surface Ig is lacking. However, carbohydrate contents and structures have been reported for their secreted counterparts (Robinson et al., 1973; Baenziger and Kornfeld, 1974). The H chain carbohydrate for MOPC 104E secreted μ chain is 13.3%. This figure translates into 9700 g carbohydrate per 73,000 g protein. The carbohydrate content of mouse myeloma IgM (MOPC 104E) is 11.7% for the H_2L_2. There is some discrepancy as to the number of oligosaccharide units per heavy chain. Five oligosaccharide units have been reported (Hickman et al., 1977), however the possibility of an additional oligosaccharide unit in the variable region has been reported (Anderson, personal communication, 1979).

Melcher and Uhr (1977) have shown that immunoglobulin buoyant density is an indicator of carbohydrate content. Buoyant density determinations were used to compare the carbohydrate contents of intracellular, membrane and secreted immunoglobulins. These determinations were performed in the presence of detergent. Membrane IgM

from detergent lysates of spleen cells was less dense than either intracellular or partially reduced secreted IgM. Underglycosylation or detergent binding could account for the decreased density observed in membrane IgM. The former explanation was discounted since incorporation of terminal sugar has been demonstrated in membrane IgM (Vitetta and Uhr, 1974) and the electrophoretic mobilities of membrane and secreted μ chains are similar. The low density of membrane IgM was thus attributed to detergent binding. Detergent binding is a property of integral membrane proteins (Helenius and Simons, 1975). Information regarding the content, structure, and placement of carbohydrate on mouse membrane IgM has not been reported.

Carbohydrate analysis of IgD myeloma proteins has been reported (Spiegelberg et al., 1970; Jefferis et al., 1975). The absolute amounts of carbohydrate varied in these studies. However, both reports agreed that the δ chains are rich in carbohydrate and have on the average 15% carbohydrate. The calculated carbohydrate content for the intact IgD molecule is 11% using a median molecular weight of 184,000 daltons of which carbohydrate is 20,000 daltons (Jefferis et al., 1975). IgD has a high sialic acid content which seems to be variable when compared to other immunoglobulin classes (Jefferis and Metthews, 1977).

Only three glycopeptides were recovered from peptide maps of serum δ chains (Spiegelberg et al., 1970) suggesting that all the carbohydrate is attached at three sites. One site was localized in the hinge region and contained all the galactose and N-acetylgalactosamine. The other two sites were located in the Fc fragment of the H chain and contained N-acetylglucosamine, mannose and the terminal sugars.

The structure, content or placement of carbohydrate on mouse membrane IgD has not been reported. However in the buoyant density gradient experiments previously described, mouse membrane IgD had a higher density than mouse membrane IgM indicating that IgD has appreciable amounts of carbohydrate or that IgD binds much less detergent (Melcher and Uhr, 1977). These data are consistent with the fact that human IgD is known to have a higher percentage carbohydrate content than does human IgM (Perry and Milstein, 1970).

Mouse membrane IgM has a lower functional affinity for lentil lectin than does mouse membrane IgD (Kubo et al., 1970). The observed differences in functional affinity for lectin binding have been attributed to the number of oligosaccharide units or to differences in carbohydrate sequences. Differences in carbohydrate sequences are supported by binding studies of membrane IgM and IgD to Concanavalin A Sepharose, a mannose binding affinity adsorbent (Hunt and Marchalonis, 1974). In these studies IgD was retained less well by the affinity adsorbent.

Antigen Receptors and Lymphocyte Triggering

Interaction of an antigen with a lymphocyte leads to division and differentiation and culminates in the formation of an antibody-secreting plasma cell. The mechanisms by which antigen binding is translated into a functional response remain to be determined. There are two theories to explain the induction of antibody secreting precursor cells; these are the one signal hypothesis and the two signal hypothesis. In the one signal hypothesis, the Ig receptor on the surface of the B cell lymphocyte binds the antigen. However, this binding does

not lead to an intracellular signal. The bound antigen serves as a site for binding of a T cell factor, or T cell. The bound T cell factor, thus concentrated on the surface of the responding lymphocyte binds another molecule on the surface of the cell. It is this latter binding that provides a single intracellular signal, which is sufficient for stimulation (Coutinho and Möller, 1975). In the two signal hypothesis, the binding of antigen to an Ig receptor on the surface of the B lymphocyte leads to the production of one intracellular signal. A second intracellular signal is generated when a factor derived from a T cell interacts with some molecule on the surface of the B cell undergoing stimulation. The simultaneous presence of both intracellular signals is necessary for division and differentiation to occur (ibid). One of the basic unanswered questions of cellular immunology concerns the process of activation of the B cell lymphocyte following antigenic stimulation.

In the above hypotheses, the roles for the Ig receptor are quite different. In the two signal hypothesis, the Ig is directly involved in generating a signal. The Ig receptor must undergo some change upon binding antigen, which can be translated to a membrane alteration either through a permeability change or by altering the activity of membrane proteins on the inner surface of the membrane. In the one signal hypothesis the Ig serves only to provide a site for focusing of binding of the T cell factor, or T cell. All that is required of this model is that the Ig be attached in some manner to the cell surface.

Membrane Attachment

There are three mechanisms by which membrane attachment might occur (Ramasamy, 1976). First, it is possible that the membrane Ig differs in structure from its secreted counterpart in such a way that only the membrane species develops a stable interaction with the plasma membrane. Structural differences could be manifested through changes in the primary amino acid sequence or perhaps through differences in the carbohydrate content or composition of the membrane and secreted Igs. In the former, one might envision a membrane H chain with a different or additional amino acid sequence at the C-terminal. A sequence with hydrophobic character could directly anchor the membrane species into the lipid bilayer. Such a mechanism has been invoked to explain the binding of other proteins, such as glycophorin, to the plasma membrane (Marchesi et al., 1976).

Studies with anti-Ig sera and enzymatic surface labeling techniques support C-terminal involvement in the attachment process. Fu and Kunkel (1974) have reported that the C-terminal portion of the H chain of cell surface IgM on human B lymphocytes is buried in the membrane. Their conclusion was based upon the observation that specific antigenic sites of the cell surface IgM were not available for reaction with the anti-sera. Using the lactoperoxidase surface labeling technique, H to L chain labeling ratios have been used as an indicator of the availability of specific amino acid residues (i.e., tyrosine) for iodination. Marchalonis et al. (1972) have reported that at least part of the C-terminal end of the membrane IgM H chain is buried. In more recent studies (Seon and Pressman, 1979), the H to L chain ratio for cell

surface IgM was smaller than that of a number of partially reduced secreted IgMs, further indicating that a portion of the membrane IgM is not readily available for iodination.

The mobilities of mouse membrane and secreted IgM H chains have been compared utilizing polyacrylamide gel electrophoresis in the presence of SDS (Melcher and Uhr, 1976). The mobility of mouse μ chain is slightly less than that of the secreted μ chain. The mobility difference is highly reproducible. A 1700 dalton molecular weight difference has been estimated between mouse membrane and secreted μ chains. Membrane μ chains have been shown to be larger than their serum counterparts in other species as well. Haimovich (1977) has reported a 10,000 dalton difference between bovine membrane and secreted μ chains. In addition, human membrane δ chains are also larger than their serum counterparts (Finkelman et al., 1976; Warr and Marchalonis, 1976). Cell surface δ chain has been identified on both human cord blood and peripheral lymphocytes, and it appears to have a mobility similar to mouse membrane IgM H chain with a molecular weight at or near 73,000. In comparison, the molecular weight of human serum δ chain is 63,000 (Spiegelberg, 1977). Further, membrane δ chains from several other species are larger than δ chains from human serum (Ruddick and Leslie, 1977; Finkelman et al., 1976; Warr and Marchalonis, 1976). Rat membrane δ chain has an apparent molecular weight of 73,000 daltons and exhibits a minor 65,000 molecular weight species which is probably a partially degraded δ chain.

That the size difference could be related to membrane attachment has prompted investigation of membrane and secreted C-terminal sequences. Secreted IgM H chains have been found to terminate in an extra domain

sequence of 19 amino acid residues (Watanabe et al., 1973; Milstein et al., 1975) which is not sufficiently hydrophobic to act as an integral membrane component. Membrane H chains could possibly carry a hydrophobic C-terminal polypeptide section instead of, or in addition to, the C-terminal stretch present in secreted H-chains. Based on computer analysis of possible nucleic acid sequences coding for serum H chains, mechanisms have been proposed for the attachment of membrane active amino acid sequences to the serum H chain (Melcher, 1978a).

McIlhinney et al. (1978) have investigated the possibility of C-terminal differences between membrane and secreted H chains by exploiting the fact that a tyrosine residue is present at the C-terminus in all examined secreted IgM H chains. The general structure of a C-terminal octapeptide released by CNBr cleavage is (ser-asp-thr-ala-gly-thr-cys-tyr-COOH). Since it is improbable that a hydrophobic extension on the membrane H chain would coincidently carry a C-terminal tyrosine residue, surface and secreted H chains were examined to see whether a C-terminal tyrosine could be cleaved from membrane H chain by treatment with carboxypeptidase. Their finding that both membrane and secreted H chains have a C-terminal tyrosine residue led them to conclude that it is unlikely that there is an extra C-terminal hydrophobic piece.

In contrast, Williams et al. (1978) have presented evidence for a difference in C-terminal amino acid sequences between membrane and secreted H chains. In similar experiments, carboxypeptidase digestion and peptide analysis have been applied to compare the C-terminal primary structure. As expected, the release of C-terminal tyrosine was seen with internally labeled secreted H chains, however only a

small amount of tyrosine was released from internally labeled non-secreted H chains. Instead other hydrophobic amino acids - phenylalanine, valine and leucine - were released. This data suggested that tyrosine was not the C-terminal residue of the membrane H chain. Their inability to isolate the C-terminal octapeptide of the cell surface H chain also indicated that these chains possess a different C-terminal structure from those that are secreted.

Yuan et al. (personal communication, 1978) have compared the trypsin chymotrypsin digests of H chains isolated from cell associated and secreted IgM. At least two peptide differences were demonstrated between cell associated and secreted IgM H chains. The location of the polypeptide differences are not known and can only be elucidated fully by primary sequence determinations. However, these results are consistent with those of Williams et al. suggesting an extra peptide may be present at or near the C-terminal of the membrane-bound H chain.

Membrane and secreted structural and size differences could also be manifested through differences in carbohydrate content or composition. Carbohydrate differences might very well influence the attachment to the plasma membrane. There is a report suggesting that membrane Ig is deficient in or lacks altogether terminal sialic acid and the branch sugars fucose and galactose (Melchers and Andersson, 1973). Others report the detection of branch sugars via the incorporation of radioactive sugars, although at low levels (Vitetta and Uhr, 1974). Melcher (1978b) has determined that newly synthesized mouse IgM is membrane active within 15 minutes after synthesis. This finding indicates that the attachment of the intracellular precursor to membrane Ig to cell membranes may be an early event in the ontogeny of the plasma membrane species.

It is thus unlikely that the terminal sugars are involved in membrane attachment since these are added later (Melchers, 1973). The extent of involvement of carbohydrate in membrane attachment remains for the most part unexplored.

A second possible mechanism for attachment is that membrane Ig is anchored to the plasma membrane either by a covalent or non-covalent interaction with another integral membrane protein. There is some evidence to support the latter of these contentions. Fc receptors, proteins that bind the Fc portion of many Igs, are present on many cell types of the immune system. It has been suggested that the Fc receptor of IgG may serve as the attachment protein (Ramasamy et al., 1974). Lymphocyte membrane receptors recognizing IgM have been reported in normal and malignant cells (Moretta et al., 1975; Ferrarini et al., 1977; Hardin et al., 1979). It is possible that one of these receptors functions as an attachment protein. A model for this type of interaction has been described for the binding of IgE to a protein in the membrane of rat mast cells and basophilic leukocytes (Conrad et al., 1975).

The final mechanism proposes that the membrane Ig is synthesized on a different subcellular site to secreted Ig and that this governs the localization of the receptor on the plasma membrane. To date there is no evidence to support this mechanism.

Scope of Study

The following questions remain to be answered concerning cell surface Ig. First, the mechanism by which Ig is attached to the plasma membrane remains to be determined. A number of pertinent differences

between cell surface and secreted Igs have already been observed. Among these is a difference in size of the two Ig molecules (Melcher and Uhr, 1976) which has been attributed to either carbohydrate content differences or peptide sequence differences (Ramasamy, 1976). The use of isopycnic density gradient centrifugations in CsCl detergent containing gradients has been shown to be sensitive to Ig carbohydrate content differences (Melcher and Uhr, 1977). The carbohydrate content differences of mouse membrane H chains and mouse and human secreted H chains of various classes have been compared using modifications of the technique previously described by Melcher and Uhr. Using buoyant density, the carbohydrate content of mouse membrane IgD H chain was estimated.

The size of mouse membrane IgD H chain remains unclear. As normally isolated, mouse membrane δ chain is smaller than mouse membrane μ chain. However, there is a recent report suggesting that the smaller δ chain may be a proteolytic fragment of a larger 'native' δ chain with a size comparable to that of mouse membrane μ chain (Sitia et al., 1977). An increased susceptibility to proteolytic degradation has been reported for human δ chain (Spiegelberg, 1972). To test whether mouse membrane δ chain is a proteolytic fragment of a larger 'native' δ chain, proteolysis inhibitors were incorporated into the spleen cell preparation, labeling and membrane Ig isolation procedures. The mobilities of the mouse membrane μ and δ chains were compared by SDS-PAGE.

The fragmentation of mouse membrane IgM and IgD by chemical or enzymatic methods has not been thoroughly studied. The conservation of cleavage sites between cell surface IgM and IgD and secreted Igs

was studied by examining the fragment sizes upon treatment with chemical and enzymatic cleaving agents.

CHAPTER II

MATERIALS AND METHODS

Materials

Materials used in these studies were obtained from the following sources: Sigma Chemical Co. (lactoperoxidase, α-chymotrypsin, papain, 2,5-diphenyloxazole (PPO), cytochrome c and guanidine-HCl); Fischer Scientific Co. (2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and CsCl); Particle Data Laboratories Ltd. (Nonidet P40); Pierce (CNBr); Beckman Instruments Inc. (Bio-Solv^{TM} solubilizer); Kodak (Royal X-Omat x-ray film, KLX DEV x-ray developer and FIX x-ray fixer); and Worthington (pepsin). Six to twelve week old inbred Balb/c mice were purchased from the Jackson Laboratory.

Minimal essential medium (MEM) ingredients and fetal calf serum (FCS) were purchased from Grand Island Biological Company. MEM for in vivo radio-labeling incubations was prepared as previously described (Melcher and Uhr, 1973; Vitetta and Uhr, 1972).

The following radioisotopes were obtained from the Amersham/Searle Corporation: Na^{125}I, carrier free; Na^{131}I, carrier free and L-[4,5-^{3}H]-leucine, specific activity 48 Ci/mmole. [^{14}C]-lactoperoxidase was prepared by the acetylation of lactoperoxidase with [^{14}C]-acetic anhydride.

MOPC 21 γ-globulin was purchased from Bionetics. MOPC 104E IgM and MOPC 315 IgA were prepared from ascites fluid obtained as a gift

from Drs. M. Boesman-Finkelstein and D. Hart. Human myeloma IgD (Delio) was obtained as a gift from Dr. J. D. Capra.

Rabbit anti-mouse IgM (raised against MOPC 104E IgM, with μ and λ activities) and rabbit anti-mouse IgA (raised against TEPC 15 IgA with α and κ activities) were obtained from Drs. M. Boesman-Finkelstein and J. Uhr respectively. Goat antiserum specific for the Fc portion of rabbit IgG was obtained as a gift from Dr. D. Hart.

Proteolytic inhibitors used in these studies: phenyl methyl sulfonyl fluoride (PMSF), aprotinin (10-20 TIU/ml), ethylene glycol N,N'-tetraacetic acid (EGTA), ε-amino caproic acid (EACA) and L-1-tosyl-amide-2-phenyl ethyl chloromethyl ketone (TPCK) were obtained from Sigma Chemical Co. PMSF and TPCK solutions were prepared by first making a 10 mM solution of each inhibitor in 95% ethanol; these solutions were then diluted into phosphate buffered saline (PBS) (0.15 M NaCl, 0.015 sodium phosphate, pH 7.2) to obtain the final concentration used in the experiments. 0.1 mM EGTA was prepared by dissolving the appropriate amount of solid into PBS, then the pH of the solution was adjusted to slightly alkaline with 2N NaOH to facilitate solubilization. ε-amino caproic acid solutions were 1-2% in PBS.

Methods

Preparation, Surface Iodination and Lysis of Mouse Spleen Cells

Six to twelve week old Balb/c mice were sacrificed by cervical dislocation. The spleens were perfused with cold PBS. After removal, spleens were trimmed of excess connective tissue and teased into PBS to

make a single cell suspension. The spleen cell suspension was transferred through a clean prewetted 100 mesh stainless steel screen into a 50 ml polypropylene centrifuge tube. The volume in the tube was brought to 20-30 ml with cold PBS and the suspension was spun at 3,000 rpm in a Beckman J-21 centrifuge (Palo Alto, Calif.) for 10 minutes. The cell pellet was resuspended in cold PBS, diluted and respun. The washed cells were resuspended in 1.0 ml of cold PBS. Ten µl of this cell suspension was added to 1.0 ml of 0.04% trypan blue/PBS and an aliquot of this mixture placed in a hemocytometer to determine cell concentration and cell viability with a microscope.

To surface label a cell suspension of 1×10^8 cells/ml with 1 mCi of either ^{125}I or ^{131}I the following materials were added in order: 100 µg lactoperoxidase in 100 µl PBS, 5 µl of 1 mM NaI, 1 mCi $Na^{125}I$ or $Na^{131}I$ (50 µl of 0.02 mCi/µl in 0.1 mM NaOH), 25 µl of 0.03% H_2O_2 in PBS. The labeling mixture was allowed to react for 5 minutes, then a second addition of 25 µl of 0.03% H_2O_2 was made. The 5 minute incubation was repeated. During the second incubation period the count rate at a known distance from Geiger-Müller (G-M) tube was observed. At the end of the second 5 minute incubation the labeled cell suspension was diluted to 20-30 ml with cold PBS and spun at 3,000 rpm for 10 minutes. The supernatant was decanted into the ^{125}I or ^{131}I radioactive waste and the cells were resuspended in 2-5 ml of cold PBS. The count rate was measured again using the G-M tube. Sufficient cell surface iodination yielded a count rate approximately one third of the rate previously measured. The cell suspension was then diluted to 20-30 ml with cold PBS and centrifuged. The cells were washed two additional times with PBS.

Cells were lysed by resuspending the final cell pellet in 1.6 ml cold PBS and adding 0.4 ml of 5% (v/v) Nonidet P40 (nonionic detergent). Cells were allowed to lyse for 15 minutes at room temperature and were then spun at 5,000 rpm for 15 minutes. The nuclear pellet was discarded. The supernatant (lysate) was the ^{125}I or ^{131}I labeled surface protein preparation.

Radioiodination of Myeloma Proteins and Cytochrome c

MOPC 21 IgG, MOPC 104E IgM, MOPC 315 IgA and human myeloma IgD (Delio) were radioiodinated with either ^{125}I or ^{131}I in solution with lactoperoxidase (Melcher and Uhr, 1977). The labeling mixture was prepared by addition of the following materials in order to a 12 x 75 mm plastic tube (Falcon): 200 µg of the protein to be radioiodinated, 20 µg lactoperoxidase in PBS, 1 mCi $Na^{125}I$ or $Na^{131}I$ (50 µl of 0.02 mCi/µl in 0.1 mM NaOH), 25 µl of 0.03% H_2O_2 in PBS. The labeling mixture was adjusted to a final volume of 0.25 ml with PBS. The reaction mixture was incubated for 15 minutes, then an additional 10 µg lactoperoxidase in PBS added. After 20 minutes, the reaction mixture was chilled. The labeling reaction was stopped by adding 50 µl of 10 mM NaN_3 followed by the addition of 500 µg unlabeled carrier protein (generally human γ-globulin) and 0.2 ml PBS.

The radioiodinated myeloma proteins were precipitated by the addition of 0.60 ml saturated Na_2SO_4. The precipitate was spun down at 2,600 rpm in a Sorvall GLC-2 for 10 minutes. The supernatant was discarded and the pellet resolubilized in 0.5 ml PBS. The precipitation was repeated by the addition of 0.5 ml saturated Na_2SO_4 and centrifuged

as above. This step was repeated two more times. The final pellet was dissolved in 1.0 ml 8 M urea, 0.2 M Tris-HCl, pH 8 in preparation for reduction and alkylation.

Cytochrome c was radioiodinated in solution as described for the myeloma proteins. 500 µg of cytochrome c was added as unlabeled carrier protein after the initial labeling reaction. The reaction mixture was dialysed against 0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate and 10% glycerol at room temperature overnight. After dialysis the labeled cytochrome c was applied directly to polyacrylamide gels as a size marker.

Incubation of Mouse Spleen Cells with ^{3}H Amino Acid(s)

Incubation medium was prepared as described in the materials section. Mice were sacrificed and the spleens removed as previously described; however the spleens were not perfused. Spleens were trimmed in medium and a spleen cell suspension was prepared, washed and counted as previously described. The cell concentration was adjusted to 4×10^{7} cells/ml. A tube containing the radioactive amino acid(s) at twice the final concentration (for ^{3}H, 20 µCi/ml was used as the final concentration) was prepared in medium. The cells and the radioactive amino acid(s) were preincubated separately at 37°C for 10 minutes. Cells were added to the radioactive amino acid(s) at time zero. The cells plus amino acid(s) were incubated at 37°C for 4 hours. At two hours into incubation, the mixture was made 10% in FCS to maintain the viability of the cells. At the end of the incubation period the cell suspension was spun at 1,000 rpm for 10 minutes. The supernatant

contained the ^{3}H labeled secreted immunoglobulins.

Immunoprecipitation of Membrane and Secreted Immunoglobulins

The ^{125}I or ^{131}I labeled membrane IgM in the 1% Nonidet P40 lysate and ^{3}H labeled secreted IgM were reacted with rabbit anti-mouse IgM. Typically 5 µl of rabbit anti-mouse IgM per 1 x 10^{7} cells/ml in the labeling procedures was used. The required amount of rabbit anti-mouse IgM was added to 0.25 ml 0.1% BSA, in 10 mM NaI/PBS prior to the reaction with the iodinated or ^{3}H-labeled IgMs. After addition of antiserum the samples were incubated at 37°C for 15 minutes. Complexes were recovered by precipitation with goat anti-rabbit Ig serum. The ratio of goat anti-rabbit Ig to rabbit anti-mouse IgM was 9:1.

Radiolabeled membrane IgD was recovered from the supernatant of the IgM precipitation by the addition of rabbit anti-mouse IgA and goat anti-rabbit Ig. The membrane IgM, IgD and secreted IgM immunoprecipitates were washed once with 0.1% BSA, in 10 mM NaI/PBS, twice with cold PBS and dissolved in 1.0 ml 8 M urea, 0.2 M Tris-HCl, pH 8 in preparation for reduction and alkylation.

Immunoprecipitation with Staphylococcal Protein A Antibody Adsorbent

Preparation of the staphylococcal adsorbent has been described previously (Kessler, 1976). Shortly before use the adsorbent was centrifuged (2000 x g, 20 minutes), resuspended and incubated for 15 minutes in NaCl-EDTA-Tris (NET, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, and 0.02% NaN_3) buffer, pH 7.4, containing 0.5% (v/v) Nonidet P40.

Then the adsorbent was washed once in NET buffer containing 0.05% Nonidet P40 and finally resuspended to a 10% (v/v) suspension in the latter buffer. In the immunoprecipitation, 0.2 ml of the 10% suspension was used per 5 µl of antiserum. As in the sequential immunoprecipitation of labeled mouse membrane IgM and IgD, the required amount of rabbit anti-mouse IgM was first added to the iodinated spleen cell lysate and incubated on ice for 5 minutes. The required amount of 10% (v/v) staphylococcal suspension was added, incubated for 10 minutes on ice, and spun at 2,600 rpm in a Sorvall GLC-2 for 10 minutes. The adsorbent pellet contained the mouse membrane IgM. The supernatant was immunoprecipitated with the required amount of rabbit anti-mouse IgA and staphylococcal adsorbent as above. The pellets from the centrifugation step were washed three times with 0.5% Nonidet P40 NET.

Labeled membrane immunoglobulins were released from the adsorbent by treatment with electrophoresis sample buffer, in the presence or absence of 2-mercaptoethanol. The immunoglobulins were recovered by pelleting the adsorbent (2,600 rpm, 10 minutes) and removing the supernatant. The adsorbent released samples were ready for gel electrophoresis.

Reduction and Alkylation of Membrane and Secreted Immunoglobulins

The labeled Ig samples were reduced with 0.15 mM 2-mercaptoethanol for 1 hour at room temperature and alkylated with 0.27 mM iodoacetamide for 30 minutes at room temperature. Samples were dialysed against 1 M propionic acid at 4°C overnight in preparation for purification of the heavy chains by gel filtration chromatography. Dialysis solutions

contained 0.1 mM PMSF in order to retard proteolysis.

Immunoglobulin Heavy Chain Purification By Gel Filtration Chromatography

Individual reduced and alkylated Ig samples, in 1 M propionic acid, were layered on a Sephadex G-100 gel filtration column (60 cm x 1 cm) (Pharmacia) to effect separation of immunoglobulin heavy (H) and light (L) chains. The column was eluted with 1 M propionic acid; 0.5 ml fractions were collected using an ISCO model 328 fraction collector. The ^{125}I or ^{131}I containing fractions were counted on a Packard Auto-gamma scintillation counter to determine the position of the heavy chain peaks. The ^{3}H labeled H chain-containing fractions were determined by counting an aliquot of each fraction dissolved in toluene/Bio-Solv^{TM}/PPO in a liquid scintillation counter.

CsCl Gradient Centrifugation of Immunoglobulin Heavy Chains

Individual H chain peaks in 1 M propionic acid from the gel filtration chromatography step were pooled and dialysed against 6 M guanidine-HCl, 0.2 M Tris-HCl, pH 8. These solutions were 0.1 mM in PMSF. Polyallomer centrifuge tubes were filled with 2.0 ml of 2.32 M CsCl in 6.0 M guanidine-HCl. The samples used were purified ^{3}H, ^{125}I or ^{131}I labeled H chains. The H chains to be compared (one labeled with ^{125}I, the other with ^{131}I) were mixed. ^{3}H labeled spleen cell secreted μ chain and ^{125}I MOPC 104E secreted μ chain were added to separate polyallomer centrifuge tubes. Included in each of these latter tubes was a [^{14}C]-lactoperoxidase marker. All samples were brought to 2.0

ml with 6.0 M guanidine-HCl, 0.2 M Tris-HCl, pH 8 and layered on the CsCl solution. A layer of paraffin oil was laid over the aqueous sample. The tubes were centrifuged for 65 hours at 35,000 rpm in an SW 50.1 rotor (Beckman Instruments, Palo Alto, Calif.) at 5°C. The tubes were then punctured at the bottom and 4 drop fractions collected from the middle of the gradient. For analysis of ^{125}I and ^{131}I cpms, fractions were counted directly. Correction was made for ^{131}I spillover into the ^{125}I channel; virtually no spillover was seen in the reverse direction. The [^{14}C]-lactoperoxidase marker and ^{3}H labeled H chain radioactivities were recovered from the gradient fractions by 10% TCA precipitation. The ^{14}C and ^{3}H containing precipitates were collected on glass fiber filters by vacuum filtration, dried, added to 10 ml of toluene/PPO and the filters counted in a liquid scintillation counter. ^{3}H counts were corrected for spillover of ^{14}C. Densities were determined on fractions of a dummy gradient by pycnometry.

The differences in peak position, expressed as the number of fractions between the peaks, were determined by fitting the data (cpms) to a Gaussian distribution by computer. To avoid distortion of the peak positions, due to trailing on the less dense side of the gradients, only the dense half of the distribution plus five fractions past the observed peak position were fit. Actual data were normalized to % radioactivity in the peak fraction and are plotted as such on the figures. A minimum of sixty data points were taken for each gradient, but only every fifth data point is shown in the figures for purposes of clarity.

Incorporation of Proteolysis Inhibitors Into Preparation and Isolation of Mouse Membrane H Chains

Preparation of inhibitor solutions has been described in the materials section. The inhibitors (PMSF, aprotinin, EGTA, EACA, and TPCK) were used either singly or in combinations. Inhibitors or a combination of inhibitors were introduced at the earliest point in the preparation of spleen cells by perfusing the intact spleen(s) with PBS containing the inhibitor(s). Once inhibitor(s) were introduced into an experiment, all subsequent solutions involved in the procedure contained that inhibitor or combination of inhibitors.

SDS-Polyacrylamide Slab Gel Electrophoresis

Electrophoresis in the presence of the detergent, sodium dodecyl sulfate, followed that described by Laemmli (1970). Lyophilized samples or immunoprecipitation pellets were dissolved in 0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromphenol blue as a tracking dye. Samples electrophoresed under non-reducing conditions were dissolved in the above sample buffer in the absence of 2-mercaptoethanol. Prior to electrophoresis samples were heated at 100^{o}C for 2-3 minutes. Slab gel electrophoresis was carried out with a current of 3 mA until the bromphenol blue dye marker penetrated the running gel, then increased to 15 mA until the bromphenol blue reached the bottom of the running gel.

Fluorography and Autoradiography

Radiolabeled proteins were visualized on polyacrylamide slab gels

by fluorography as described by Bonner and Laskey (1974). The volume (in cm^3) of the slab gel was calculated. The slab gel was soaked in 20 volumes of dimethyl sulfoxide (DMSO) for 30 minutes. This step was repeated with fresh DMSO. The slab gel was then soaked in 4 volumes of a 22.2% solution of 2,5 diphenyloxazole/DMSO for 3 hours. The volume of this solution was critical since it determines the final % of 2,5 diphenyloxazole in the slab gel.

The slab gel was soaked in 20 volumes of distilled water for 1 hour, and then soaked overnight in a 1% aqueous solution of glycerol before drying. Drying of the slab gel was performed at room temperature. The dried slab gel was placed on flashed Kodak Royal X-Omat x-ray film (Laskey and Mills, 1975) and incubated at $-70^{o}C$ for an appropriate exposure time. The fluorogram was developed using Kodak x-ray developer and fixer.

In preparation for autoradiography, slab gels were fixed in a solution of 25% isopropanol/10% acetic acid/water for 1 hour. The slab gel was stained with a solution of 25% isopropanol/10% acetic acid/0.1% Coomassie Brillant Blue/water for 1-2 hours and destained with 10% acetic acid. Before drying the slab gel was soaked overnight in a 10% methanol/1% glycerol/water solution. The slab gel was dried at room temperature. For autoradiography, the slab gel was placed on flashed x-ray film, incubated and developed as described for fluorography.

CNBr Cleavage of Immunoglobulin Heavy Chains

The CNBr reaction and cleavage procedure has been described pre-

viously (Gross, 1967; Cahnmann et al., 1966). Individually labeled mouse membrane and mouse and human secreted heavy chain peaks from gel filtration chromatography were lyophilized and resuspended in 1.0 ml 70% formic acid in a glass stoppered test tube. Crystalline CNBr was added to each sample for a final concentration of approximately 0.15 M. The samples were incubated in the dark at room temperature for a minimum of 24 hours. The cleaved heavy chains were then diluted 3 fold with distilled water and lyophilized. The samples were resuspended in electrophoresis sample buffer in preparation for gel electrophoresis.

Enzymatic Cleavages of Membrane and Secreted H Chains

Procedures for peptide mapping on polyacrylamide gels have been reported (Cleveland et al., 1977). Individual labeled mouse membrane, secreted, and human secreted heavy chain peaks from gel filtration chromatography were lyophilized and resuspended in 100 µl of electrophoresis sample buffer containing 0.125 M Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol and 0.0001% bromphenol blue. For chymotrypsin cleavage of heavy chains, a 1 mg/ml solution of the enzyme in the above sample buffer was also prepared. Cleavage with chymotrypsin was performed at 37°C for 1 hour using an enzyme concentration of 100 µg/ml. After 1 hour the cleavage reaction was stopped by making each sample 10% in 2-mercaptoethanol, 2% in SDS and boiling for 2-3 minutes. The samples were ready for polyacrylamide slab gel electrophoresis.

Pepsin and papain cleavages were performed on intact mouse membrane Igs released from Staphylococcal Protein A by treatment with electrophoresis sample buffer as described for chymotrypsin cleavage. Pepsin

and papain solutions of 1 mg/ml were also prepared in the above sample buffer. Samples to be cleaved with pepsin were loaded into the sample wells of a polyacrylamide slab gel, enzyme was added for a final concentration of 100 µg/ml and the samples were electrophoresed. Papain cleavage was performed in a similar manner, however enzyme was added to a final concentration of 50 µg/ml.

CHAPTER III

ISOPYCNIC DENSITY GRADIENT CENTRIFUGATION EXPERIMENTS

Introduction

Isopycnic density gradient centrifugation in non-denaturing solutions has been used previously to investigate the properties of proteins (Cox and Schumaker, 1961), to investigate the de novo biosynthesis of enzymes (Filner and Varner, 1967) and to estimate the carbohydrate content of glycoproteins (Melcher and Uhr, 1977). Isopycnic density gradient centrifugation under denaturing conditions has been used in the separation of proteins and nucleic acids from chromatin (Sonnenbichler et al., 1977) and preparatively to isolate glycoproteins (Robinson and Monsey, 1971). It has not, however, been used as an analytical tool, despite the use of buoyant density in estimating changes in the extent of glycosylation of proteins.

The studies described here utilize isopycnic density gradient centrifugation performed in the presence of a strong denaturant, guanidine-HCl. The buoyant densities of labeled immunoglobulin membrane and secreted heavy chains have been compared in order to answer several questions concerning membrane immunoglobulins. First, are there differences in the carbohydrate contents of membrane and secreted immunoglobulin heavy chains?

The mobility of mouse membrane IgM H chain is slightly slower than that of secreted IgM H chain on sodium dodecyl sulfate containing polyacrylamide gels (Melcher and Uhr, 1976). A 1700 molecular weight difference has been estimated between mouse membrane and secreted IgM H chains. The observed molecular weight difference could be due to either primary amino acid sequence differences or carbohydrate differences (Ramasamy, 1976). The extra size of the membrane H chain has been implicated in membrane attachment.

Second, what is the carbohydrate content of mouse membrane δ chain? The carbohydrate content of mouse membrane δ has not been estimated although the molecule must have appreciable amounts of sugar (Melcher and Uhr, 1977).

The density relationship of membrane and secreted immunoglobulin heavy chains in the absence of detergent is a third consideration of these experiments. In non-denaturing, detergent containing gradients partially reduced secreted immunoglobulin was more dense than its membrane bound counterpart (Melcher and Uhr, 1977). The decreased density of membrane immunoglobulin was attributed to its ability to bind detergent strongly, thus lowering its density. Membrane bound immunoglobulins are integral membrane proteins (Melcher et al., 1975) and detergent binding is a property of integral membrane proteins (Helenius and Simons, 1975). The buoyant density comparisons described here have been performed in the absence of detergent. Although nonionic detergent was used in the preparation of cellular lysates, repeated extensive dialysis and gel filtration purification of the heavy chains is thought to have removed any residual detergent.

Results

A typical Sephadex G-100 gel filtration column profile for the separation of labeled heavy chains from light chains is seen in Figure 2. In order to determine the resolution of the gel filtration step, selected fractions from the G-100 profile were lyophilized and electrophoresed on a 7.5% SDS-polyacrylamide slab gel. The labeled proteins on the gel were visualized by fluorography. As seen in Figure 3, the gel filtration separation of the reduced and alkylated labeled immunoglobulins yielded adequate separation of the heavy and light chains. Reduced and alkylated mouse membrane IgM is shown. Fractions taken from region A of the G-100 profile yielded a single heavy chain band on the slab gel. An intermediate region (B) was observed before the light chain containing region (C) of the profile appeared. Only those fractions located in the heavy chain peak region (A) were used in the heavy chain buoyant density comparisons.

The H chains to be compared were centrifuged to equilibrium in the same tube containing 6.0 M guanidine-HCl, 0.2 M Tris-HCl and CsCl at pH 8. The use of two isotopes was advantageous in the comparison of peaks in the gradient. ^{131}I and ^{125}I labeled H chains in the same centrifuge tube could be counted directly after fractionation of the gradient via gamma scintillation counting. Initial experiments were done to determine if any isotope effects were present when using the double labeling technique. Mouse membrane μ chains were labeled in separate reactions with ^{131}I and ^{125}I, combined in the same tube, centrifuged to equilibrium in a CsCl gradient under denaturing conditions, fractionated and counted. As seen in Figure 4, the membrane μ

Figure 2. Sephadex G-100 Column Profile of Reduced and Alkylated ^{125}I Mouse Membrane IgM

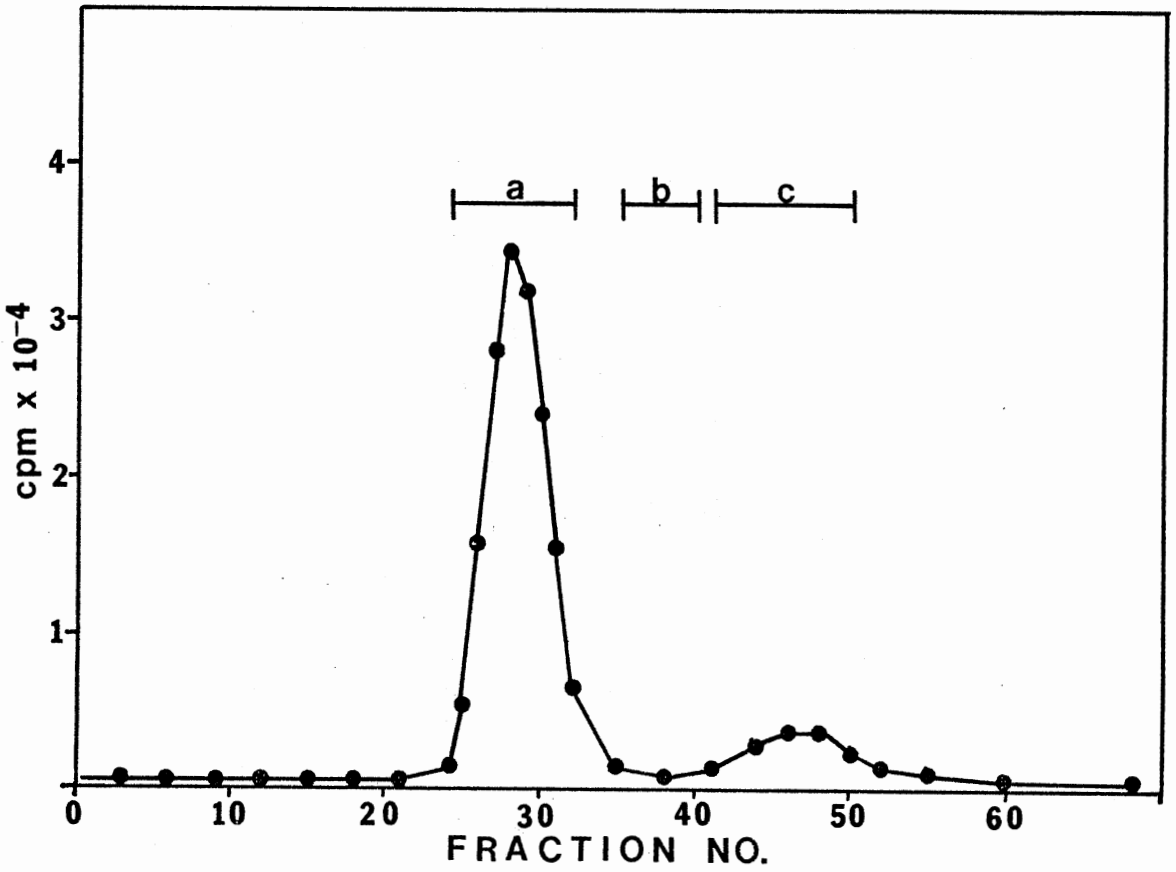

a
b
c
cpm x 10⁻⁴
4
3
2
1
0
10
20
30
40
50
60
FRACTION NO.

Figure 3. ^{125}I Mouse Membrane μ and Light Chain Separated on a 7.5% SDS-polyacrylamide Slab Gel. a, b and c represent fractions from Sephadex G-100 column purification step applied to the slab gel.

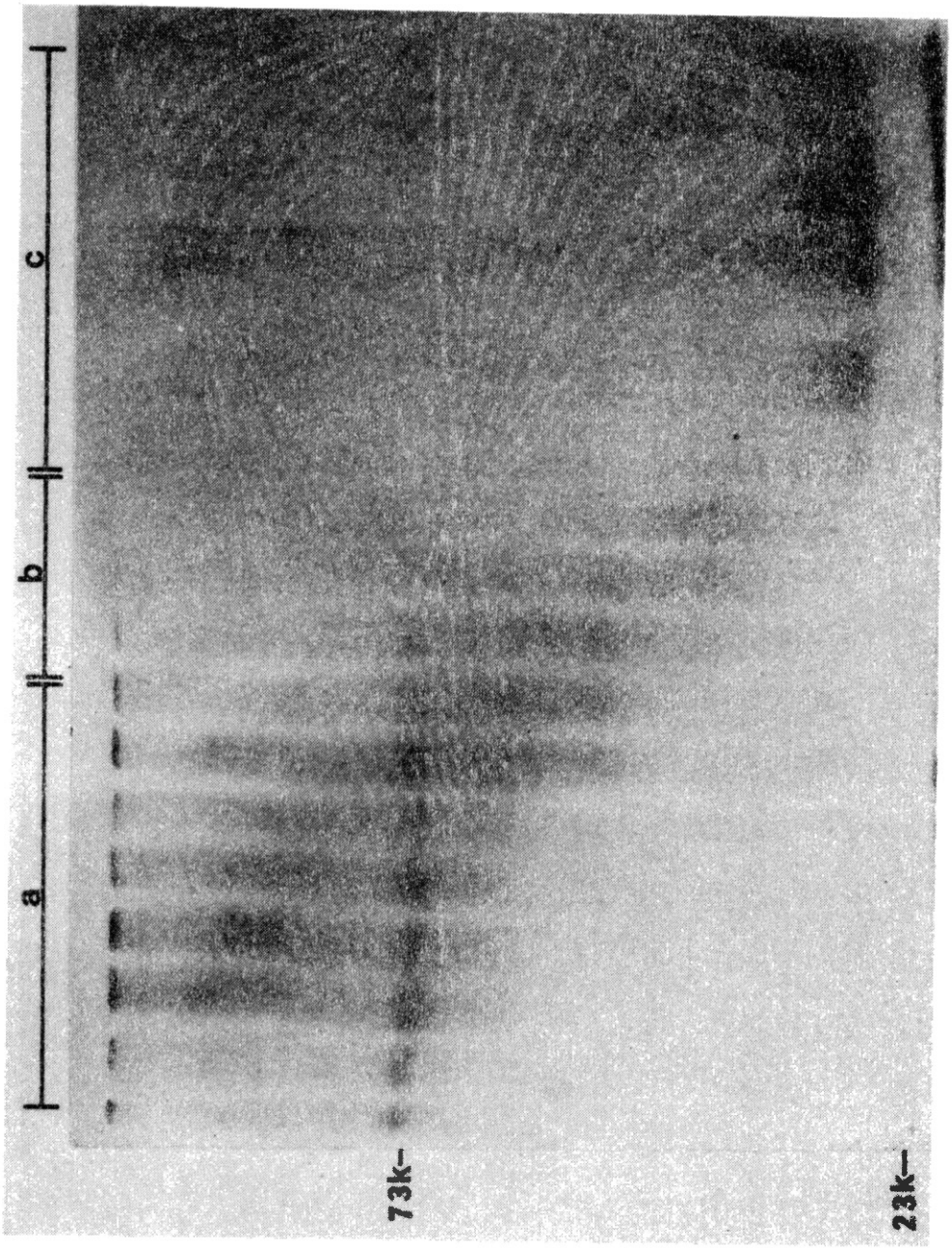
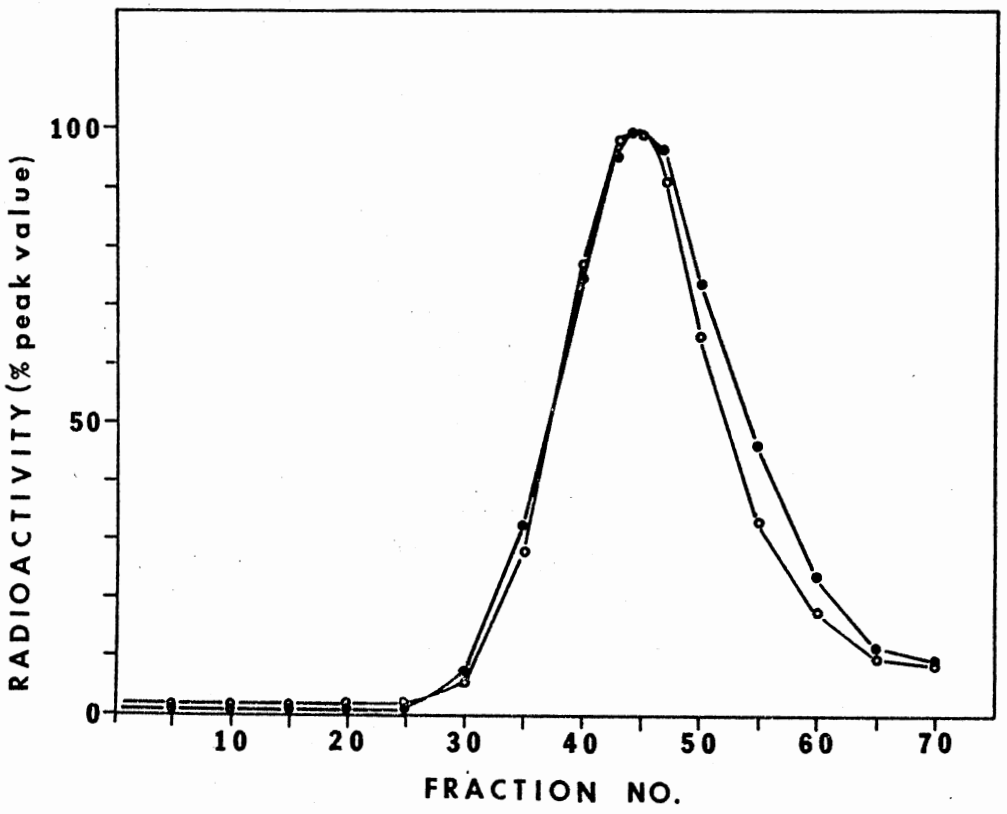

a
b
c
73k—
23k—

Figure 4. Comparison of Densities of ^{125}I Labeled Mouse Membrane μ Chain (Closed Circles) and ^{131}I Labeled Mouse Membrane μ Chain (Open Circles) in CsCl Guanidine-HCl


RADIOACTIVITY (% peak value)
100
50
0
10
20
30
40
50
60
70
FRACTION NO.

chains, although labeled with different isotopes of iodine, have the same positions on the gradient. Interchange of ^{131}I or ^{125}I labels does not affect the equilibrium density of the protein in the gradient.

The buoyant density of a hydrodynamic particle is a function of the buoyant densities of its component parts (Ifft and Vinograd, 1966). For Ig, these parts include the polypeptide chains, the oligosaccharide chains, and the water and ions bound to these components. In order to make useful comparisons among immunoglobulins it is necessary that the buoyant density of the polypeptide (with its associated water and ions) not be significantly different from one immunoglobulin to the other (Melcher and Uhr, 1977). To determine if the method used in these experiments is sensitive to changes in H chain carbohydrate content, mouse membrane IgM and IgD H chains have been compared with secreted myeloma MOPC 104E IgM, MOPC 315 IgA and MOPC 21 IgG H chains. The carbohydrate contents of these myeloma proteins have been estimated.

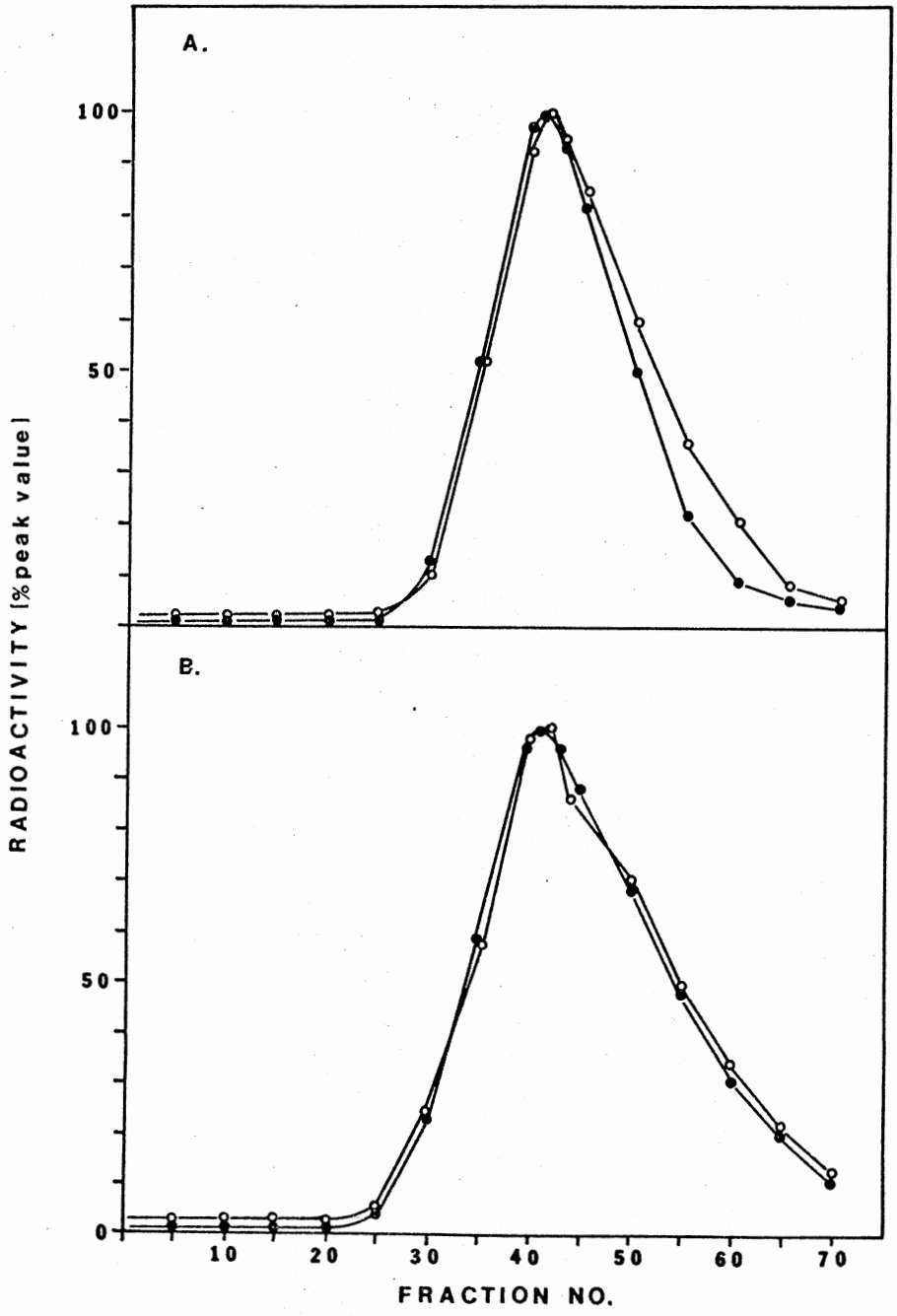
The H chain carbohydrate content of MOPC 104E IgM is 13.3% (Robinson et al., 1973). Based on the hexose content of several mouse myeloma IgA proteins and the total carbohydrate content of human IgA (Underdown et al., 1971, Jaffe et al., 1971), the α chain carbohydrate content can be estimated to be 10.5%. The carbohydrate content of γ chain has been calculated to be about 4% based upon IgG sugar content of 3% (Niedermeyer et al., 1971).

In the first determination, ^{125}I membrane μ was compared with ^{131}I MOPC 104E secreted μ. As seen in Figure 5A, the densities of membrane μ chain and secreted MOPC 104E μ chain are indistinguishable. As a control, the isotopes were reversed (i.e. ^{131}I membrane μ versus

Figure 5. Comparison of Densities of Mouse Membrane and MOPC 104E μ Chains in CsCl Guanidine-HCl

A. ^{125}I membrane μ chain (closed circles) versus ^{131}I MOPC 104E secreted μ chain (open circles).

B. ^{131}I membrane μ chain (open circles) versus ^{125}I MOPC 104E secreted μ chain (closed circles).


A.
B.
100
50
0
RADIOACTIVITY (% peak value)
10
20
30
40
50
60
70
FRACTION NO.

^{125}I MOPC 104E μ chain), and the same result was observed (Figure 5B).

In order to determine if myeloma secreted μ chain is a valid analogue of normal secreted μ chain, the densities of these two H chains were compared. ^{3}H-labeled μ chain secreted by normal spleen cells and ^{131}I MOPC 104E myeloma μ chain were centrifuged to equilibrium in separate tubes. Included in each gradient tube was [^{14}C]-lactoperoxidase, as reference protein. After centrifugation and fractionation, the [^{14}C]-lactoperoxidase profiles of each gradient were superimposed and the density profiles of the H chains were plotted together (Figure 6). The densities of normal versus myeloma secreted μ chains appear to be quite similar and thus MOPC 104E secreted μ chain seems to be a valid analogue of the normal secreted chain with respect to carbohydrate content. Some error is inherent in the use of marker alignment for peak comparisons. In addition there may be a different effect on the buoyant density of ^{3}H substitution and ^{125}I or ^{131}I substitution. The specific activity (atom ^{3}H/labeled molecule) of the secreted H chains could not be determined; thus whether a density difference was expected cannot be predicted.

Next, ^{131}I membrane μ chain and ^{125}I membrane δ chain were compared. Membrane δ chain is more dense than membrane μ chain with a two fraction difference in the observed peak positions (Figure 7). A gradient density distribution is also shown in Figure 7. Since membrane μ and MOPC 104E secreted μ chains have equal densities (Figure 5), membrane δ should be more dense than MOPC 104E secreted μ chain. This result is confirmed in Figure 8, in which ^{125}I membrane δ chain is compared with ^{131}I MOPC 104E secreted μ chain. A two fraction difference is observed, with the membrane δ chain being more dense.

Figure 6. Comparison of Densities of ^{125}I MOPC 104E μ Chain (Closed Circles) and ^{3}H Secreted μ Chain (Open Circles) in Separate Tubes in CsCl Guanidine-HCl. The arrow represents the point of alignment of the two gradients using [^{14}C]-lactoperoxidase as marker.

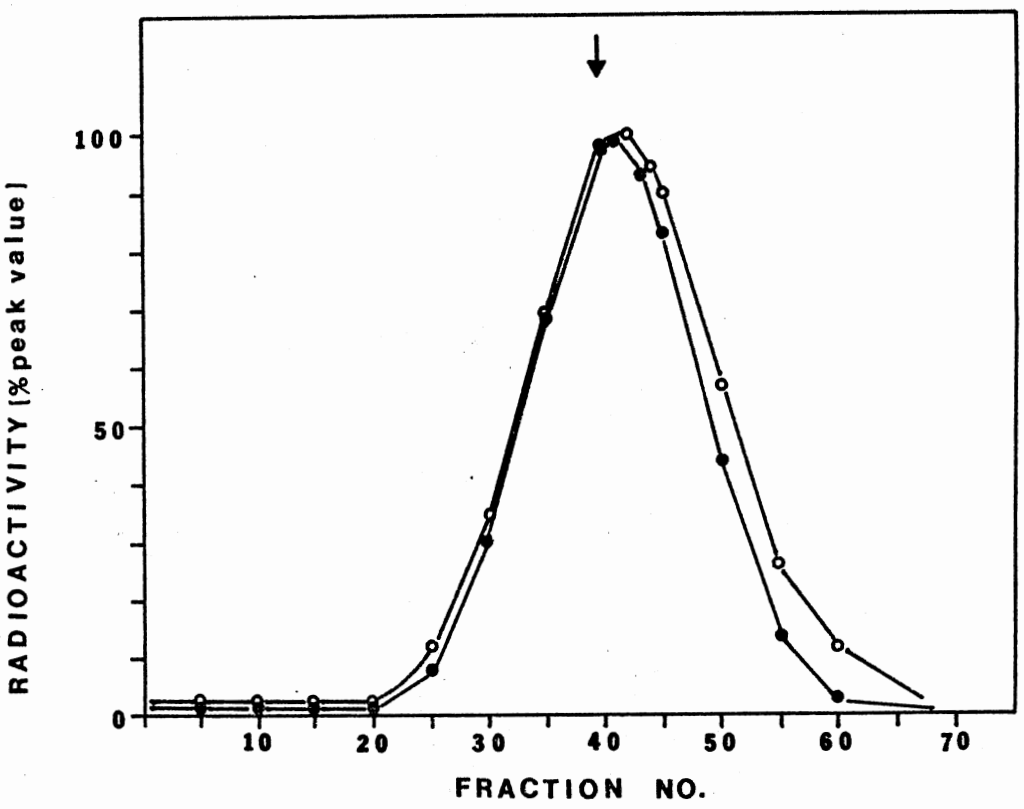

RADIOACTIVITY (% peak value)
100
50
0
10
20
30
40
50
60
70
FRACTION NO.

Figure 7. Comparison of Densities of ^{125}I Membrane μ Chain (Closed Circles) and ^{131}I Mouse Membrane δ Chain (Open Circles) in CsCl Guanidine-HCl

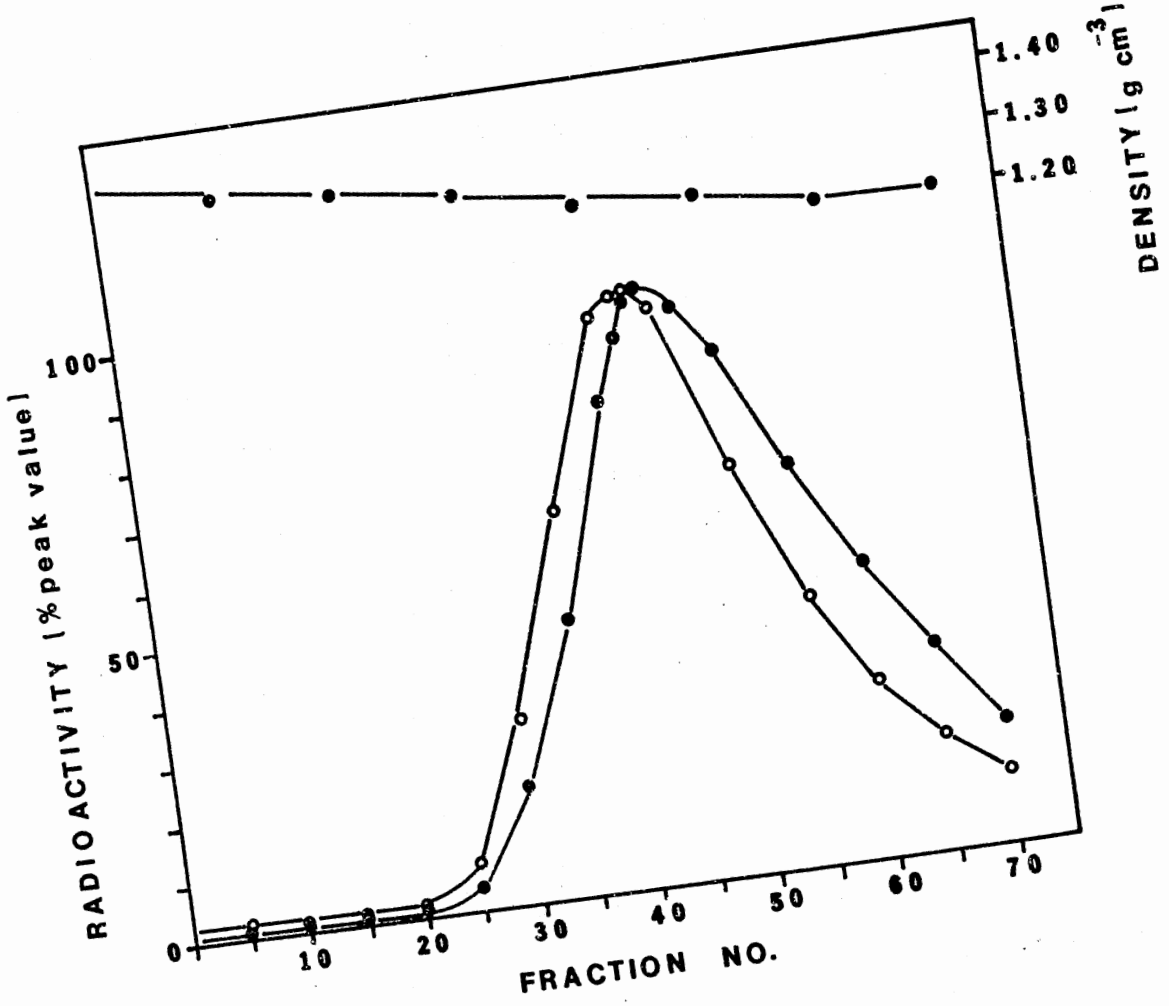
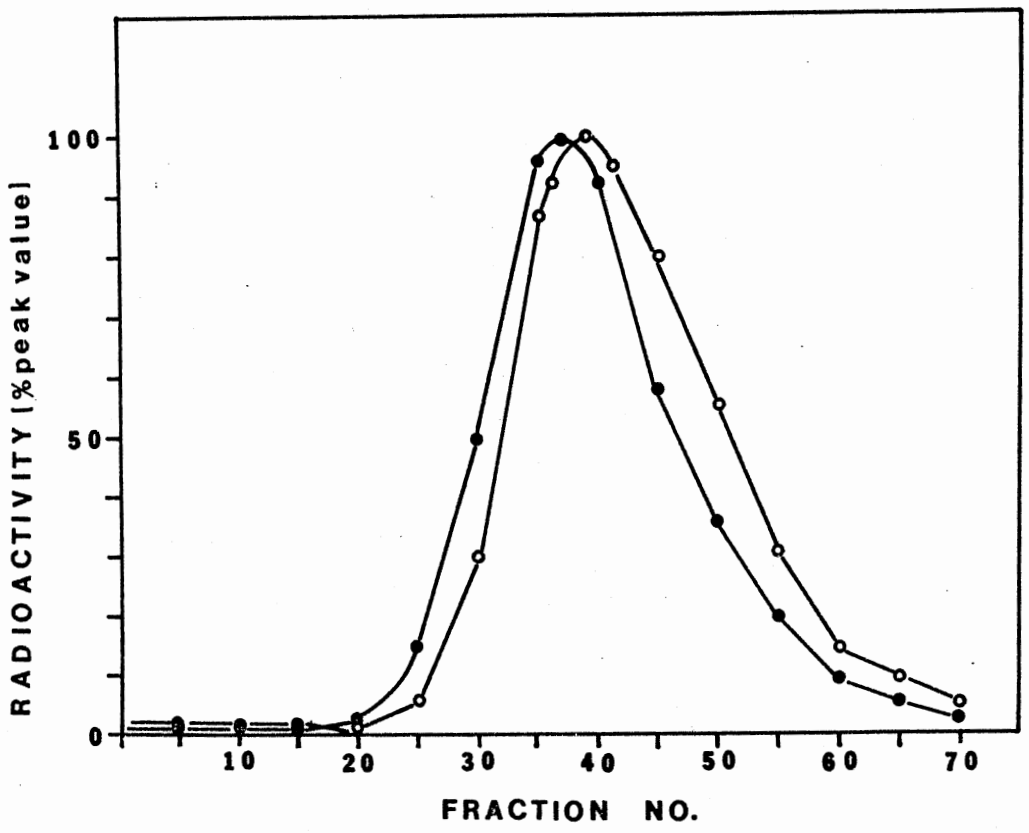

1.40
1.30
1.20
DENSITY (g cm⁻³)
100
50
0
RADIOACTIVITY (% peak value)
10
20
30
40
50
60
70
FRACTION NO.

Figure 8. Comparison of Densities of ^{125}I Membrane δ Chain (Closed Circles) and ^{131}I MOPC 104E μ Chain (Open Circles) in CsCl Guanidine-HCl.


RADIOACTIVITY (%peak value)
100
50
0
10
20
30
40
50
60
70
FRACTION NO.

The results of a comparison of ^{125}I mouse membrane δ chain and ^{131}I human myeloma δ chain are seen in Figure 9. The membrane δ chain of mouse and human δ chain peaks appear to be coincident. The carbohydrate content of this particular human δ chain is not known. A variation in the carbohydrate content of human IgDs has been reported (Spiegelberg, 1972; Perry and Milstein, 1970).

The sensitivity of the method in detecting carbohydrate content differences among H chains was most readily seen in the comparisons of membrane proteins against MOPC 315 α and MOPC 21 γ chains. In the comparison of ^{131}I membrane μ chain with ^{125}I MOPC 315 α chain, the greater density of membrane μ is seen as a two fraction difference over the secreted α chain (Figure 10A). With an estimate of membrane μ chain carbohydrate (13.3%) and the knowledge of secreted α chain sugar content (10.5%), the two fraction difference reflects a carbohydrate content difference of approximately 3%. As expected the comparison of ^{131}I membrane δ chain with ^{125}I MOPC 315 α chain yielded a four fraction difference, with membrane δ being the denser of the two immunoglobulin heavy chains (Figure 10B).

Pronounced density differences were observed in the comparisons of membrane μ and δ chains with MOPC 21 γ chain. ^{131}I membrane μ chain was more dense than ^{125}I MOPC 21 γ chain by three to four fractions (Figure 11A). A seven fraction difference was observed in the ^{131}I membrane δ chain versus ^{125}I MOPC 21 γ chain comparison (Figure 11B). Considerable trailing of radioactivity on the less dense side of the γ chain peaks occurred. The trailing was not investigated. Trailing could be caused by (1) breakdown of iodinated γ chains, (2) dilution of γ chains by IgG antibodies used in the isolation of membrane μ and δ

Figure 9. Comparison of Densities of ^{125}I Mouse Membrane δ Chain (Closed Circles) and ^{131}I Human Myeloma Secreted δ Chain (Open Circles) in CsCl Guanidine-HCl

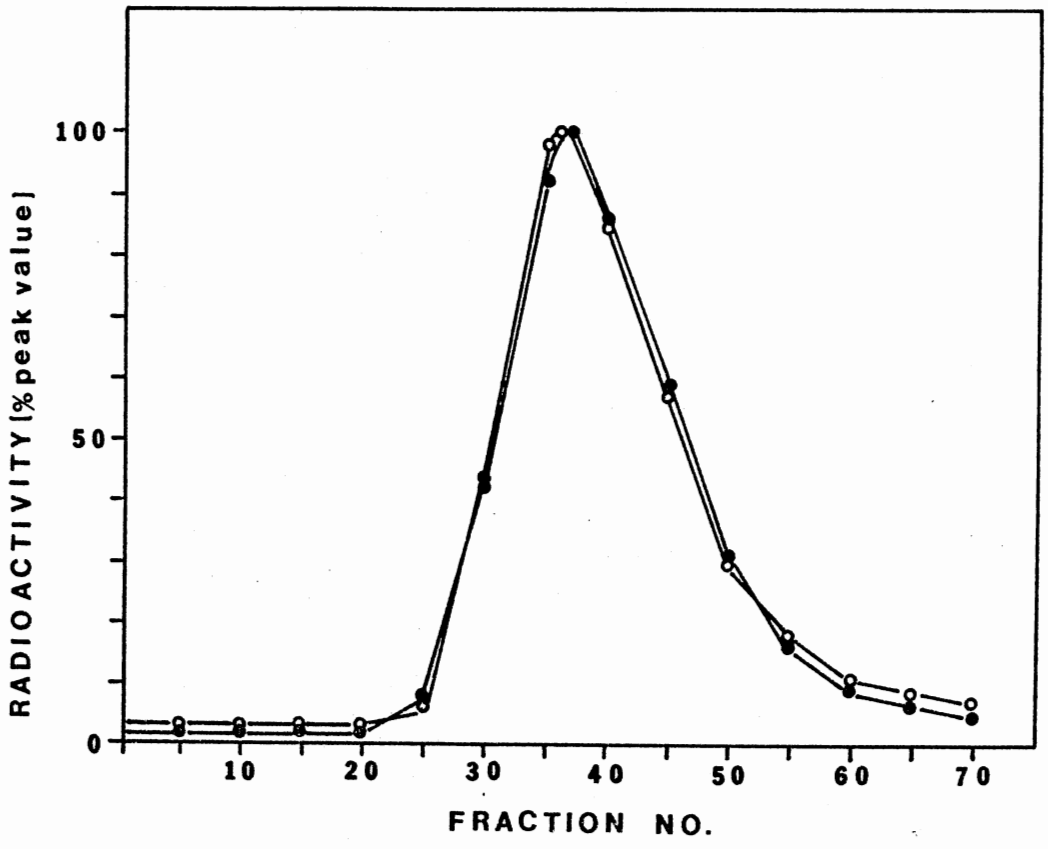

RADIOACTIVITY (% peak value)
100
50
0
10
20
30
40
50
60
70
FRACTION NO.

Figure 10. Comparison of Densities of Mouse Membrane H Chains with MOPC 315 α Chain in CsCl Guanidine-HCl

A. ^{125}I MOPC 315 α chain (closed circles) versus ^{131}I mouse membrane μ chain (open circles).

B. ^{125}I MOPC 315 α chain (closed circles) versus ^{131}I mouse membrane δ chain (open circles).

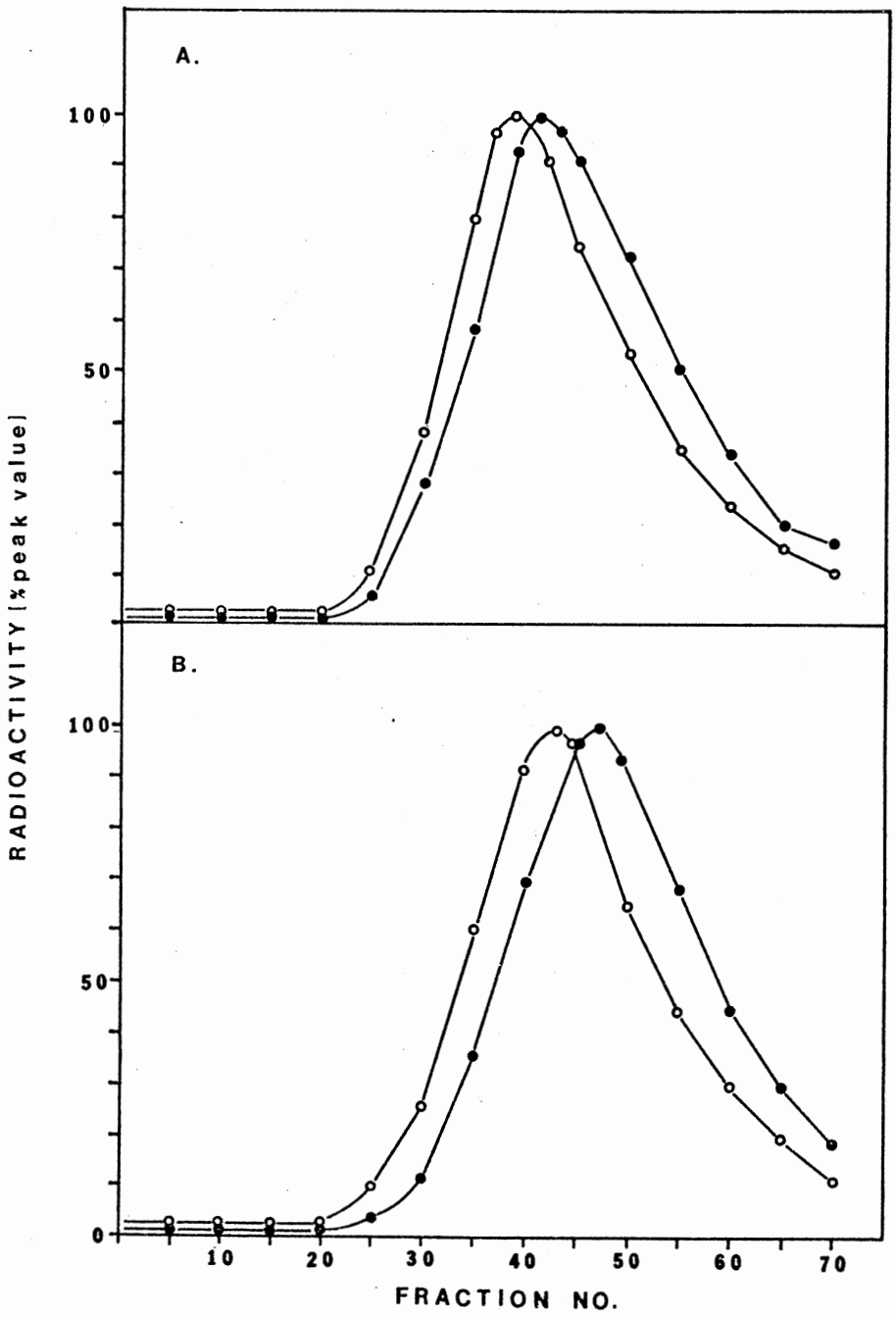
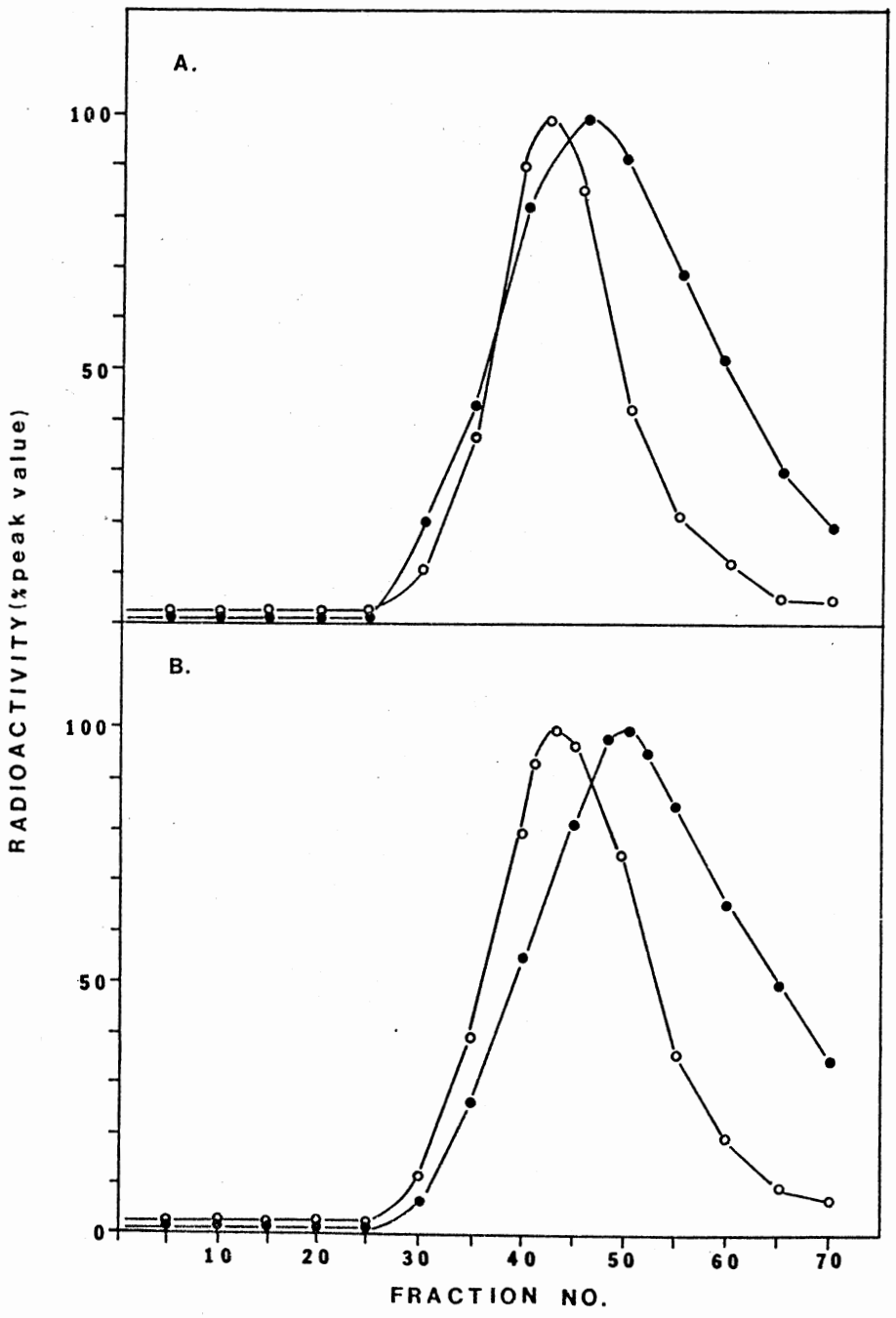

A.
B.
100
50
0
RADIOACTIVITY (%peak value)
10
20
30
40
50
60
70
FRACTION NO.

Figure 11. Comparison of Densities of Mouse Membrane H Chains with MOPC 21 γ Chain in CsCl Guanidine-HCl

A. $^{125}_{131}$I MOPC 21 γ chain (closed circles) versus ^{131}I mouse membrane μ chain (open circles).

B. $^{125}_{131}$I MOPC 21 γ chain (closed circles) versus ^{131}I mouse membrane δ chain (open circles).


A.
B.
100
50
0
RADIOACTIVITY(%peak value)
10
20
30
40
50
60
70
FRACTION NO.

chains, (3) deterioration of resolution near the top of the gradient. The latter explanation is favored since the density distribution curve (Figure 7), as expected (Ifft and Vinograd, 1966), tends to flatten out near the top of the gradient.

The percentage carbohydrate content, the estimated densities and peak position differences expressed in number of fractions for the H chain comparisons are summarized in Table II. It should be pointed out that the precision of determining densities is considerably less than the precision obtained by comparison of two proteins in the same gradient. The difference in observed densities of the H chain preparations are in the order predicted from the knowledge of their carbohydrate contents. The H chain preparations are seen to increase in density in the order γ, α, secreted μ equal to membrane μ, membrane δ equal to human δ.

The buoyant densities of immunoglobulins have been determined in phosphate-buffered CsCl gradients (Melcher and Uhr, 1977; Lifter and Choi, 1978). An apparent decrease in the H chain densities relative to Ig densities in non-denaturing gradients was observed in these experiments. The decrease in observed protein density was presumed to be due to binding of the guanidine-HCl to the protein. The increases in partial specific volume upon denaturation of proteins with guanidine-HCl, due to binding of guanidinium ion to peptide bonds and aromatic side chains (Lee and Timasheff, 1974) are sufficient to account for the decreased densities observed.

When the reciprocal of the peak densities determined from the CsCl-guanidine-HCl gradients are plotted versus the carbohydrate content of the respective heavy chains (Figure 12), a linear relationship is found.

TABLE II

HEAVY CHAIN DENSITY COMPARISONS

	% Carbohydrate	Density[a]	Peak Fractions Difference[b]
A. Compared with membrane μ	13.3	1.270	
MOPC 21 γ	4.0	1.260	3-4
MOPC 315 α	10.5	1.268	2
MOPC 104E μ	13.3	1.270	0
Mouse Membrane δ	15.5[c]	1.272	2
B. Compared with membrane δ	15.5[c]	1.272	
MOPC 21 γ	4.0	1.260	7
MOPC 315 α	10.5	1.268	4
Human δ Delio	15.5[c]	1.272	0

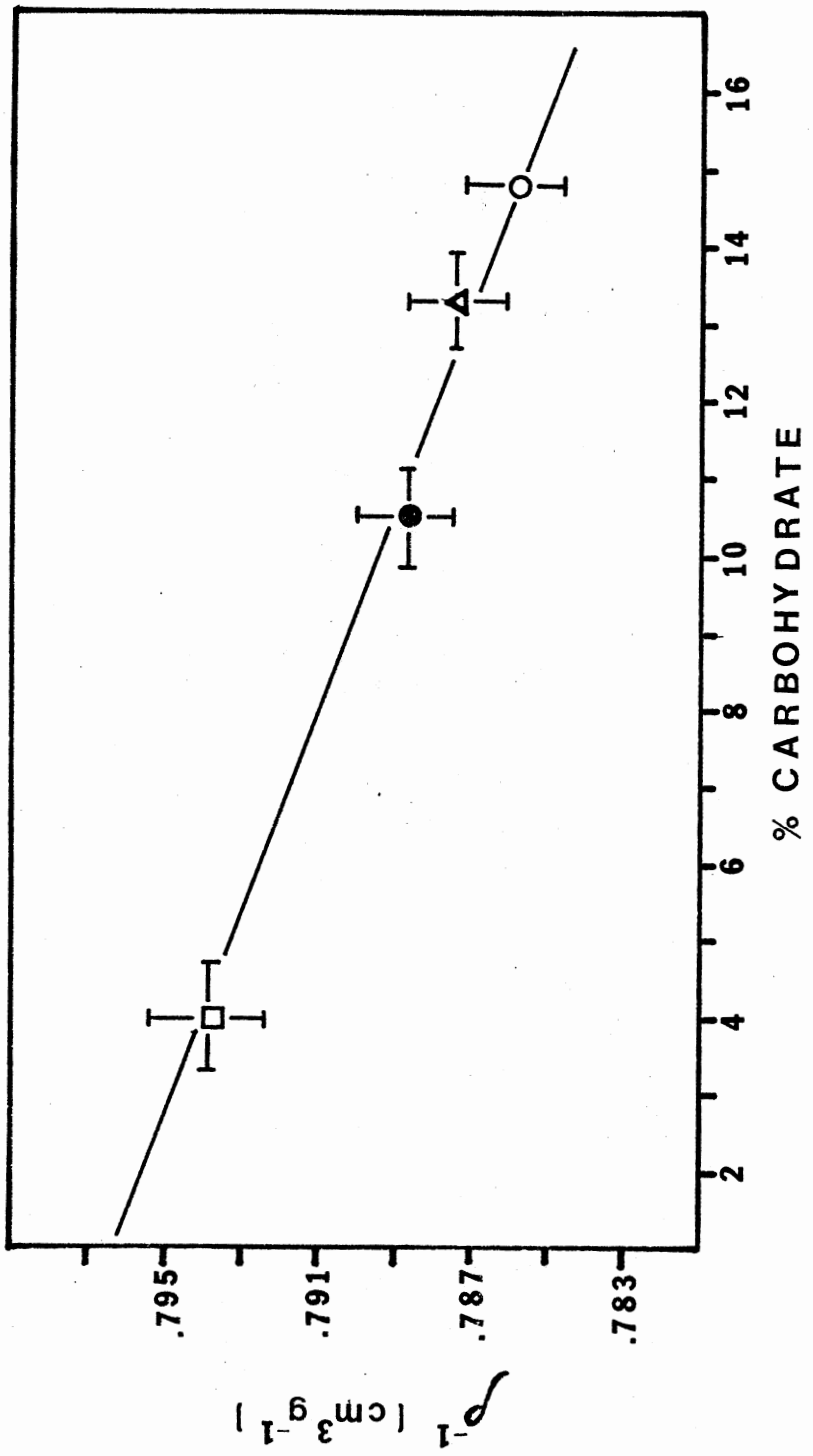
[a] Gram/cm^{-3}, MOPC 21 γ density has an uncertainty of ± 0.002/gram cm^{-3}, density estimations of remaining H chains have uncertainties of ± 0.001 gram cm^{-3}.

[b] Approximate peak fractions difference from peak positions determined by computer fitting gradient data to a Gaussian distribution.

[c] Estimated from Figure 12.

Figure 12. Plot of H Chain Carbohydrate Contents Versus Reciprocal Densities

Densities determined by equilibrium gradient centrifugation in CsCl guanidine-HCl. H chains of IgG (open square), IgA (closed circle), secreted IgM (open triangle) and mouse membrane IgD (open circle) are plotted. Horizontal error bars reflect the uncertainty in assignment of values of carbohydrate content. Vertical error bars reflect error in density measurements.


.795
.791
.787
.783
2
4
6
8
10
12
14
16
% CARBOHYDRATE
ρ⁻¹ [cm³ g⁻¹]

The vertical error bars reflect the error in density measurements; the horizontal error bars reflect the uncertainty in the assignment of values for the carbohydrate content of the respective heavy chains. This relation can be used to estimate the carbohydrate content of H chains for which this is not known. From Figure 12 the carbohydrate content of murine membrane δ chain was estimated as 15.5%, that of human δ chain Delio as 15.5%, and that of murine membrane μ chain as 13.3%.

CHAPTER IV

PROTEOLYSIS OF MOUSE MEMBRANE IgD

Introduction

The actual size of mouse membrane δ chain remains, for the most part, unclear. Mouse membrane δ chain as normally isolated has a molecular weight of 65,800, as determined by SDS-polyacrylamide gel electrophoresis, compared to 73,000 determined for mouse membrane μ chain (Melcher and Uhr, 1976). Sitia et al. (1977) have reported a mouse membrane δ chain with a mobility similar to that of human membrane δ chain of molecular weight 73,000 on SDS-polyacrylamide gels. The possibility that the normally isolated mouse δ chain is a proteolytic fragment of a larger 'native' membrane δ chain has been postulated by this same group. The proteolytic fragmentation of human IgD has been well documented (Fahey et al., 1968; Spiegelberg, 1972; Goyert et al., 1977). Membrane μ and δ chains of several other mammalian species have comparable mobilities on SDS-polyacrylamide gels (Ruddick and Leslie, 1977; Finkelman et al., 1976). In contrast, Warr and Marchalonis (1976) have reported that the mobility of mouse membrane δ chain is significantly faster than that of human membrane δ chain on SDS-polyacrylamide gels.

My approach to this problem, that is investigating the possibility that mouse membrane δ chain is larger in size than normally isolated,

has been the incorporation of various proteolytic inhibitors into the preparation and isolation procedures of mouse membrane Igs. The mobilities of the mouse membrane μ and δ chains were then compared by electrophoresis on SDS-polyacrylamide gels.

Results

Prior to studying the degradation of mouse membrane IgD, the effect of the various proteolytic inhibitors on the lactoperoxidase labeling reaction was determined. Individual samples containing bovine serum albumin (BSA) were iodinated via the lactoperoxidase method as previously described for the myeloma proteins in methods. The iodination was performed in the presence of proteolysis inhibitors (or combinations thereof) used in the subsequent experiments. The iodinated BSA samples were precipitated with 10% trichloroacetic acid, collected on glass fiber filters, washed, dried and counted for ^{125}I radioactivity. Recovered radioactivities of BSA samples iodinated in the presence of proteolytic inhibitor(s) were equal to the radioactivity of a BSA sample iodinated in the absence of proteolytic inhibitor(s) (data not shown). It was concluded that the proteolytic inhibitor(s) used in these experiments had no effect on the labeling reaction.

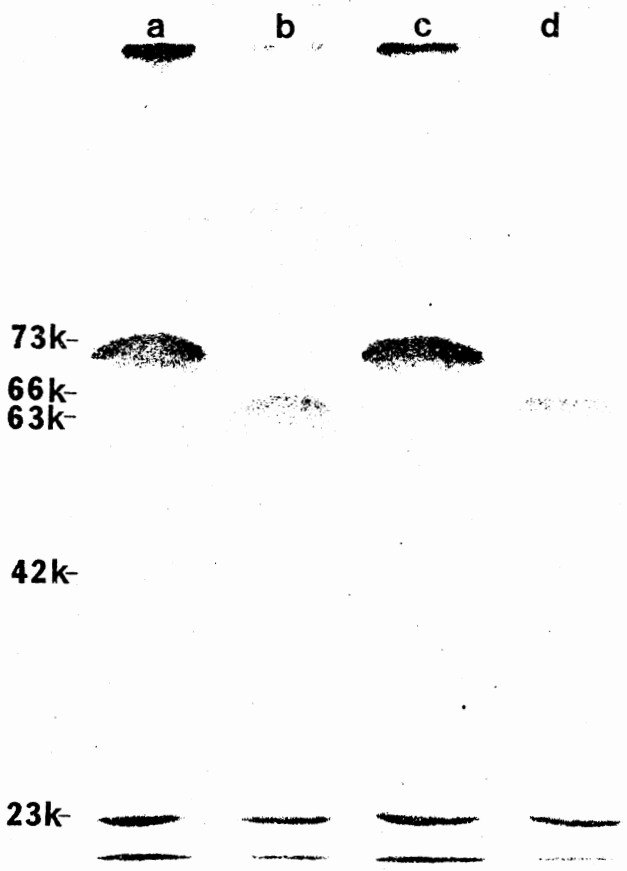
Sulfonyl halides, e.g. phenyl methyl sulfonyl fluoride (PMSF), react with many of the serine proteases such as α-chymotrypsin and trypsin. These compounds sulfonate the hydroxyl group of a specific serine residue in the active site and thereby inhibit enzymatic activity. PMSF has been shown to be an effective inhibitor of serine proteases in the concentration range of 0.1 to 1.0 mM (Fahrney and Gold, 1963). In the initial experiment 0.1 mM PMSF was included in the pre-

paration of ^{125}I mouse membrane μ and δ chains. The mobilities of mouse membrane μ and δ chains were compared, in the presence and absence of PMSF, on a 7.5% SDS-polyacrylamide slab gel (see Figure 13). The presence of PMSF (lanes a and b) did not affect the mobility of the mouse membrane δ chain. The membrane H chains prepared in the absence of PMSF are shown in lanes c and d. The membrane δ chain moved faster than the membrane μ chain and had an apparent molecular weight of approximately 66,000 as compared to 73,000 for mouse membrane μ chain. Secondary bands of approximately 70,000 and 63,000 were observed in the μ and δ lanes, respectively, in the presence and absence of PMSF. These bands were thought to be degradation products of the higher molecular weight species. In addition to the major heavy chain bands, a band of 40-45,000 molecular weight and the 23,000 molecular weight L chain were observed. The 40-45,000 molecular weight band has been tentatively identified as lymphocyte actin. Actin has been shown to be a major component of mouse spleen cells and also appears as a nonspecific contaminant of immune precipitates (Barber and Delovitch, 1979). Although the actin is a cytoplasmic component and the cells have been surface iodinated, the appearance of labeled actin can be explained by the iodination of cytoplasmic contents of ruptured cells. This experiment was repeated with an increased concentration of PMSF (1.0 mM) and identical results were obtained (data not shown).

Next, 0.1 mM EGTA was incorporated into the preparation and isolation of the mouse membrane H chains. EGTA, a chelating agent, was added to bind divalent metal cations since these are required for some enzymatic activities (Webster and Prado, 1970). As in the PMSF experiment, the relative mobilities of the membrane μ and δ chains remained

Figure 13. SDS-PAGE Analysis of Mouse Membrane H Chains Prepared in the Presence of 0.1 mM PMSF

a. Mouse membrane μ chain + PMSF
b. Mouse membrane δ chain + PMSF
c. Mouse membrane μ chain - PMSF
d. Mouse membrane δ chain - PMSF


a
b
c
d
73k-
66k-
63k-
42k-
23k-

unchanged (data not shown).

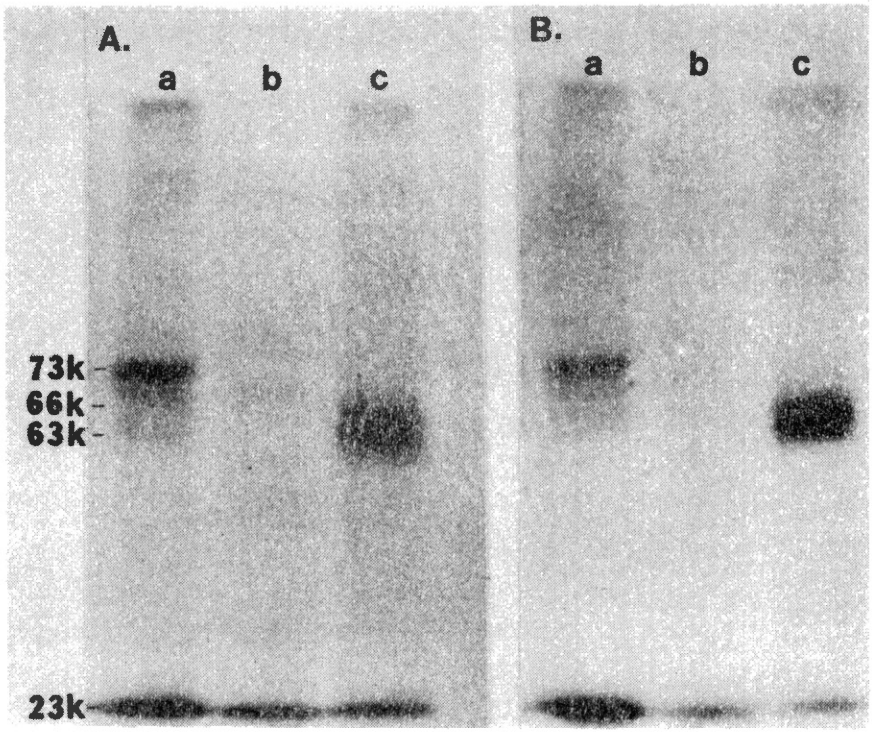
Aprotinin is a naturally occurring inhibitor isolated from bovine organs of serine proteases. The inhibitor is a peptide of 58 amino acid and structural analysis suggests that it is ideally shaped to cover the active site of serine proteases (Kassell, 1970). The addition of aprotinin to reagents used during the preparation and isolation procedures did not change the relative mobilities of the membrane μ and δ chains (data not shown). The concentration of aprotinin used was 100 kallikrein inhibitor units/ml. By definition, 900 kallikrein inhibitor units (KIU) are equal to one trypsin inhibitor unit (TIU), where one trypsin inhibitor unit will decrease the activity of two trypsin units by 50%.

The aprotinin experiment was repeated using an increased concentration of inhibitor. Panero et al. (1978) have reported that aprotinin, at high concentrations, increased the percentage of human neonatal IgD-positive lymphocytes in the presence of maternal serum. The concentration of aprotinin was increased to 1000 KIU/ml in PBS for the initial preparation of mouse spleen cells then further increased to 3000 KIU/ml for the preparation of the detergent lysate and the immunoprecipitation steps. The mobilities of the membrane μ and δ chains are compared, in the absence (A) and presence (B) of the increased concentration of aprotinin, as shown in Figure 14. Lanes a in Figure 14A and B represent reduced membrane IgM recovered from a detergent lysate by reaction with rabbit anti-mouse IgM (RAM-IgM), as described in Methods, in the absence or presence of aprotinin, respectively. In order to insure complete clearance of membrane IgM, the detergent lysate was reacted a second time with RAM-IgM. As seen

Figure 14. SDS-PAGE Analysis of Mouse Membrane H Chains Prepared in the Presence of Aprotinin

A. a. Mouse membrane μ chain - aprotinin
   b. Second RAM-IgM clearance - aprotinin
   c. Mouse membrane δ chain - aprotinin

B. a. Mouse membrane μ chain + aprotinin
   b. Second RAM-IgM clearance + aprotinin
   c. Mouse membrane δ chain + aprotinin


A.
a
b
c
73k
66k
63k
23k
B.
a
b
c

in lane b, the membrane IgM was completely cleared since a molecular weight band of 73,000 was not observed in the reduced preparations with or without aprotinin. In Figure 14A and B, lane c represents reduced mouse membrane IgD recovered by reacting the lysate with rabbit anti-mouse IgA after clearance of membrane IgM in the absence or presence of aprotinin. The increased concentration of aprotinin did not affect the relative mobilities of the membrane δ and membrane μ chains. The major membrane δ chain bands were 66,000 and 63,000 with no trace of a heavy δ chain. The 23,000 molecular weight band is L chain.

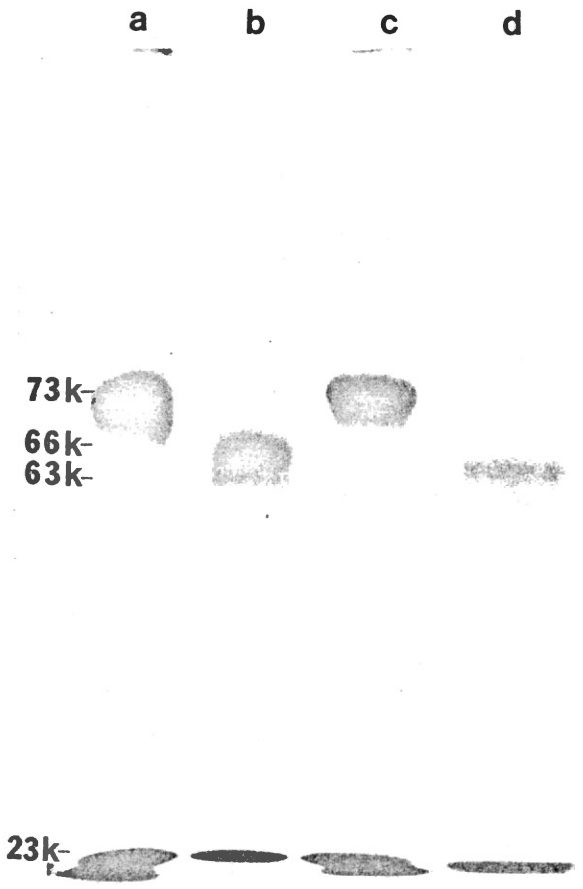
A combination inhibitor experiment is shown in Figure 15, lanes a and b. 1.0 mM PMSF, 0.1 mM EGTA and 100 KIU/ml aprotinin were introduced into the experiment at the onset. No change in the relative mobilities of the membrane μ and δ chains was observed.

TPCK at a concentration of 1.0 mM was the next proteolytic inhibitor incorporated into the preparation procedure. TPCK is an irreversible inactivator of proteolytic enzymes with chymotrypsin-like activity. Trypsin is not affected by the inhibitor. Inhibitor studies with chymotrypsin suggest that TPCK alkylates a histidine residue located at the active center of the enzyme irreversibly inactivating it (Schoellmann and Shaw, 1963). The mobilities of the membrane μ and δ chains are compared in Figure 15, lane c and d. The presence of TPCK did not alter the mobilities of the membrane H chains.

The incorporation of ε-amino caproic acid (EACA) into the preparation procedure was of special interest since it has been reported that EACA prevents the 'spontaneous' degradation of human myeloma IgD (Spiegelberg et al., 1970; Goyert et al., 1977). EACA has also been shown to be effective in preventing the degradation of surface IgD on

Figure 15. SDS-PAGE Analysis of Mouse Membrane H Chains Prepared in the Presence of PMSF, EGTA, Aprotinin and TPCK

a. Mouse membrane μ chain + PMSF, EGTA, aprotinin
b. Mouse membrane δ chain + PMSF, EGTA, aprotinin
c. Mouse membrane μ chain + TPCK
d. Mouse membrane δ chain + TPCK


a
b
c
d
73k-
66k-
63k-
23k-

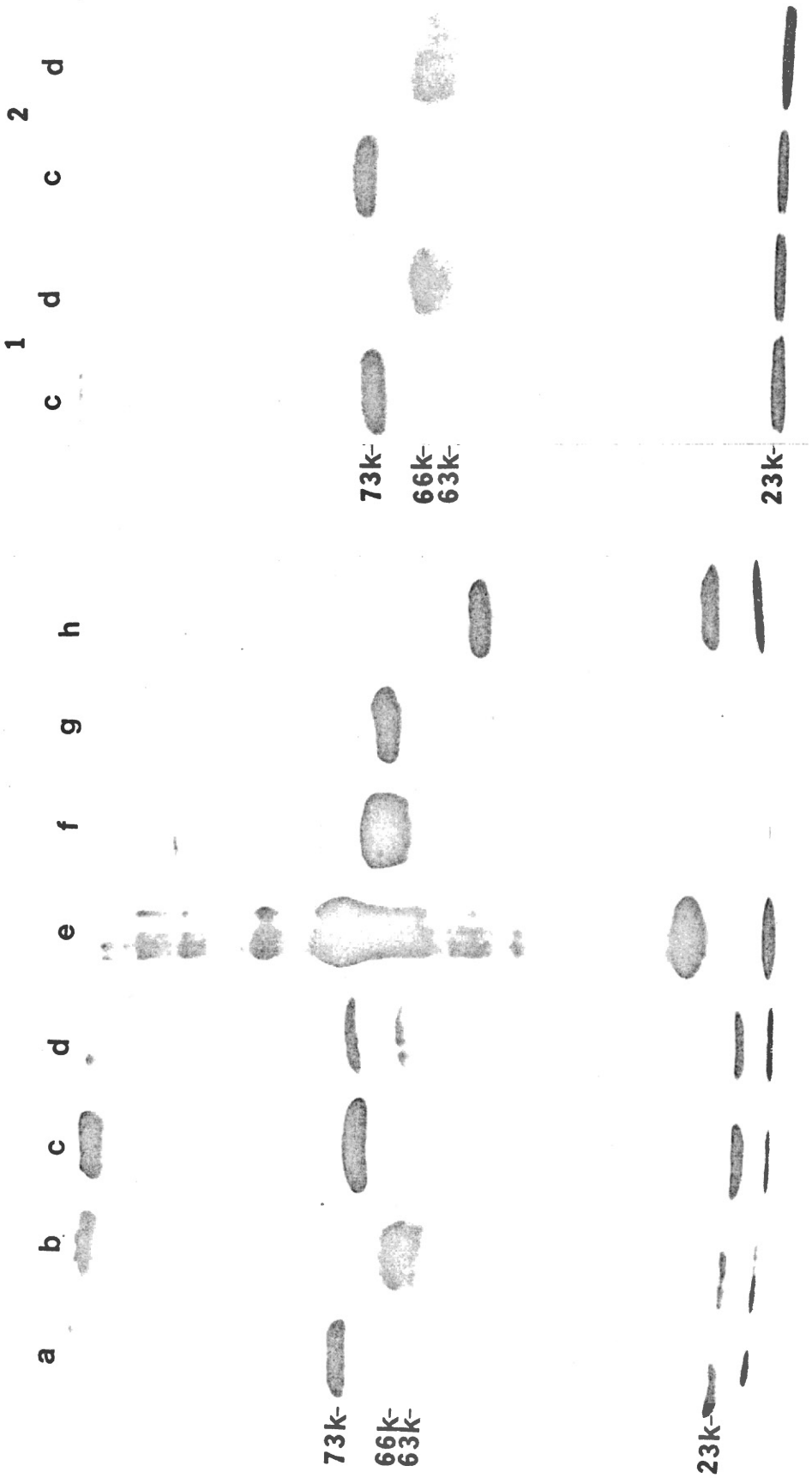
human neonatal lymphocytes by maternal serum (Panero et al., 1978). A 1% solution of EACA in PBS was used in these experiments since this concentration was effective in preventing 'spontaneous' degradation of human myeloma IgD. Several experiments were done with 2% EACA in PBS, however the 1% increase in concentration had a marked effect on mouse spleen cell viability. Generally a greater than 90% cell viability was observed with 1% solutions of EACA, as determined by trypan-blue exclusion. In the presence of 2% EACA spleen cell viability dropped to less than 50%.

In the initial experiment, 1% EACA seemed to produce a mouse membrane δ chain with a mobility comparable to that of mouse membrane μ chain, see Figure 16, lanes c and d. Preparation of mouse H chains in the absence of EACA is shown in the same figure, lanes a and b. The presence of EACA also seemed to be effective in preventing the H chain degradation products normally observed at least in this particular experiment. However, this experiment was repeated several times and a membrane δ chain with a mobility comparable to that of membrane μ chain was not reproducibly observed in the presence of EACA. The results of two repeat experiments are also seen in Figure 16. The most likely explanation for the 73,000 molecular weight band in the membrane δ lane would be incomplete clearance of the spleen cell detergent lysate of all membrane IgM prior to the immunoprecipitation of the membrane IgD. Lanes e through h in Figure 16 are ^{125}I-labeled MOPC 104E μ, human δ (Delio), MOPC 315 α and MOPC 21 γ heavy chains used as molecular weight markers. The literature molecular weight values for the marker proteins are 73,000, 64,000, 55-58,000 and 51,000, respectively including H chain carbohydrate (Robinson et al.,

Figure 16. SDS-PAGE Analysis of Mouse Membrane H Chains Prepared in the Presence of EACA Plus Myeloma H Chain Markers

a. Mouse membrane μ chain - EACA
b. Mouse membrane δ chain - EACA
c. Mouse membrane μ chain + EACA
d. Mouse membrane δ chain + EACA
e. MOPC 104E μ chain
f. Human δ chain (Delio)
g. MOPC 315 α chain
h. MOPC 21 γ chain

1. Repeat experiment + EACA
2. Repeat experiment + EACA


a
b
c
d
e
f
g
h
73k-
66k-
63k-
1
c
d
2
c
d
73k-
66k-
63k-
23k-
23k-

Spiegelberg, 1977; Nisonoff et al., 1975). The estimation of molecular weights, especially those of glycoprotein, via SDS-polyacrylamide gel electrophoresis has been shown to be less than reliable. Melcher and Uhr (1976) have shown that the molecular weights observed for membrane H chains on SDS-polyacrylamide gels are a function of the acrylamide concentration used in the gel. At acrylamide concentrations of less than 10% observed molecular weights of membrane heavy chains were higher suggesting a slowing effect due to attached carbohydrate. However, the slowing effect is overcome at acrylamide concentrations of 10% or greater. Some disparity was seen in the apparent molecular weights of the marker proteins on the 7.5% acrylamide gel when compared to literature values. Observed molecular weights for MOPC 104E μ, human δ (Delio) and MOPC 21 γ heavy chains were consistent with their literature values. MOPC 315 α chain had an observed molecular weight of approximately 65,000, somewhat higher than the generally accepted value of 55-58,000.

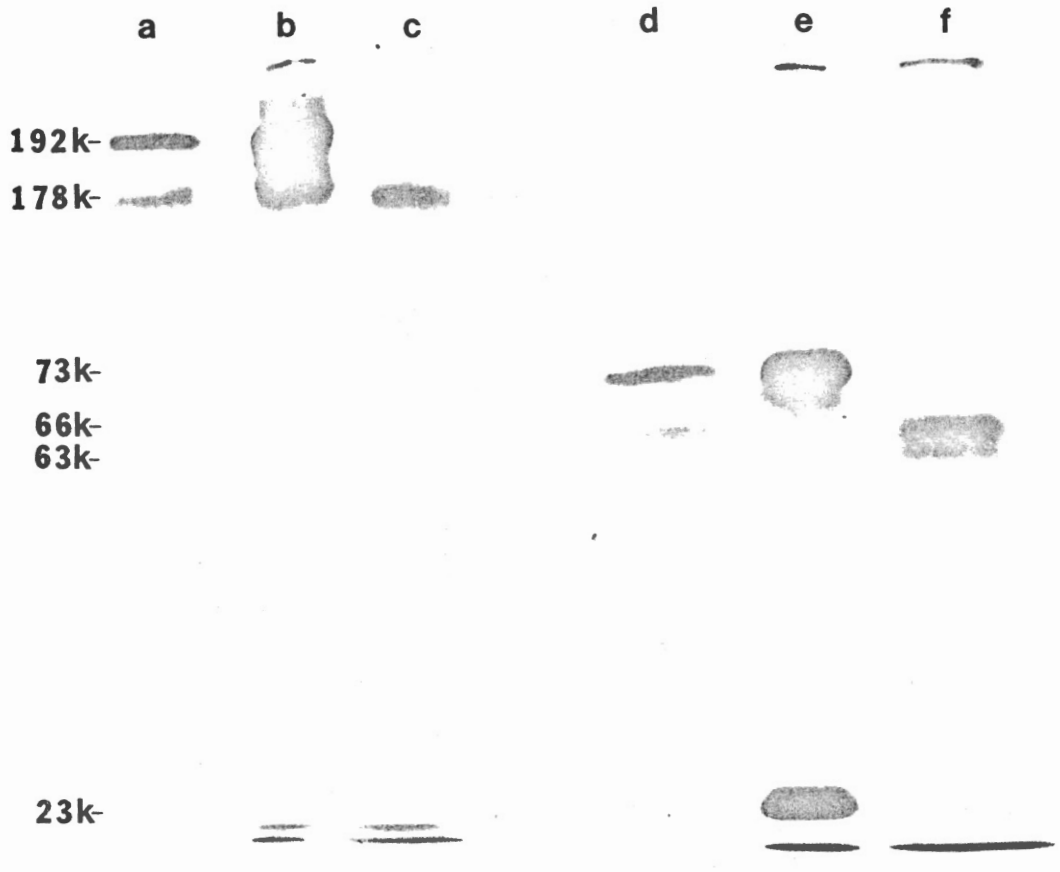
The possibility that the degradation of mouse membrane IgD could result from the method used in the isolation of the membrane species was also tested. Generally iodinated mouse membrane IgM and IgD were recovered from a mouse spleen cell detergent lysate by reaction with rabbit anti-mouse IgM and IgA sera respectively followed by goat anti-rabbit Ig serum. The ratio of goat serum to rabbit anti-serum was 9:1. Thus for every 50 μl of rabbit anti-serum introduced 0.45 ml of goat serum was added. The addition of rabbit serum and large amounts of goat serum in the recovery of the membrane Igs could possibly introduce serum proteases into the experiment as well. In order to determine if the degradation of mouse membrane IgD could be related to the use of

rabbit and goat serum in the isolation procedure an alternative isolation method was used.

Protein A, a polypeptide found on the surface of Staphylococcus aureus that binds rabbit Ig (Langone et al., 1977), was substituted for goat serum in the recovery of the membrane Igs. An experiment done in the presence of 1% EACA using Protein A in the recovery of the membrane proteins is shown in Figure 17. Intact membrane IgM and IgD recovered from a mouse spleen cell detergent lysate by reaction with rabbit anti-mouse IgA and Protein A are shown in lane a. The recovery of intact membrane IgM from a spleen cell detergent lysate by reaction with rabbit anti-mouse IgM and Protein A is shown in lane b. After clearing the lysate of membrane IgM, the intact membrane IgD was recovered by reacting with rabbit anti-mouse IgA and Protein A, as seen in lane c. The intact membrane proteins were removed from the Protein A in the absence of reducing agent as described in Methods. Treatment of the recovered intact membrane Igs with reducing agent yielded H and L chains. Lane d contains membrane μ and δ chains, a secondary δ band and L chain with observed molecular weights of 73,000, 66-63,000 and 23,000, respectively. Molecular weights of the intact membrane IgM and IgD were assigned simply by totaling molecular weights of H and L chains. Membrane μ chain and L chain are seen in lane e. Membrane δ chain, the secondary δ fragment and L chain are seen in lane f. Important to note is the comparison of the H chains in lanes e and f. In the presence of 1% EACA and in the absence of goat serum the relative mobilities of the mouse membrane μ and δ H chains remain the same. One must conclude that recovery of the membrane IgD with

Figure 17. SDS-PAGE Analysis of Mouse (Unreduced and Reduced) Membrane H Chains Prepared in the Presence of EACA and Isolated Using Heat Killed *Staphylococcus aureus*

a. Unreduced mouse membrane IgM and IgD
b. Unreduced mouse membrane IgM
c. Unreduced mouse membrane IgD
d. Reduced mouse membrane IgM and IgD
e. Reduced mouse membrane IgM
f. Reduced mouse membrane IgD


a
b
c
d
e
f
192k-
178k-
73k-
66k-
63k-
23k-

goat serum does not contribute to proteolysis of membrane δ chain if indeed it does occur. One might also conclude that the use of rabbit anti-mouse serum in the recovery of membrane IgD does not contribute to proteolysis of membrane δ chain since only small amounts of this antiserum are used.

CHAPTER V

CHEMICAL AND ENZYMATIC CLEAVAGE OF MOUSE MEMBRANE AND SECRETED IMMUNOGLOBULINS

Introduction

H chains from several myeloma Ig classes have been completely or at least partially sequenced. The complete amino acid sequence of MOPC 21, a mouse myeloma IgG, has been reported (Adetugbo, 1978). The amino acid sequences of IgM H chains from two different mammalian species have been reported (Putnam et al., 1973; Capra and McCumber, 1979). In addition, the partial amino acid sequences of mouse MOPC 104E and ABPC 22 IgM H chains are also known (Hood, personal communication, 1979; Capra, personal communication, 1979). The partial amino acid sequences of MOPC 315 and MOPC 47A IgA H chains and the complete amino acid sequence of human IgA_1 H chain have been reported (Robinson and Appella, 1977; Francis et al., 1974; Liu et al., 1976).

Capra and McCumber (1979) have compared the overall amino acid sequence homologies of human, mouse and canine IgM H chains. The μ chains from the three different species displayed a remarkably high overall sequence homology (80%). In addition, when particular sequences in the constant domains were compared a greater than 95% sequence homology was observed. Some amino acid sequences are also preserved in variable regions as well. For example, in the comparison of the first

150 N-terminal amino acids of three IgA H chains (human α_1, mouse 315 and 47Aαs) a 45-60% sequence homology is observed. In contrast, the amino acid sequences of different Ig classes are not as highly conserved. Putnam et al. (1976) have compared the structural homology of human IgA, IgG, IgM and IgE H chains. A 25-30% sequence homology was observed between α, γ, μ, and ε chains. Partial amino acid sequences have been reported for human myeloma IgDs (Spiegelberg, 1975; Perry and Milstein, 1970). No sequence information is presently available for mouse membrane IgM or IgD.

Possible similarities in the placement of cleavage sites on mouse and human H chains have been explored utilizing chemical and enzymatic cleavage techniques. If cleavage sites are conserved between two Ig classes one might expect peptide fragments of similar sizes. Similar fragmentation patterns thus could be used as an indicator of identical Ig class since it appears that amino acid sequences of a single Ig class are conserved, even among different species. In contrast, one might expect different cleavage patterns from two different Ig classes since it has been shown that amino acid sequences are not as highly conserved between classes. A high overall amino acid sequence homology between two H chains does not necessarily imply a conservation of specific cleavage sites however. If one examines the amino acid sequences of two human IgM H chains (Ou and Gal) a 96% sequence homology is observed between residues 122 and 567. Of the four methionine residues present from 122 to 567, four are conserved in both chains. In contrast, if one examines the amino acid sequences of human IgM (Ou) and canine IgM (Moo) H chains an 80% sequence homology is observed between residues 122 and 567. However, none of the seven methionine residues present in

the canine H chain are conserved in the human H chain.

The number of cleavage fragments would also be an important consideration. If a single coincident fragment was observed between the cleavage patterns of two H chains, it would probably not indicate conservation of cleavage sites since fragments of similar size could originate from different parts of the H chain. As the number of coincident fragments increased the probability that the fragments were derived from different parts of the H chains would become less. In addition, even if fragments of two H chains have similar mobilities on SDS-polyacrylamide gels, conservation of cleavage sites could not be positively concluded. The resolution of the fragments on polyacrylamide gels is not good enough for one to be absolutely sure the fragments are of the same size. In the experiments reported here, the cleavage patterns of mouse membrane IgM and IgD H chains have been compared with those of mouse myeloma IgM, IgA and IgG H chains as well as those of human myeloma IgD H chain.

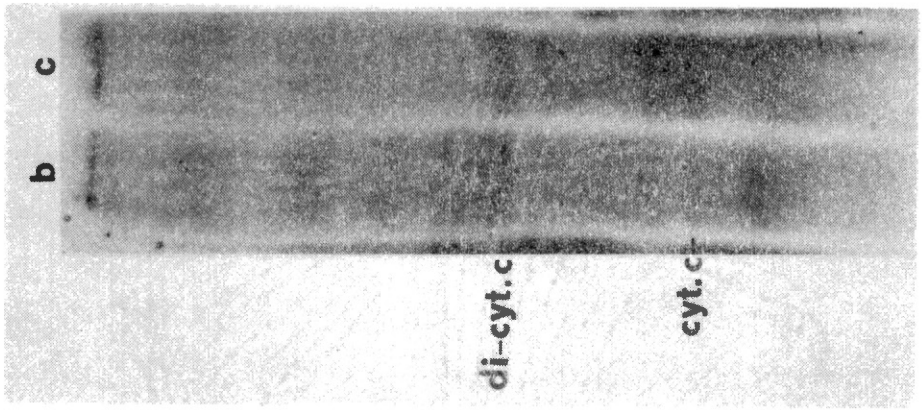
Results

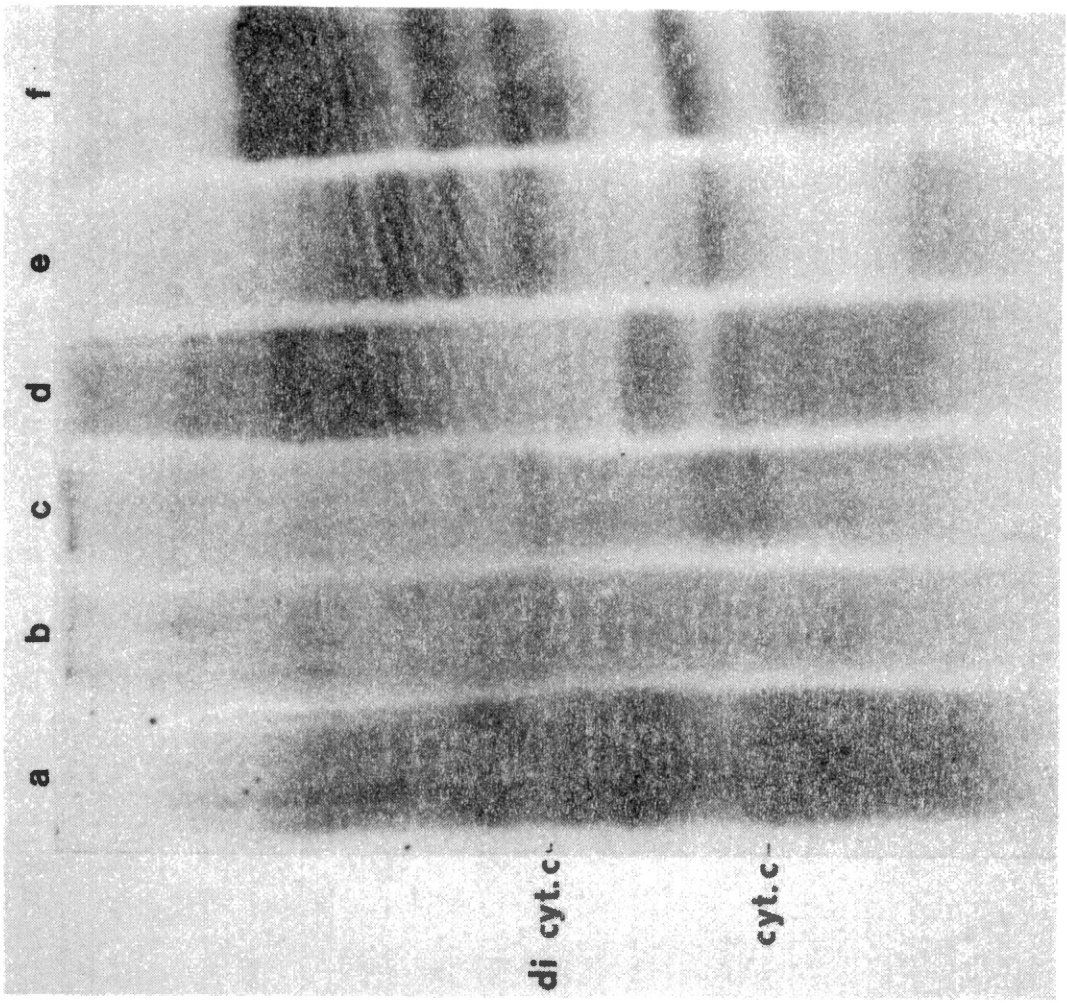
CNBr Cleavage of H Chains

Mouse membrane μ and δ chains, mouse myeloma μ, α, and γ chains and human δ chain (Delio) were labeled, column purified and cleaved with cyanogen bromide (CNBr), electrophoresed on a 15% polyacrylamide slab gel and the resulting fragments visualized by fluorography. The results of a CNBr cleavage of the membrane and secreted H chains are seen in Figure 18. In order to facilitate direct comparison of the fragmentation patterns, the individual lanes of a CNBr fluorogram were scanned

Figure 18. SDS-PAGE Analysis of CNBr Cleavage Fragments of Mouse Membrane, Mouse Myeloma and Human Myeloma H Chains

a. MOPC 104E μ chain
b. Mouse membrane μ chain
c. Mouse membrane δ chain
d. Human δ chain (Delio)
e. MOPC 315 α chain
f. MOPC 21 γ chain


b
c
di-cyt.c
cyt.c


a
b
c
d
e
f
di cyt.c
cyt.c

with a Quick Scan densitometer (Helena Laboratories) (Figure 19). Also shown in Figure 19 are size estimates assigned to the H chain cleavage fragments.

Size estimates for the H chain CNBr fragments were obtained by using cytochrome c dimer and monomer as size markers, electrophoresed in lanes adjacent to the cleaved H chains, and available amino acid sequence data. Size estimates seen on Figure 19 are in units of amino acid residue equivalents. The amino acid sequence data available were first used to calculate the size of the CNBr fragments or fragment combinations possible for the mouse myeloma H chains. Single or combination fragments were searched to determine which fragments contained carbohydrate moieties. In the event a fragment contained carbohydrate, the molecular weight of the carbohydrate was obtained using oligosaccharide structures reported by Baenziger and Kornfeld (1974). The molecular weight(s) of the oligosaccharide(s) per fragment were then divided by 120, the average amino acid molecular weight, to obtain amino acid residue equivalents. The amino acid residue equivalents for the carbohydrate were then added to the number of amino acid residues present in the CNBr fragment to obtain the final size estimate. Of the H chains examined in these experiments, MOPC 21 γ chain is the only one whose complete amino acid sequence is known. MOPC 104E μ chain has been partially sequenced and the sizes of its CNBr fragments are known. The approximate sizes of the myeloma γ and μ fragments were first estimated by using the cytochrome c size markers. Then the amino acid sequence data were searched for a possible fragment, corrected for sugar present, of approximately that size.

In order to obtain the best size estimates for mouse membrane μ

Figure 19. Densitometer Tracings of a Fluorogram of Fragments Produced by CNBr Cleavage of Mouse Membrane, Mouse Myeloma and Human Myeloma H Chains

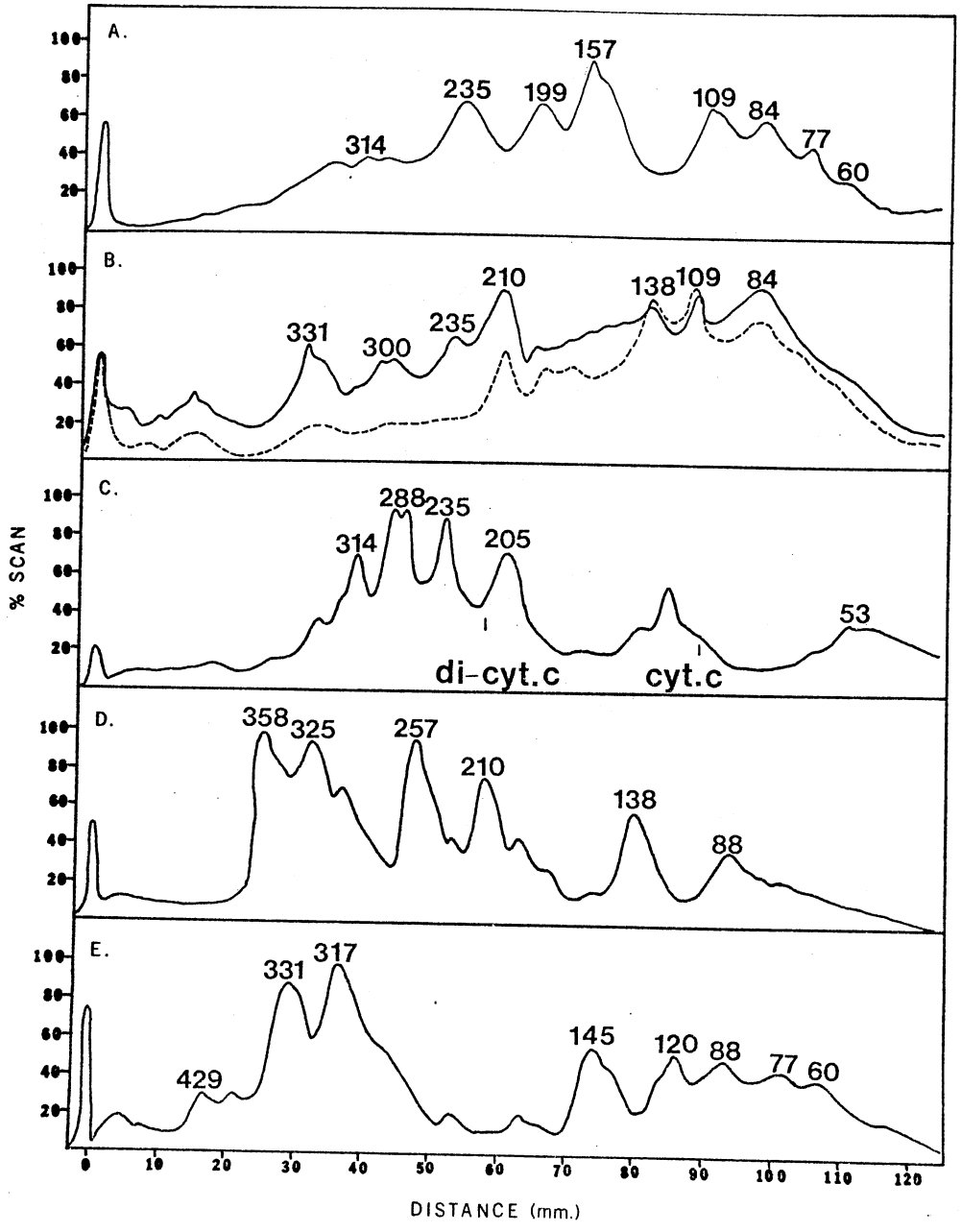
A. MOPC 104E μ chain

B. Mouse membrane μ and δ chains. Solid line represents μ chain, dashed line represents δ chain

C. MOPC 315 α chain

D. MOPC 21 γ chain

E. Human δ chain (Delio)


A.
157
235
199
109
84
77
60
314
B.
210
138
109
84
331
300
235
C.
288
235
314
205
53
di-cyt.c
cyt.c
D.
358
325
257
210
138
88
E.
331
317
145
120
88
77
60
429
% SCAN
DISTANCE (mm.)

and δ chains, and in part MOPC 315 α and human δ chains, a plot of amino acid residue equivalents assigned to the fragments of MOPC 104E μ and MOPC 21 γ chains versus fragment migration into the gel was made. This plot was used to estimate the sizes of the membrane μ and δ chain fragments without the aid of amino acid sequence data. In addition, the plot was used to assign the best size estimates to MOPC 315 α chain CNBr fragments (in conjunction with amino acid sequences for mouse MOPC 315 and 47A and human α chains) and to human δ chain CNBr fragments (in conjunction with limited CNBr cleavage data reported by Kocher and Spiegelberg (1979)). It was assumed that some homology exists between mouse and human α chains. Proof of this assumption awaits the completion of either of the mouse α chain sequences.

In Figure 18, the fragmentation pattern of MOPC 104E μ chain is shown in lane a. The fluorogram densitometer tracing and estimated fragment sizes are seen in Figure 19, panel A. According to the partial amino acid sequence, complete CNBr cleavage should yield nine fragments of 164, 109, 84, 62, 62, 47, 20, 14 and 8 amino acid residues in length respectively. The cleavage of the MOPC 104E μ chain was not complete since eight to nine fragments with estimated sizes of 314, 235, 199, 157, 109, 84, 77 and 60 amino acid residue equivalents respectively were detected. Only a few of these correspond to products expected from complete cleavage.

The cleavage patterns of mouse membrane μ and δ chains are shown in Figure 18, lanes b and c respectively. The fragments are compared in the fluorogram densitometer tracing displayed in Figure 19, panel B. The mouse membrane H chains have similar fragmentation patterns, although like MOPC 104E μ chain, the cleavage of these H chains was incomplete.

Coincident fragments are observed at greater than 429, 331, 210, 138, 109 and 84 amino acid residue equivalents. A possible area of non-correspondence exists at 199. The background in the membrane μ chain pattern, especially from 199 to the bottom of the gel, makes the assignment of possible differences in this area uncertain.

The sizes of the coincident membrane μ and δ fragments deserve additional consideration. Peptide fragments can be derived from variable domains alone; they can be a combination of variable and constant domains; or they can be completely derived from the constant domains of the H chain. It is impossible to pinpoint the origin of the fragments observed, however some generalization can be made. CNBr fragments of 110 amino acid residues or less can be derived completely from variable or constant domains, since a domain is approximately 110 amino acid residues in length, or can be a combination of variable and constant domains. Consider for example the two fragments of MOPC 104E μ chain at 109 and 84, which also happen to be seen in the membrane μ and δ chains. The 109 fragment can be completely derived from the constant region of the H chain (residues 391 to 500) or can be a combination of variable and constant domain peptides (residues 34 to 143, no cleavage at methionine 81). It is unlikely that the 109 fragments are derived solely from the H chain variable region since three methionines (residues 20, 34 and 81) are present in this region. The fragment seen at 84 could originate completely from the constant region (residues 143 to 227) or be an incomplete cleavage fragment of the variable region of the H chain (residues 1 to 81, no cleavage at methionines 20 and 34). One would expect to see some degree of homology in peptides derived from the variable region of two different Ig classes since different Ig classes

derive their variable regions from the same population of variable region sequences.

Fragments greater than 110 amino acid residues have only two possible sources. First the fragments may be a combination of variable and constant domains or second they may originate entirely from within the constant region of the H chain. The fragments seen in the MOPC 104E μ chain at 314, 235 and 199 amino acid residue equivalents could fit either of these possibilities. Fragments 314 and 199 originate entirely within the constant region of the H chain (residues 227 to 500 and 391 to 562 respectively, plus amino acid residue equivalents for carbohydrate). Fragment 235 originates from a combination of variable and constant domains (residues 20 to 227, plus amino acid residue equivalents for carbohydrate). That fragments coincident in the mouse membrane μ and δ chain patterns are larger than 110 amino acid residues indicates that they are at least in part derived from constant domain sequences. The homology implied by these coincidences is greater than one would expect for H chains of two different classes. Proof of structural homology requires a comparison of the amino acid sequences of the coincident membrane μ and δ fragments. Sequence information on mouse membrane H chains has not been reported however.

The comparison of the fragmentation patterns of mouse membrane and MOPC 104E μ chains is of interest. Although some similarities exist in the fragments at 235, 109 and 84 amino acid residue equivalents, for the most part the patterns are less similar than one would expect for H chains of the same Ig class.

The cleavage pattern of mouse MOPC 315 α chain is seen in Figure 18, lane e and Figure 19, panel C. Seven MOPC 315 α chain fragments

were observed, size estimates were assigned to six. The estimated fragment sizes assigned were 314, 288, 280, 235, 205 and 53. Fragments 314, 288, 280, 235 and 53 originate entirely from within the constant region of the H chain sequence used to estimate the sizes (estimates include amino acid residue equivalents for carbohydrate). Fragment 205 is a combination of variable and constant region sequences plus carbohydrate. A fragment with an approximate size of 138-109 was observed. However, based upon the amino acid sequence data used to assign the fragment sizes, a fragment of this size was not expected. The mouse and human α chains apparently have differences in the placement of methionines along their H chains. The fact that Igs of different classes yield different cleavage patterns is most readily seen in the comparison of MOPC 104E μ and MOPC 315 α chains. Although coincident fragments are seen at 314 and 235, the patterns are otherwise very dissimilar. The MOPC 315 α chain fragment pattern is also different from those of mouse membrane μ and δ chains. The α and membrane μ chains do have coincident fragments at 235 and 138-109 while the membrane δ chain only shows a coincident fragment with the α chain in the 138-109 area. The positions of the cytochrome c dimer and monomer of 208 and 104 amino acids in length are also shown in panel C.

The incomplete CNBr cleavage pattern of MOPC 21 γ chain is seen in Figure 18, lane f and Figure 19, panel D. Ten CNBr fragments with sizes of 161, 49, 49, 48, 35, 34, 30, 14, 10 and 10 were expected from a complete cleavage of the mouse γ chain. However, the six major fragments with estimated sizes of 358, 325, 257, 210, 138 and 88 amino acid residue equivalents were observed. Several minor fragments were also observed as shoulders of the 325, 257 and 210 γ chain fragments. The

difference in the CNBr cleavage patterns among different classes are again readily apparent in the comparison of MOPC 315 α and MOPC 104E μ fragments with MOPC 21 γ fragments.

The cleavage patterns of mouse membrane μ and δ chains show some similarities when compared to MOPC 21 γ chain. Coincident fragments are seen at 210, 138 and near 84, the remaining membrane μ and δ fragments are different from those of γ chain.

The CNBr cleavage pattern of human myeloma δ (Delio) chain (Figure 18, lane d, Figure 19, panel E) shows five major fragments and three minor fragments. The estimated sizes assigned to the major fragments were 331, 317, 145, 120 and 88 amino acid residue equivalents. The minor fragments were assigned values of 429, 77 and 60. There are some similarities between the mouse myeloma H chains and human δ chain, but for the most part the cleavage patterns are different. The comparison of the human δ chain cleavage pattern with those of mouse membrane μ and δ chains are nearly the same. Similarities do exist in the patterns at 331 and 88-84, however the remainder of the fragments are different.

There are several possible pitfalls in comparing fragmentation patterns that are incomplete. The fact that smaller fragments are not observed in some of the H chain cleavage patterns (e.g., MOPC 315 and 21) could be a consequence of incomplete cleavage or a lack of label in those peptides. Whether complete CNBr cleavage of mouse membrane, mouse myeloma and human myeloma H chains would yield fragmentation patterns more or less similar to one another than those displayed here is not known. In repeat experiments, the H chain CNBr fragmentation patterns were also incomplete and were similar to those shown. The reason complete cleavage of the H chains was not obtained is also unknown since cleavage condi-

tions were essentially the same as those used by others to effect complete cleavage of Ig H chains (Anderson, personal communication, 1979). Human γ chain was cleaved with different CNBr concentrations (0.15 to 7.5 mM) and varied cleavage times. Regardless of the concentration of CNBr at all times tested, the cleavage patterns remained unchanged. Thus it seems unlikely that an insufficient concentration of CNBr caused the incomplete cleavage of the membrane and myeloma H chains.

Chymotrypsin Cleavage of H Chains

The similarity of mouse membrane μ and δ chains is further supported by cleavage patterns obtained by limited proteolysis with chymotrypsin. Mouse membrane μ and δ chains, MOPC 104E μ and MOPC 315 α chains were subjected to limited proteolysis with the enzyme chymotrypsin as described by Cleveland et al. (1977). Limited proteolysis was used because of the high number of possible chymotryptic cleavage sites present within Ig H chains. If the partial amino acid sequence data for MOPC 104E μ chain is examined for possible chymotryptic cleavage sites (i.e., phenylalanine, tryptophan and tyrosine residues), 48 such sites exist and would yield a large number of fragments upon complete enzymatic digestion. However, only those fragments containing a tyrosine residue would be detected by fluorography.

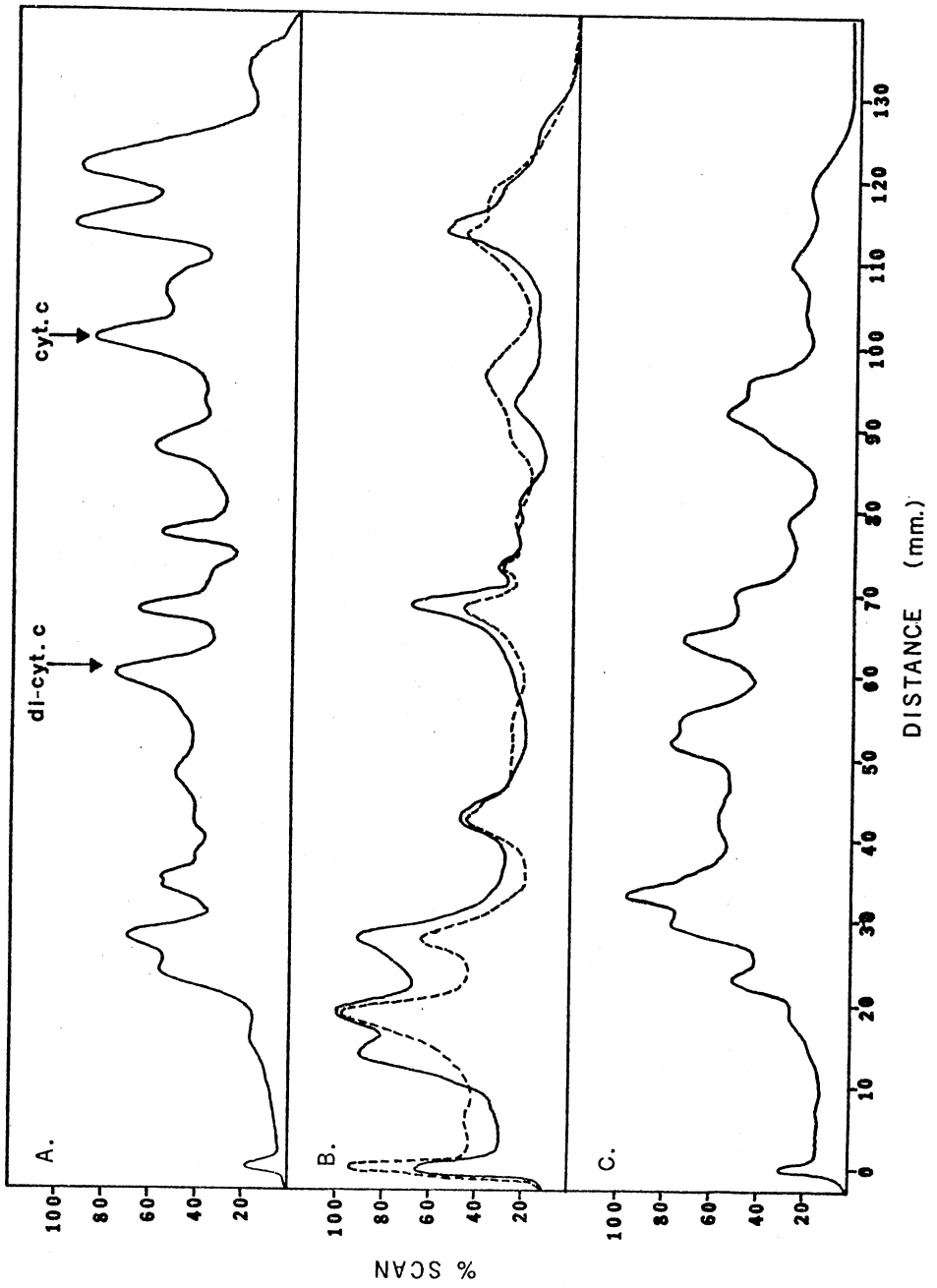
The results of the limited chymotryptic cleavage of mouse H chains are seen in Figure 20. The cleavage patterns of the mouse H chains are displayed as densitometer tracings of a fluorogram. The limited chymotryptic digest of MOPC 104E μ chain is seen in panel A. Approximately 15 bands were seen in the cleavage pattern of this μ chain. The positions of the size markers (cytochrome c dimer and monomer) have

Figure 20. Densitometer Tracings of a Fluorogram of Fragments Produced by Limited Chymotrypsin Proteolysis of Mouse Membrane and Mouse Myeloma H Chains

A. MOPC 104E μ chain

B. Mouse membrane μ and δ chains. Solid line represents μ chain, dashed line represents δ chain

C. MOPC 315 α chain


A.
B.
C.
di-cyt. c
cyt. c
% SCAN
DISTANCE (mm.)

also been indicated in panel A. No attempt was made to assign sizes to the chymotryptic fragments other than the size in relation to the size markers. Fragments with mobilities faster than cytochrome c dimer are probably 208 amino acid residues in length or less, while fragments slower than the dimer are greater than 208 amino acid residues in length.

The composition of mouse membrane μ and δ chain chymotryptic fragments is shown in panel B. Although the cleavage of the μ and δ chains is not complete there is more conservation in the chymotryptic cleavage sites than one would expect from two Ig H chains of different classes. Near the origin there is a fragment observed in the membrane μ chain pattern that is not present in the membrane δ chain pattern. However after this initial fragment difference, nearly all of the fragments seen in membrane μ and δ chains are coincident. An area of non-correspondence exists in a region near the cytochrome c marker. Fewer chymotryptic peptides were seen in the membrane H chains than in the MOPC 104E μ chain. The smaller number of membrane H chain fragments could reflect a difference in the iodination of cell surface Igs as compared to iodination of myeloma Igs in solution with lactoperoxidase. The smaller number of membrane H chain peptides could also be due to a different conformation of the membrane H chain in solution that restricts the cleavage by chymotrypsin. A few of the membrane μ and δ chain and MOPC 104E μ chain fragments are of similar mobilities.

The chymotryptic cleavage of MOPC 315 α chain is shown in panel C. The cleavage pattern of the MOPC 315 α chain is different from that of the myeloma H chain from a different class. There are more fragments present in the MOPC 104E μ chain. This could be partially explained by the extra domain present in the μ chain however. Differences in the

chymotryptic patterns of the membrane H chains and MOPC 315 α chain also exist.

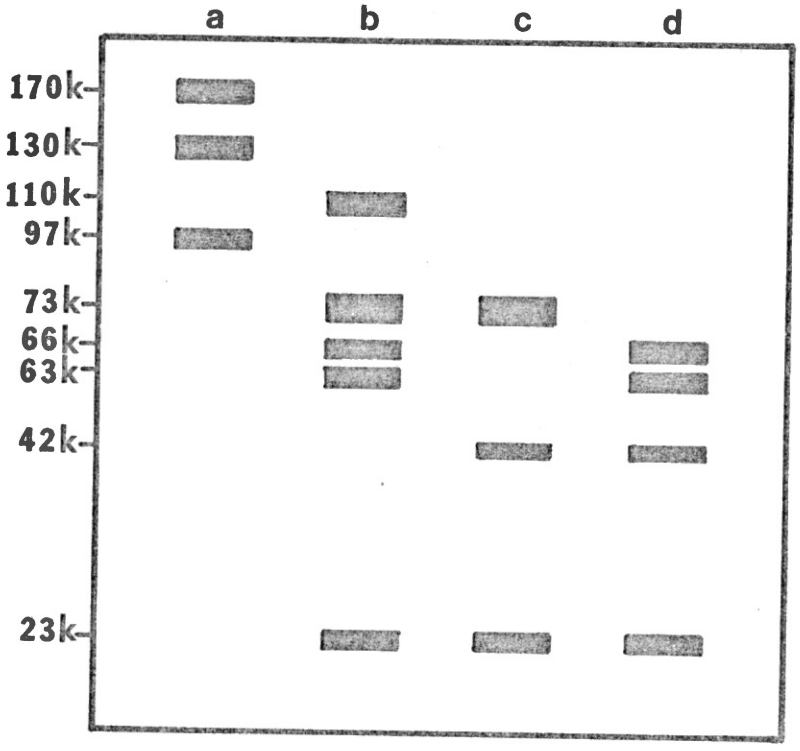
As in the CNBr experiments, the comparison of incomplete chymotryptic cleavage patterns has its pitfalls. Whether the H chains compared would appear to be more or less similar to one another if completely cleaved at all sites is unknown. If the H chains were completely cleaved, the ability to detect the fragments would depend upon how completely the H chain was iodinated. The fact that all H chains were processed and treated in an identical fashion suggests that the similarities and/or dissimilarities seen among the H chains reflect actual differences in structure.

Pepsin Cleavage of Mouse Membrane IgM and IgD

Mouse membrane intact IgM and IgD molecules were cleaved with pepsin. Pepsin attacks and cleaves intact Igs on the C-terminal side of the inter-heavy chain disulfide bond(s) and produces an $(Fab)_2$ fragment and an Fc fragment. After cleavage the fragments were electrophoresed on a 7.5% SDS-polyacrylamide slab gel and the fragments visualized by fluorography. Figure 21, lanes a and b show unreduced mouse membrane IgM and IgD that have been cleaved with pepsin respectively. The reduced mouse membrane IgM and IgD H chains, 42K (actin) and L chain bands used as molecular weight markers (not treated with pepsin) are shown in lanes c and d respectively. Cleavage of intact membrane IgM produced three fragments with estimated molecular weights of 170K, 130K and 97K. The pepsin fragments expected have molecular weights of 130K for the $(Fab)_2$ fragment and 65K or 33K for the Fc fragment. The size of the Fc fragment depends upon its ability to remain intact. The 130K

Figure 21. SDS-PAGE Analysis of Fragments Produced by Pepsin Cleavage of Intact Mouse Membrane IgM and IgD

a. Mouse membrane IgM + pepsin
b. Mouse membrane IgD + pepsin
c. Reduced mouse membrane IgM - pepsin
d. Reduced mouse membrane IgD - pepsin


a
b
c
d
170k
130k
110k
97k
73k
66k
63k
42k
23k

fragment could be the $(Fab)_2$ fragment. A 65K or 33K fragment was not observed. The Fc fragment was probably further degraded. This is consistent with reports of the further degradation of the Fc fragment of IgG by pepsin (Porter, 1959). Likewise the 97K fragment probably represents a degradation product of the 130K fragment. The 170K band is probably intact membrane IgM or a degradation product of the intact IgM. The lack of appropriate molecular weight markers in this area does not allow positive identification of this band.

Pepsin cleavage of intact membrane IgD produced five fragments with estimated molecular weights of 110K, 73K, 66K, 63K and 23K. Estimated sizes of the fragments expected by treatment of membrane IgD with pepsin are unknown. The 110K and 73K fragments likely represent the $(Fab)_2$ fragment and its degradation product. The size of the Fc fragment seen for IgD would be smaller than that expected for IgM since the latter has an additional constant domain. Although additional bands were observed at 66K, 63K and 23K, these were attributed to reduction of the intact molecule to H and L chains. If the 130K and 110K fragments represent intact $(Fab)_2$ fragments of membrane IgM and IgD respectively, one must conclude that the pepsin cleavage sites are different for the two molecules.

Papain Cleavage of Mouse Membrane IgM and IgD

Mouse intact membrane IgM and IgD were cleaved with papain. The papain fragments were reduced and electrophoresed on a 15% SDS-polyacrylamide slab gel and the fragments visualized by autoradiography on flashed x-ray film. Papain attacks and cleaves intact Igs on the N-terminal side of the inter-heavy disulfide bond(s) and produces two

Fab and one Fc fragment. In the mouse membrane IgM, the three fragments would have similar molecular weights of approximately 65K. Reduction of the Fab fragment would produce an L chain and the H chain portion of the Fab fragment (the Fd fragment) having molecular weights of 23K and 24K respectively. The Fc fragment when reduced would produce two similar fragments of approximately 33K each. The mouse membrane IgD would be expected to yield similar Fab and reduction fragments. The Fd fragment could vary in size depending upon the length of the IgD H chain hinge region however. The Fc fragment of the membrane IgD and its reduction products would likely be smaller since the δ chain lacks the extra domain seen in the membrane μ chain. Experiments have been reported in which mouse membrane IgD was more susceptible than membrane IgM to papain cleavage when the molecules were cleaved while attached to the cell surface (Vitetta and Uhr, 1976; Bazin et al., 1978). In solution, the membrane Igs were equally susceptible to cleavage by papain however (Vitetta and Uhr, 1976).

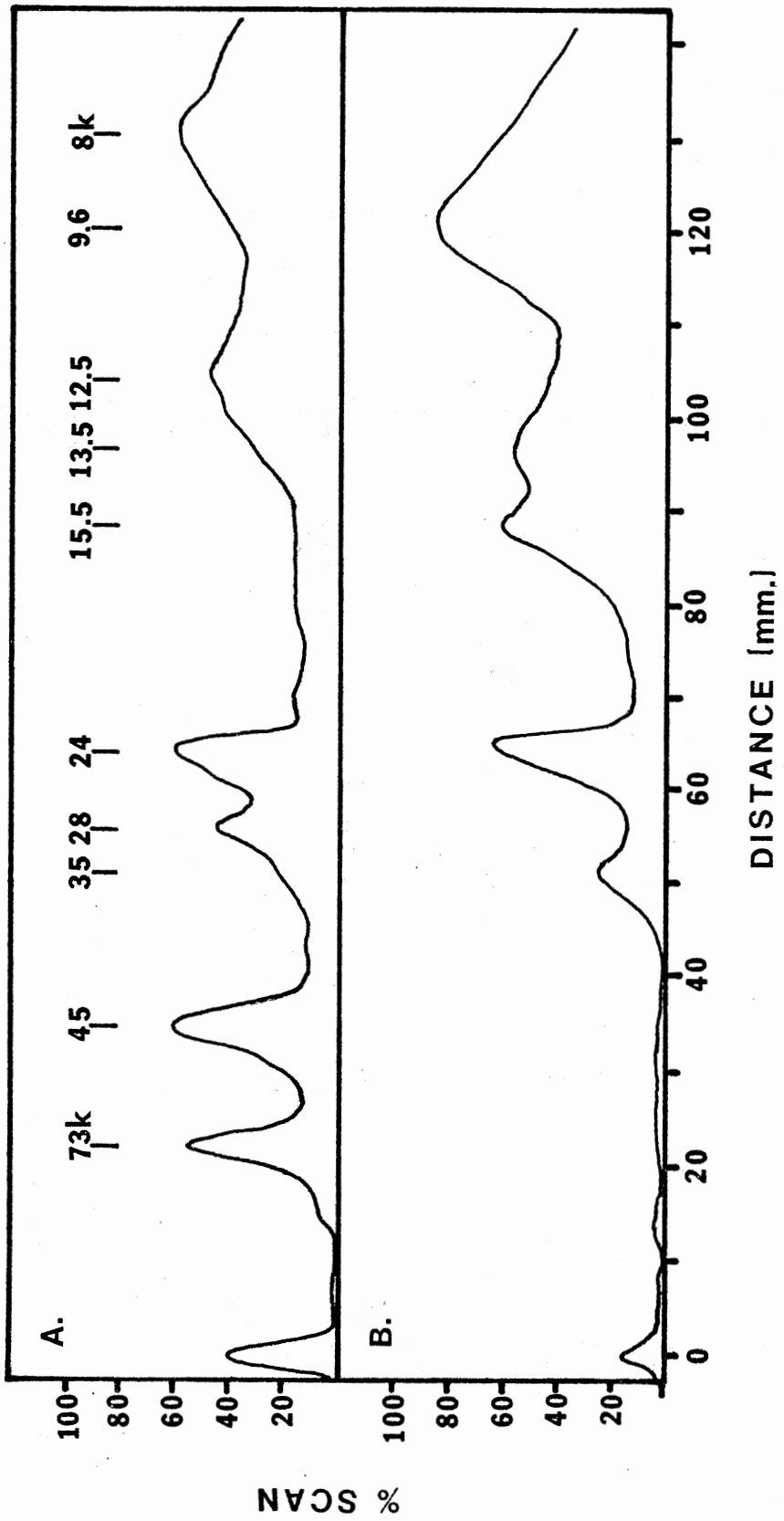
The fragments produced by papain cleavage and reduction of mouse membrane IgM and IgD are shown in Figure 22 as densitometer tracings of an autoradiogram. A set of standard molecular weight markers were used to estimate the sizes of the fragments on the slab gel. Labeled membrane H and L chains, not treated with papain, were also used as molecular weight markers.

Six major fragments were produced from mouse membrane IgM (panel A) with molecular weights of 73K, 45K, 28K, 23K, 12.5K and 8K. The 73K and 23K bands are probably H and L chains. The 45K fragment is probably the reduced Fc fragment while the 28K fragment is the Fd portion of the H chain. The 12.5K and 8K fragments are probably further degradation

Figure 22. Densitometer Tracings of an Autoradiogram of Fragments Produced by Papain Cleavage and Reduction of Mouse Membrane IgM and IgD

A. Mouse membrane IgM

B. Mouse membrane IgD


A.
B.
73k
45
35
28
24
15.5
13.5
12.5
9.6
8k
100
80
60
40
20
0
20
40
60
80
100
120
% SCAN
DISTANCE (mm.)

products of the Fd and reduced Fc fragments.

The papain cleavage pattern for mouse membrane IgD is seen in panel B. Five major fragments were produced with molecular weights of 35K, 23K, 15.5K, 13.5K and 9.6K. Lin and Putnam (1979) have reported that papain cleavage of human myeloma IgD produces two Fab fragments and an Fc fragment of 47K and 80K respectively. Reduction of these fragments would produce the Fd fragment (24K), L chain (23K) and two fragments from the reduced Fc (about 40K each). In contrast, the Fd fragment for mouse membrane IgM and IgD of molecular weight 37K has been observed by others (Vitetta and Uhr, 1976). The Fd fragment thus has a size range of 24K to 37K, while the only estimate of the reduced Fc fragment is 40K. The 35K fragment seen in the cleaved and reduced membrane IgD could either be the Fd or the reduced Fc fragment. If the 35K fragment is the reduced Fc fragment, then the Fd fragment would have a similar mobility on the gel to that of L chain and not be detected as a discrete band. The possibility also exists that either one of these fragments could be further degraded to produce the lower molecular weight fragments seen at 15.5K, 13.5K and 9.6K.

An observation when comparing the reduced papain cleavage products of the membrane IgM and IgD is the lack of intact H chain and of the larger 45K IgM-like fragment in the IgD. The membrane proteins were treated in solution with the same concentration of enzyme and for equal times then reduced. It appears that the membrane IgD is more susceptible to cleavage in solution. These results are in conflict with data reported previously by Vitetta and Uhr (1976).

CHAPTER VI

DISCUSSION AND CONCLUSIONS

CsCl buoyant density centrifugation under denaturing conditions is capable of distinguishing molecules with slightly different carbohydrate contents. Of the heavy chains tested the μ and α chain were closest to one another in carbohydrate content, having 13.3 and 10.5% carbohydrate, respectively. The difference of 2.8% in carbohydrate content was sufficient to produce a two fraction difference in peak position after centrifugation in CsCl containing guanidine-HCl. The technique is capable of distinguishing distributions whose peaks differ by less than one fraction when both polypeptides are included in the same gradient. When they are in separate tubes, the error in aligning markers or density measurements substantially increases the error in carbohydrate content estimation (Table II and Figure 12). With both polypeptides in the same tube, a difference of 1% in carbohydrate content is detectable, but molecules that differ in carbohydrate content by less than 0.5% can not be distinguished. This technique may prove useful in the density comparisons of other proteins, such as cell surface glycoproteins of normal and malignant cells (Sherblom, Buck and Carraway, ms in preparation, 1979). It has the advantage of very small requirements of material, but does require that the molecule be radiolabeled and be capable of being freed of other labeled material such as by immunoprecipitation.

The sensitivity of the technique is sufficient to allow certain

conclusions to be drawn about membrane μ and membrane δ chains. First a higher carbohydrate content is not the cause of the larger size of membrane μ chains when compared with their secreted counterparts. For polypeptides of low carbohydrate content, such as Ig H chains, the mol. wt. determined by comparison with non-glycosylated polypeptides in SDS-PAGE is indistinguishable from that obtained by summation of the mol. wt. of peptide and carbohydrate portions. Thus, under these conditions an equal weight of carbohydrate or protein will contribute the same to the mobility of the chain. Therefore, the apparent 1700 dalton difference (calculated as peptide) may also be a 1700 dalton difference in carbohydrate content. Assuming that the apparent 1700 dalton difference in size of membrane and secreted μ chains is entirely due to a difference in carbohydrate content, the membrane μ chain should contain 15.0%. The serum μ chain is known to have 13.3% carbohydrate. The difference, 1.7% is within the capability of the method for distinguishing density distributions. On the other hand, if the extra size is due to peptide sequence, then the membrane μ chain should have 13.0% carbohydrate. The small 0.3% difference should result in a difference of peak position of less than a quarter of a fraction, a difference not able to be determined.

Since the densities of membrane μ and secreted μ chains were indistinguishable, they must be very similar in carbohydrate content. An alternate interpretation, that membrane μ contains extra carbohydrate and also components of low density, such as lipid or detergent is deemed unlikely. First the two components would have to fortuitously counterbalance each other exactly in their effect on the density. Second undenatured mouse (Melcher and Uhr, 1977) and chicken (Lifter and Choi,

1978) membrane immunoglobulins had lighter densities than their secreted counterparts probably due to the binding of detergent molecules to the immunoglobulins. If this detergent had not been removed by the procedures used, then the resultant heavy chains should have had a substantially lighter density in guanidine-HCl-cesium chloride gradients. Since this was not observed, it can be concluded that dialysis against propionic acid, gel filtration in propionic acid, and dialysis against guanidine-HCl were effective in removing the material responsible for the lighter density. This suggests that the material was non-covalently bound detergent or lipid and further supports the concept that membrane immunoglobulins are integral membrane proteins. Although the carbohydrate contents of membrane and secreted μ chains are thus similar, the carbohydrate compositions and the peptide sequence location of carbohydrate chains need not be the same.

Not only do the present results imply that membrane IgM does not have substantially more sugar than secreted IgM, but they also suggest that membrane IgM does not have substantially less sugar than the secreted IgM. Based on incorporation studies with radioactive sugar precursors, it has been suggested that membrane IgM lacks the terminal sugars, fucose and galactose (Melcher and Andersson, 1973). This raised the possibility that this IgM was attached to the membrane because it was not fully glycosylated. Other workers (Vitetta and Uhr, 1974) were however able to demonstrate the incorporation of radioactive fucose into membrane IgM, albeit at low levels. Since no substantial under-glycosylation of the membrane IgM was detected, the lack of full glycosylation cannot be responsible for anchoring of IgM to the membrane. It is, however, entirely possible that full glycosylation plays a role in the

exteriorization of the membrane IgM. It has been observed that the addition of inhibitors of glycosylation such as 2-deoxyglucose and tunicamycin inhibits the secretion of some, but not all, immunoglobulins (Eagon and Heath, 1977; Hickman and Kornfeld, 1978; Hickman et al., 1977). Whether glycosylation plays a role in the exteriorization of membrane IgM awaits further experimentation.

An important correllary of the conclusion that there is no difference in the sugar content of membrane and secreted IgM is that they may differ in the length of the μ chain. Independent experiments of Yuan et al. (personal communication, 1978) have shown that there are differences in chymotryptic tryptic peptide profiles between membrane and secreted μ chains. It is likely that the putative extra peptide sequence is attached to the C-terminal end of the μ chain. The C-terminal ends of heavy chains appear to be attached to the membrane (Fu and Kunkel, 1974; Hough et al., 1977). Further, Williams et al. (1978) have reported amino acid sequence differences at the C-terminal end of human membrane and secreted μ chains. On the other hand, it has been reported (McIlhinney et al., 1978) that both membrane and secreted μ chains have tryosine as the C-terminal amino acid. A likely position for the attachment of a different and longer C-terminal sequence has been identified (Melcher, 1978b). Two possible roles for this postulated extra C-terminal sequence can be suggested. One, it may provide a structure that can be strongly bound by some integral membrane protein. The interaction imagined could be similar to the binding of IgE to a protein in the membranes of rat mast cells and basophilic leukocytes (Conrad et al., 1975). The possibility that the extra peptide sequence is itself hydrophobic and provides a structure capable of direct interaction with membrane lipids

is favored. The preliminary data of Williams et al. (1978) are consistent with a hydrophobic stretch of amino acids.

Mouse membrane δ chain has a greater percentage carbohydrate (15.5%) content than membrane μ chain (13.3%). These results are consistent with the findings that human IgD has a greater percentage carbohydrate content than human IgM (Perry and Milstein, 1970) and thus further support the identification of this mouse immunoglobulin as IgD. The density of membrane IgD in non-denaturing detergent containing gradients is 0.005 g/cm^3 lighter than that of MOPC 104E IgM, an IgM that bears a glycostylated light chain and thus has a carbohydrate content of 11.7%. Based on the present estimate of the δ chain carbohydrate content of 15.5%, it is calculated that the intact IgD should have a carbohydrate content of 11.5%, very close to that of MOPC 104E IgM. The large difference in density between these two molecules in non-denaturing detergent-containing gradients suggests that IgD, like IgM, must bind appreciable quantities of detergent, a characteristic of integral membrane proteins.

A 'native' mouse membrane δ chain of a size comparable to that of mouse membrane μ chain or human membrane δ chain was not observed in experiments in which various proteolytic inhibitors were incorporated into the preparation and isolation of mouse membrane H chains. These results are consistent with reports from other laboratories in which mouse membrane δ chain has a faster mobility on SDS-polyacrylamide gels than either mouse membrane μ chain of human membrane δ chain (Melcher et al., 1974; Abney and Parkhouse, 1974; Lisowska-Bernstein and Vassalli, 1975; Pernis et al., 1975; Finkelman et al., 1976; Warr and Marchalonis, 1976). However these results are in direct conflict with work reported by Sitia et al. (1977) in which mouse membrane δ chain had a mobility

comparable to that of mouse membrane μ chain and human membrane δ chain on SDS-polyacrylamide gels.

The experimental procedures used in preparation, radioiodination and lysis of mouse spleen cell suspensions in both studies were essentially the same. The techniques used to recover the mouse spleen cell surface IgM and IgD from cellular detergent lysates were different. In the experiments reported here, the membrane Igs were recovered by sequential immunoprecipitation with rabbit anti-mouse IgM and IgA sera respectively, followed by goat anti-rabbit Ig or heat-killed bacteria. Rabbit anti-mouse IgM and IgA sera immobilized on a Sepharose affinity adsorbent were used to recover the membrane Igs by Sitia et al. (1977). It is unlikely that the method of recovery is important in maintaining a 'native' membrane δ chain, if indeed one does exist. Affinity adsorbents used by others to isolate mouse membrane H chains from detergent lysates (Warr and Marchalonis, 1976), resulted in membrane H chains of sizes comparable to those reported here. It is also unlikely that the use of rabbit and goat antisera in the recovery of the membrane H chains is involved in proteolysis of a 'native' membrane δ chain. The substitution of Protein A, a Staphylococcal adsorbent, for goat antiserum in the recovery of the membrane H chains failed to produce a membrane δ chain with a mobility comparable to that of membrane μ chain on SDS-polyacrylamide gels.

The possibility exists that the proteolytic inhibitors used in these experiments were completely ineffective in preventing the degradation of membrane δ chain. The failure to prevent proteolysis could be related to the concentration of the inhibitors used, although the concentration of PMSF and TPCK were on the same order as those shown to be effective by others (Fahrney and Gold, 1963; Hart and Streilein, 1976). Of special

interest were the experiments in which aprotinin and EACA were incorporated into the preparation and isolation procedures of the mouse membrane H chains. 1% EACA, the same concentration used in these experiments, has been shown to be effective in preventing the proteolytic fragmentation of human IgD (Goyert et al., 1977). Further, it has been reported that aprotinin and EACA reduce the proteolytic effects of maternal serum on human neonatal lymphocytes (Panero et al., 1978). Both aprotinin and EACA used at concentrations effective in preventing proteolysis in the human system were not effective in producing a 'heavy' mouse membrane δ chain. In one EACA experiment a mouse membrane μ-like H chain did appear in the membrane δ lane on the SDS-polyacrylamide slab gel. However, it was attributed to the failure to clear all of the mouse membrane IgM from the cellular detergent lysate and was not observed in subsequent repetitions of the same experiment.

There is a possibility that the 'so called' fragmentation of mouse membrane δ chain could be prevented by a proteolytic inhibitor since not all proteolytic inhibitors available were tried. Further, proteolytic enzymes not affected by the proteolytic inhibitor(s) used might also exist. It is important to note that in the experiments of Sitia et al. (1977) the 'heavy' mouse membrane δ chain was observed in the absence of any proteolytic inhibitors incorporated into the experimental procedures. Diisopropylfluorophosphate has been incorporated into membrane H chain preparation procedures (Finkelman et al., 1976); however results were similar to those reported here. That secondary bands were generally observed in the mouse membrane μ and δ chain lanes on the polyacrylamide gels might also support the ineffectiveness of the proteolytic inhibitors used.

If the fragmentation of the mouse membrane δ chain does occur, it must be confined to the N or C-terminal of the H chain. If the mouse membrane δ chain were cleaved at the hinge, one would expect at least two fragments with molecular weight of approximately 30-33K on SDS-polyacrylamide gels under reducing conditions. The δ chain observed in these experiments was always in the 66-63K range.

Whether proteolysis of the mouse membrane δ chain occurs naturally in vivo has not been determined. If it did, incorporation of proteolytic inhibitors into the preparation and isolation procedures of the membrane H chains would probably not be effective.

In contrast, if the protease inhibitors used were effective in inhibiting all proteolytic enzyme activities and mouse membrane δ chain does have a size larger than normally isolated, the larger 'native' membrane δ chain should have been detected. That the larger 'native' δ chain was not detected leads to the conclusion that the 66K membrane δ chain could be the 'native' species on mouse spleen cell membranes. An important piece of information that is not as yet known is the size of mouse serum δ chain. Serum IgD has only recently been reported in the mouse (Woods et al., 1979). One would expect that mouse membrane δ chain would be larger than its serum counterpart. This would be consistent with bovine and mouse membrane and serum μ chains and human membrane and serum δ chains (Haimovich, 1977; Melcher and Uhr, 1976; Finkelman et al., 1976).

A possible explanation for why Sitia et al. (1977) have been able to detect the 'heavy' mouse membrane δ chain and others have not is that two forms of mouse membrane μ chains exist, one being serologically different from the other. One form of mouse membrane μ chain would be cleared from

detergent lysates with rabbit anti-mouse μ chain serum. The other form, not being reactive with the rabbit anti-mouse μ chain serum, would be detected in the rabbit anti-mouse IgA immunoprecipitation. The fact that 'heavy' mouse membrane δ chain was observed when anti-mouse IgD serum was substituted for rabbit anti-mouse IgA serum (Sitia et al., 1977) does not support the possibility of two membrane μ chain forms. However, class identification using antisera that are thought to be class or species specific may be less than reliable. Mouse membrane δ chain must possess some antigenic determinants in common with mouse membrane μ chain since increased amounts of anti-μ chain serum will immunoprecipitate mouse membrane IgD (Lisowska-Bernstein and Vassalli, 1975). It has also been shown that chicken anti-human δ serum recognizes an immunoglobulin on the surface of rat lymphocytes (Ruddick and Leslie, 1977). More recently, a rat myeloma line has been found to secrete an IgD-like protein, antiserum to which recognizes a membrane protein on the surface of rat and mouse spleen cell lymphocytes (Bazin et al., 1978).

I sought to explore the conservation of specific cleavage sites on mouse and human H chains utilizing chemical and enzymatic cleavage techniques. If the placement of cleavage sites along two H chains is conserved, similar fragmentation patterns would be expected. Of course similar fragmentation patterns, at best, would indicate possible conservation of cleavage sites and further structural homology would only be implied. Specific cases can be cited in which conservation of chemical cleavage sites (e.g. methionine residues) parallels a high amino acid sequence homology between two H chains of the same class (Putnam et al., 1973; Watanabe et al., 1973). A high amino acid sequence homology between two H chains of the same class does not insure the conservation

of chemical cleavage sites however (Capra and McCumber, 1979; Putnam et al., 1973).

Mouse membrane μ and δ chains produced similar cleavage patterns when treated with CNBr and chymotrypsin (which cleave at methionine and aromatic amino acid residues respectively) even though in both cases the cleavage was incomplete. The number and sizes of the coincident fragments in the membrane μ and δ cleavage patterns indicate more homology than one would expect for two H chains of different Ig classes. Although the origins of the coincident fragments are not known, it seems unlikely that many coincident fragments could be derived from different parts of the two H chains cleaved. In addition, the sizes of the coincident fragments in the membrane μ and δ chain patterns indicate that they are at least in part derived from constant domain sequences. Some degree of homology would be expected between two H chains through fragments derived from their variable regions sequences. However, only a few fragments of 110 amino acid residues or less were observed due to incomplete cleavage.

The fragment patterns of H chains of the same class that should have produced the most similar cleavage patterns, in fact, did not. The fragment patterns of mouse membrane and MOPC 104E μ chains did produce some fragments that appear to migrate in identical positions but for the most part the patterns were different. The possibility exists that mouse MOPC 104E μ chain is not typical of the IgM class. The MOPC 104E μ chain is unusual among mouse V_H sequences in that it has a methionine residue at position 20. Analysis of the partial amino acid sequence information available for mouse MOPC 104E and mouse ABPC 22 μ chains produced an 85% sequence homology however (Capra, personal communication, 1979; Hood, personal communication, 1979). Ideally, the cleavage patterns of mouse

spleen cell secreted and mouse spleen cell membrane μ chains should have been compared. The inability to observe the ^{3}H labeled spleen cell secreted μ chain versus iodinated spleen cell surface μ chain cleavage fragments via fluorography made this comparison not possible. The cleavage patterns of mouse membrane and human secreted δ chains were different. This may be expected since the placement of methionine residues in Ig constant regions have not been highly conserved during evolution (Putnam et al., 1973; Capra and McCumber, 1979; Adetugbo, 1978; Cunningham et al., 1970; Rutishauser et al., 1970). That the cleavage patterns of mouse membrane μ and δ chains were different from those of myeloma μ and δ chains does not rule out the possibility that the membrane H chains are actually of the IgM and/or IgD classes however.

Whether or not the class distinctions assigned to the mouse membrane IgM and IgD H chains are entirely correct remains unanswered. It is possible that the two mouse membrane H chains could be almost identical in structure yet not react equally with antiserum raised against one of them. Mouse membrane IgM and IgD H chains apparently have similar antigenic determinants since increased concentrations of anti-μ chain serum will immunoprecipitate IgD (Lisowska-Bernstein and Vassalli, 1975). This suggests that when an antiserum is raised against a mouse μ chain, a heterogeneous population of antibodies reacting to that μ chain are produced. Further, the concentrations of individual antibody activities within that population are different. One might envision several major antigenic determinants to which a majority of the antibodies are made and also minor antigenic determinants toward which only small amounts of antibodies are made. Thus as the anti-μ serum concentration is increased in the recovery of mouse μ chain, more antibodies directed towards the

minor antigenic determinants are available to react with δ chain (similar to μ chain in minor antigenic determinants). The determination of major and minor antigenic determinants and the ability to raise antibody against them could depend upon the content and/or distribution of carbohydrate on the H chain or perhaps the conformation of the molecule.

The similarities observed in the membrane H chain cleavage patterns along with the fact that the two H chains are serologically alike suggest that only a single class of Ig is found on the surface of mouse spleen cells. A new class called IgB, due to its restricted appearance on the surface of B cells could be proposed. The differences seen in the antisera reactivities would be consequence of changes in conformation. IgB would exist in two conformations, one which cross reacts with anti-μ serum and the other with anti-δ serum. The δ cross reactive conformation of the β heavy chain would have to be susceptible to proteolytic cleavage in the mouse, partly so in the rat, and not at all in the human and other species. Proof that cell surface H chains are of one class would require the comparison of their amino acid sequences and/or carefully controlled serological characterization.

H chains from different Ig classes produced CNBr and chymotrypsin cleavage patterns that were different. This was expected since a search of existing amino acid sequence information yielded little or no conservation in the placement of at least methionine residues.

Cleavage of mouse membrane IgM and IgD with pepsin produced two $(Fab)_2$-like fragments of 130K and 110K respectively. These results are surprising when compared with the CNBr and chymotrypsin cleavage fragment homology in that if the amino acid sequences of the two membrane H chains are nearly identical, one would expect pepsin fragments of

similar sizes. That either 130K or 110K fragments were not produced does not rule out a similarity of the two membrane H chains. Conformational differences between the two molecules could account for the exposure of a different pepsin cleavage site on either the membrane IgM or IgD. The pepsin cleavage of the intact molecules was performed in SDS, thus the difference sizes of the membrane IgM and IgD fragments may reflect the extent of denaturation of the membrane proteins. This rationale has been used in the past to explain differences observed in the susceptibility of detergent released membrane IgM to papain and trypsin cleavage (Vitetta and Uhr, 1976; Bourgois et al., 1977). The possibility that the 110K fragment seen in the mouse membrane IgD represents a degradation product of a larger $(Fab)_2$ fragment cannot be ruled out.

The papain cleavage of mouse membrane IgM and IgD produced fragments of similar size to those expected. The fragments have not been well characterized, thus no conclusions as to their origins can be made. As in the pepsin cleavages, one would expect similar fragment sizes if the membrane H chains were nearly identical in structure. That similar sized fragments were not observed may be a consequence of the conformation of membrane IgM and IgD or may reflect actual structural differences.

IgD is more susceptible than IgM to papain cleavage when the molecules are attached to the lymphocyte surface (Vitetta and Uhr, 1976; Bazin et al., 1978). In contrast, it has been reported that detergent released membrane IgM and IgD have equal susceptibilities to papain (Vitetta and Uhr, 1976). In the papain cleavages shown here, the membrane IgD appeared to be more susceptible to cleavage in solution than IgM. Similar results have been seen for the cleavage of mouse membrane

IgM and IgD in solution with trypsin (Bourgois et al., 1977). Again the difference in papain cleavage fragments may reflect the extent of denaturation of the membrane proteins since the cleavage was performed in SDS.

The molecular weights of papain released Fab fragments from mouse membrane and human myeloma IgDs have been reported at 60K and 47K respectively (Vitetta and Uhr, 1976; Lin and Putnam, 1979). Thus there appear to be some structural differences between the mouse and human δ chains. Whether differences observed in the fragment sizes between the mouse membrane IgM and IgD reflect structural differences is not known.

I feel that some significant information has been gained in these studies concerning mouse membrane Igs. Much more needs to be done. The advances to be made in this area of research will indeed be of interest in the years ahead. The role of membrane Ig in the activation of the humoral immune response is still, for the most part, a mystery to me and I am sure to many others.

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