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CHARACTERIZATION OF THE RECEPTORS FOR IgE OF RAT BASOPHILIC LEUKEMIA CELLS

by

Ricki M. Helm

A Thesis

Submitted to the Faculty of Graduate Studies in

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ABSTRACT

The present study confirmed the findings of earlier studies that isolation of the receptors for IgE from surface-iodinated, solubilized rat basophilic leukemia (RBL) cells with the aid of IgE, and anti-IgE yields a molecule which, when analayzed by SDS-polyacrylamide gel electrophoresis on 10% gels has an apparent molecular weight of 45,000 daltons. A single molecule of the same molecular weight was also isolated using the DNP-IgE and anti-DNP-Sepharose systems. The isolation of receptors with the aid of IgE-Sepharose confirmed the presence of two receptors having apparent molecular weights of 55,000 daltons and 45,000 daltons, designated as H and R, respectively, in both exogenously (^{125}I) and endogenously (^{3}H-amino acids and ^{3}H-sugars) labelled RBL cell preparations. In addition to H and R, a third receptor was identified having an apparent molecular weight of 71,000 daltons. The binding of all three molecules to IgE-Sepharose could be inhibited by free IgE added to intact cells prior to solubilization, clearly demonstrating their surface orientation and the specificity of these three molecules for IgE.

Evidence for the glycoprotein nature of these three receptors was obtained using lectin affinity chromatography. Studies with lentil lectin and Concanavalin A established that the receptors contained mannose and/or N-acetylglucosamine residues in their carbohydrate moieties. The binding of both H and R receptors to wheat germ agglutinin (WGA) and *Ricinis communis* agglutinins (RCA) suggested that N-acetylglucosamine and galactose, respectively, were present in these molecules. The binding of R receptor to Pea and Gorse lectins, which demonstrate specificity for fucose residues, points to the presence of this sugar in R. These latter lectins bound only R and, therefore, exhibited a selectivity similar to lentil lectin which,

however, bound only small amounts of H. All other lectins could not discriminate between these molecules. Thus, the use of lectins provided some information on the carbohydrate moiety of the receptors, and provided a means of separating H and R receptors.

The incorporation of tritiated leucine and tyrosine precursors into cultured RBL cells established the protein nature of all three IgE receptors, whereas the incorporation of tritiated galactose and fucose into IgE receptors confirmed their glycoprotein nature. These studies with the tritiated amino acids revealed several molecules in addition to those showing definite specificity for IgE. Prominent among these was a molecule with an apparent molecular weight of 26,000 daltons, which continued to be present in eluates obtained from IgE-Sepharose and in receptor complex preparations isolated by DNP-IgE and anti-DNP-Sepharose. This molecule most likely is not exposed to the surface of RBL cells, as it fails to iodinate under normal circumstances; nor is it likely to be a glycoprotein, because it does not label with the tritiated sugars used in this study. The association of this 26,000 dalton protein molecule with one of the receptors (H or R) cannot be excluded.

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LIST OF ABBREVIATIONS

αMM	alpha methyl mannoside
BBS	borate buffered saline
BSA	bovine serum albumin
CLL	chronic lymphocytic leukemia
CNBr	cyanogen bromide
ConA	Concanavalin A
CPM	counts per minute
CTA	classical transplantation antigen
DAAD	direct allogenic anaphylactic degranulation
DNP	dinitrophenyl
EDTA	ethylene diamine tetraacetate
FCS	fetal calf serum
GAR	goat anti-rabbit immunoglobulin
HCL	hairy cell leukemia
HT-29	colon carcinoma cell line
LcH	Lentil lectin
MEM	minimum essential medium
NCS	Nuclear Chicago solubilizer
NP-40	Nonidet P-40 detergent
NRS	normal rabbit serum
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
P-K test	Prausnitz-Küstner test
RAR	rabbit anti-receptor immunoglobulin
RBL	rat basophilic leukemia

List of Abbreviations Cont'd.

RCA	*Ricinus communis* lectin
RER	rough endoplasmic reticulum
R_f	relative mobility
RMC	rat mast cell
SC	secretory component
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis in sodium dodecyl sulfate
SRS-A	slow reacting substance of anaphylaxis
TBS	Tris buffered saline
TCA	trichloroacetic acid
WGA	wheat germ agglutinin

CHAPTER I

INTRODUCTION

[A] HISTORICAL ASPECTS

1. Allergy and Skin Sensitization with Reagin:

The term "allergy" was originally coined to describe a specific altered reaction of an individual following contact with a foreign substance (von Pirquet, 1906). The modern concept of the reaction shown by an allergic individual is described by the stimulation of the immune system by a foreign antigen resulting in the production of antibodies which, upon combination with the inducing antigen, elicits tissue injury. Ramirez in 1919 observed the passive transfer of allergic symptoms following the accidental blood transfusion between an allergic donor and a healthy recipient. That transfer of allergic symptoms was due to the presence of a humoral factor in the serum of allergic patients was first demonstrated by the local passive transfer of specific allergen sensitivities to the skin of non-allergic individuals by the classic experiments of Prausnitz and Küstner (P-K test) (1921). Injection of allergic serum into the non-allergic individual, followed by challenge with the allergen (antigen), produced an erythema-wheal reaction at the site of injection. Subsequently, the passive transfer of immediate hypersensitivity to other allergens was observed and the nature of the specifically reacting substance, termed atopic reagin by Coca and Grove (1925) in the serum of atopic individuals, became the focal point for determining the skin sensitizing factor found in allergic patients' serum. However, the information had to await the advances made in immunochemistry during the 1950's and early 1960's to provide information upon the nature of humoral factors involved and the

nature of antibodies in general.

The actual nature and the chemical and immunological characterization of reaginic antibody required the separation of antibodies into specific classes and subclasses. These distinctions were to be made primarily on the basis of distinct antigenic characteristics. Although antibodies could be separated into distinct classes and subclasses due to differences in net charge, molecular size and other immunological properties, they shared a common feature of having a four-chain structure consisting of two pairs of different polypeptide chains — the heavy and the light chains. By the early 1960's, three classes of human serum immunoglobulins — IgG, IgM and IgA — had been recognized, aided largely by (i) the discovery that myeloma proteins and Waldenström-type macroglobulins were homogeneous proteins of molecular structure related to immunoglobulins present in normal serum and (ii) the timely invention of immunoelectrophoresis. IgG and IgM classes were found to correspond to the 7s and 19s type immunoglobulin (Ig) antibody, respectively, whereas IgA represented a new class of immunoglobulin as determined by immunoelectrophoretic studies. The physicochemical properties of reagin appeared to resemble IgA; thus, it was suggested that IgA may be associated with immediate hypersensitivity. Reaginic activity was shown to be absent in the major Ig fractions of IgG and IgM; however, substantial evidence was presented which showed that the properties of serum reagin resembled those of purified IgA isolated from atopic patients (Heremans and Vaeron, 1962; Fireman *et al.*, 1963), or from normal individuals (Ishizaka *et al.*, 1963). A fourth class of human Ig (IgD) was identified in 1964 with the discovery of yet another myeloma protein with distinct antigenic characteristics. However, it was not until the late 1960's that convincing evidence was

presented, which indicated that neither IgD nor IgA were associated with reaginic activity (Ishizaka and Ishizaka, 1966a). Discrepancies between the IgA fraction of reaginic serum which showed positive sensitizing ability and that of the IgA antibody obtained against blood group substance in serum of hay fever patients, which failed to sensitize, suggested that the reaginic property present in IgA fractions belonged to a very minor component. Using conventional immunochemical methods, it was demonstrated that reaginic antibody did not belong to either IgG, IgM, IgA nor IgD (Ishizaka and Ishizaka, 1966a; Ishizaka *et al.*, 1966a; Ishizaka and Ishizaka, 1966b).

2. Immunoglobulin E:

The first direct evidence for a new class of immunoglobulins came from experiments in which an antiserum raised against a reagin-rich fraction of allergic serum from a ragweed-sensitive patient was absorbed with normal IgG and myeloma IgA proteins. The supernatant fraction from these absorptions showed a band with γ_1 mobility in immunoelectrophoresis with the reagin-rich fraction from the ragweed-sensitive patient, but it failed to give any precipitin bands with any of the classes of immunoglobulins or myeloma proteins. This led to the suggestion of the presence of a new unique immunoglobulin class, tentatively called γ-E (Ishizaka and Ishizaka, 1966b). Results obtained from a correlation study between reaginic activity and γ-E globulin demonstrated that the skin-sensitizing activity in allergic sera was inactivated in parallel with the removal of γ-E globulin by immunoprecipitation with anti-γ-E (Ishizaka *et al.*, 1966c). Furthermore, the association between reaginic activity with γ-E was demonstrated in other allergen-reagin systems (Ishizaka and Ishizaka, 1968a). Among the most convincing pieces of evidence was the demonstration that purified

γ-E from atopic patients' sera sensitized skin for a P-K reaction and that this skin-sensitizing capacity could be removed by antibodies specific for γ-E (Ishizaka and Ishizaka, 1967). By measuring the antigen-binding activity of IgG, IgA and IgM antibodies in the serum of hay fever patients, the P-K titer of the patients' sera correlated with the γ-E antibody concentration, whereas no correlation in antigen-binding activity was obtained between the P-K titer among any of the other antibody concentrations (IgG, IgA and IgM). Thus, the quantitative correlation between γ-E and reaginic activity indicated that the major part of the skin-sensitizing antibody in allergen-reagin systems belonged to γ-E (Ishizaka *et al.*, 1967a).

The antigenic nature of γ-E demonstrated that antigenic determinants for κ and λ light chains, common to all immunoglobulins, were also associated with this Ig; however, immunoelectrophoresis indicated that it was not part of any of the other Ig classes (IgG, IgA, IgM or IgD) (Ishizaka *et al.*, 1967b).

Coincidentally, an atypical immunoglobulin, myeloma protein ND, was discovered by Johansson and Bennich (1967). This protein could not be assigned to either the IgG, IgA nor IgM classes of Ig. Chemical and physicochemical analysis of protein ND demonstrated that it contained 11% carbohydrate, sedimented as a 7.9s component by ultracentrifugation analysis and had an apparent molecular size of 196,000 daltons (Bennich and Johansson, 1968). An exchange of antisera between the Ishizakas' and Bennich's groups was arranged and a comparative antigenic analysis revealed that IgND belonged to the IgE class (Bennich *et al.*, 1969). Subsequently, the biological evidence for the structural relationship between IgND and reaginic antibody was obtained by specifically blocking the P-K reaction with myeloma protein ND (Stanworth *et al.*, 1967). Hence, a second IgE

myeloma protein was discovered in the United States (Ogawa *et al.*, 1967).

Human IgE can be cleaved by papain into Fab and Fc fragments and by pepsin into $F(ab')_2$ fragments (Bennich and Johansson, 1968; Ishizaka *et al.*, 1970). Like other immunoglobulin classes, IgE has a four-chain structure consisting of two heavy or ε chains and two light chains, κ or λ. Heating of the IgE molecules at 56°C for four hours inactivated its skin-sensitizing ability (Ishizaka *et al.*, 1970). IgE antibodies have further been shown not to fix complement (C), even 800 μg of aggregated IgE failed to fix significant amounts of C or C1a (Ishizaka *et al.*, 1970). There is, however, some evidence that aggregated IgE will fix late components of C through the alternate pathway (Ishizaka *et al.*, 1972).

3. Target Organs, Tissues and Cells of Reaginic Hypersensitivity Reactions:

As already discussed, IgE has been shown to take part in P-K reactions in the skin of normal individuals. In addition, IgE antibodies have also been shown to be responsible for the passive cutaneous anaphylaxic (PCA) reaction in monkey skin, which had been sensitized with sera from atopic patients (Ishizaka *et al.*, 1967c). A reversed type allergic reaction, in which human skin was sensitized with IgE and challenged with antibody specific for IgE, was shown to induce an erythema-wheal reaction in normal individuals, whereas antibodies specific for IgG, IgA, IgM and IgD failed to do so (Ishizaka and Ishizaka, 1968b).

One of the most significant organs affected by allergic hypersensitivity reactions is the lung. Goodfriend *et al.* (1966) demonstrated that in monkey lung passively sensitized with reaginic serum, followed by challenge with antigen, induced histamine release, and Brocklehurst (1960) was able to show the release of slow-reacting substance of anaphylaxis (SRS-A) from asthmatic lung by challenge with allergen. Subsequently, IgE was

shown to be the agent responsible for sensitizing monkey lung tissue for the release of both histamine and SRS-A (Ishizaka *et al.*, 1970). Clearly, the sensitization for reaginic hypersensitivity reactions has been shown to involve the binding of reaginic antibodies with tissue constituents and the immunoglobulin largely responsible for antigen-induced histamine release is IgE.

Sensitization of homologous species with IgE antibodies has also been observed at the cellular level. Mast cells were suggested to be the principal target cells in guinea pig ileum that were being sensitized by IgE (Humphrey and Mota, 1959; Mota, 1959). Subsequently, rat mast cells were shown to be actively sensitized by the injection of antigen with *Bordetella pertussis* and to release histamine upon challenge with the antigen (Uvnas and Thon, 1959; Perera and Mongar, 1963; Mota, 1963; Austen *et al.*, 1965). Leukocytes, isolated from atopic individuals, were shown to release histamine upon exposure to the specific allergen to which the person was sensitive (Lichtenstein and Osler, 1964). Following this, Levy and Osler (1966) were able to demonstrate that leukocytes isolated from normal individuals could be passively sensitized with reaginic serum. The sensitization was further shown to be dependent upon temperature, pH and the ionic strength of the medium.

Direct binding studies using radiolabelled IgE myeloma protein confirmed the blood basophil to be the only blood cell type capable of specifically binding IgE (Ishizaka *et al.*, 1970). In contrast, neutrophils, eosinophils, monocytes and lymphocytes failed to bind appreciable amounts of radiolabelled IgE on their surfaces. IgE was further shown to bind specifically to mast cells in the skin, bronchioles, omentum and lamina propia of the small intestine of monkeys (Tomioka and Ishizaka, 1971).

Sullivan *et al.* (1971), with the aid of electron microscopy and hybrid antibody techniques, were able to demonstrate that basophils kept at 0°C during sensitization with IgE had IgE bound in patches distributed around the entire circumference of the cell (patching), while cells kept at room temperature had IgE distributed asymmetrically over one pole of the cell (capping).

Ishizaka *et al.* (1972), using monkey lung cell suspensions and radiolabelled anti-IgE and anti-IgG in conjunction with histamine release, provided further evidence in the support of the correlation between cell-bound IgE and mediator release. Anti-IgE was shown to be firmly bound to mast cells, whereas anti-IgG bound only to neutrophils and macrophages, but not to mast cells. In addition, the release of mediators of anaphylaxis — histamine and SRS-A — was demonstrated only by treatment of the cells with anti-IgE, but not by anti-IgG. Thus, it is clear that mast cells in the tissue and the basophil in the blood are the cells binding IgE and act as specific target cells involved in IgE-mediated hypersensitivity reactions.

4. Homocytotropic Antibodies in the Rat:

It has not been clearly established whether reaginic antibodies are strictly limited to the class of IgE. Some data clearly indicate that similar properties can be attributed to certain subclasses of IgG. The existence, in several species of two major types of homocytotropic antibodies (i.e. they can only be found to bind to target cells of the same or closely related species) has been recognized. Rats, for example, are known to produce two well-defined homocytotropic antibodies whose biological properties have been extensively studied. Rat IgE has been shown to sensitize homologous skin to provoke PCA with a 24-72 hour latent period,

and to prepare peritoneal mast cells and lung tissue for the release of histamine and SRS-A upon challenge with antigen (Mota, 1964; Binaghi and Benacerraf, 1964; Stechschulte *et al.*, 1970; Orange *et al.*, 1970; Morse *et al.*, 1969). The other antibody with homocytotropic property has been identified as IgGa, a subclass of rat IgG (Orange *et al.*, 1970; Morse *et al.*, 1969; Bach *et al.*, 1971; Morse *et al.*, 1968; Orange *et al.*, 1968; Stechschulte *et al.*, 1967). The optimum conditions for *in vitro* sensitization of target cells by these two homocytotropic antibodies in the rat have been published by Bach *et al.* (1971). These studies demonstrated that IgGa could block sensitization by IgE and, therefore, the authors suggested that these two antibody classes interacted with the same receptor (Bach *et al.*, 1971). In a system in which binding of rat IgE could be measured directly (Kulczycki *et al.*, 1974; Kulczycki and Metzger, 1974), the relationship between rat IgGa and IgE was further evaluated (Halper and Metzger, 1976). The results of this experiment indicated that immune complexes of IgGa antibody and DNP-BSA (the antigen to which the IgGa was prepared) could inhibit binding of IgE; however, it proved impossible to demonstrate direct binding of monomeric IgGa by cellular receptors, nor would monomeric IgGa inhibit the binding of IgE to target cells.

In addition to IgGa antibody prepared against DNP-BSA, Halper and Metzger (1976) used monomeric IgG2a, a monoclonal Ig which was isolated by Bazin *et al.* (1973) from the serum and ascites fluid of immunocytoma-bearing rats. This paraprotein was shown to be identical (Halper and Metzger, 1976) to the IgGa antibody prepared according to the conditions described by Bloch *et al.* (1968). A 500-fold excess of either myeloma IgG2a or IgGa antibody could not inhibit the binding of IgE to rat basophilic leukemia (RBL-1) cells. Paraproteins isolated from sera of rats bearing

immunocytomas which were producing IgM, IgA, IgG1 and IgG2c were also ineffective in the inhibition of IgE-binding. As pointed out above, the only effective inhibitors of IgE binding to RBL-1 cells were found to be immune antigen-antibody complexes containing antibody of the IgG2a class. To explain the inhibition by immune complexes, Halper and Metzger (1976) postulated that the weak affinity of IgG2a was amplified by the extensive multipoint attachment of IgG2a immune complexes.

Minute amounts of IgG have been demonstrated on the surface of human basophils. However, the concentration of labelled anti-IgG needed for the identification of IgG on these cells was 100 - 1,000 times higher than that required for anti-IgE to demonstrate the presence of IgE in analogous experiments (Ishizaka *et al.*, 1972). Furthermore, anti-IgG was shown to be a much weaker histamine releaser on human basophils than anti-IgE. In addition, IgG, but not IgE, which bound to mouse mast cells, could be removed by simple washing of the cells (Vaz and Prouvost-Danon, 1969). Clearly, antibodies of the IgE class have been recognized for their high affinity for target cells (mast cells and basophils), while subclasses of IgG demonstrate a much reduced affinity for these same target cells.

[B] MAST CELLS AND BASOPHILS

1. Vasoactive Amine Releasing Cells Involved in Immediate Hypersensitivity Reactions:

The origin of mast cells and the manner of their formation have been largely based on histological and morphological studies. Mast cells have been defined as connective tissue elements which possess cytoplasmic granules that are stained metachromatically by basic aniline dyes. As a connective tissue cell, it is distinct from the closely related basophil,

which is blood-bound and myelogenous; however, they both exhibit numerous similarities. They are alike in containing cytoplasmic granules in which vasoactive amines, such as histamine and serotonin, are stored. Furthermore, both cells can be passively sensitized by reaginic antibodies and triggered by antigen to release their granules. As indicated, the prominent feature of mast cells is that their cytoplasm contain granules possessing strong affinities for cationic dyes. Heparin, a sulfated mucopolysaccharide, displays such a strong metachromasia and it is well established that mast cells contain granules with high concentrations of heparin, as well as histamine (Riley and West, 1953; Lagunoff, 1974). Mast cells have further been identified as large mononuclear cells which are normally present in tissues, especially around small blood vessels and just beneath epithelial and epidermal surfaces (Seyle, 1965; Padawer, 1963). Basophils, the blood-borne granulocyte, has also been shown to contain large amounts of stored histamine in cytoplasmic granules (Ehrich, 1953; Graham *et al.*, 1955; Valentine *et al.*, 1955).

2. Mast Cell Genesis:

Although the origin and fate of mast cells is still uncertain, it is definitely known that basophils are not the precursors of mast cells. Basophils arise in the bone marrow from the same stem cell as other polymorphonuclear leukocytes (Dvorak and Dvorak, 1975). Upon maturation, these cells normally leave the marrow and remain in the circulation. Mast cells are thus described as the vasoactive amine containing cells of the tissue and the basophils as the principal vasoactive amine containing cells of the blood. The prominent function of both cell types is that the release of these vasoactive amines once sensitized by antibody and challenged by specific antigen.

An *in vitro* approach to the question of mast cell genesis was taken by culturing suspensions of thymus cells using mouse models (Ginsburg and Sachs, 1962; Ginsburg, 1963; Ginsburg and Lagunoff, 1967). Ishizaka *et al.* (1976) applied these principles and were able to demonstrate the differentiation of mast cells by long term culture of rat thymus cells on embryonic cell monolayers, suggesting that both thymus and embryo tissues contained precursors of mast cells. These cultured mast cells contained significant amounts of histamine and could be passively sensitized with rat IgE antibody for antigen-induced histamine release. They were shown to contain metachromatic granules morphologically similar to normal peritoneal and thoracic mast cells and to possess specific receptors for IgE on their surfaces. In subsequent experiments, Ishizaka *et al.* (1977a) presented findings which indicated that the mast cells obtained from *in vitro* culture had biologic functions similar to those of normal peritoneal mast cells. However, *in vitro* cell populations were determined to be largely immature, based primarily on the measurement of histamine content and on the staining intensity of the metachromatic granules. Although these conditions provided a source of IgE-free mast cells, the time and expense, as well as the numbers of cells obtained, made cultured mast cells an unlikely source of IgE-receptor bearing cells for large scale receptor isolations.

[C] CELL SURFACE INTERACTIONS

1. Ligand-Receptor Interactions:

Since many aspects of cell behaviour are believed to be regulated at the level of the cell surface, a complete understanding of the cell surface in the regulation of cell behaviour relies ultimately not only on the isolation and structural analysis of receptors, but also upon the

knowledge of their orientation within the lipid bilayer, as well as the structure of the inner surface and its association with the cytoplasmic milieu. Recognition of a foreign substance is an essential requirement for a cell and considerable progress has been made in the identification, isolation and purification of a variety of membrane localized receptors.

By definition, a true receptor is a distinct molecular entity whose function is to bind an endogenous ligand and thereby achieve a physiological effect (Goldstein *et al.*, 1979). The resulting recognition phenomenon between receptor and ligand is referred to as a ligand-receptor interaction. The receptor should normally be a product of the cell itself and the binding of specific ligand to the receptor site should be a necessary but not necessarily a sufficient step in a subsequent sequence of changes in the cell. The receptor must further meet the qualifications of (i) saturability, i.e. the cell should possess a finite number of sites which can be filled at high ligand concentration; (ii) specificity, i.e. the cell should demonstrate a preference for binding of a known effector ligand compared to negative control; and (iii) it must demonstrate a high affinity interaction (Dorrington, 1976).

2. Plasma Membrane Structure:

According to recent theories, the plasma membrane consists of a bimolecular phospholipid layer which is interrupted by protein and glycoprotein molecules (Singer and Nicolson, 1972; Marchesi *et al.*, 1972; Winzler, 1970). It was further proposed that the lipid bilayer forms a continuous phase which is interrupted or partially interrupted by a class of relatively hydrophobic (intrinsic) proteins inserted into the bilayer and held tenaciously by hydrophobic interactions. Other proteins (extrinsic) are largely associated with the membrane by ionic or hydrogen binding

forces. They can be easily extracted by alterations in ionic strength, pH, by the use of chelating agents or by protein pertubants, whereas intrinsic proteins require much more drastic treatment for their dissociation. Investigations involved in the elucidation of detailed molecular architecture of plasma membranes indicate that glycoproteins of the cell membrane are largely intrinsic proteins anchored by hydrophobic interactions in the lipid layer with the carbohydrate groups facing the outside. The polypeptide portions may be anchored in the lipid by sequences of hydrophobic amino acid residues that transverse the bilayer, but in some cases several polypeptides may collectively form a complex that transverse the bilayer so that they are in constant contact with both external and cytoplasmic environments. They can thus provide a means of communication across the lipid bilayer either for the flow of solutes and water, or for "signals" in response to external "messengers", such as hormones, antibodies or other cells. Thus, glycoproteins play a specific role in the response of cells to environmental substances or stimuli. The response involves three types of events which involve the plasma membrane of the cell. The first can be classified as recognition (specific binding) of an extracellular molecule (hormone, antibody, lectin, soluble glycoprotein, or the glycoprotein of another cell) by the receptor. The second involves a triggering reaction (a form of communication) across the membrane, which may or may not involve the receptor directly. Finally, the third type involves interactions with the inner face of the membrane with cytoplasmic milieu. Eventually these interactions lead to a response of the cell which modulates the environment.

3. Plasma Membrane Glycoproteins:

Numerous studies are available which have established that there are

major surface components exposed to the external environment of the cell that serve as receptors for various antibodies, lectins, hormones and viruses. Although the assembly of animal cell membranes is still obscure, considerable information regarding the constituents of the plasma membrane has been obtained. Cell surface components, which mediate cellular behaviour, are frequently glycoproteins and are firmly associated with the plasma membrane, dipping into and even spanning the lipid bilayer. The close association of carbohydrates with the cell membrane has been demonstrated using cytochemical techniques, such as periodic-acid Schiff (PAS) reagent, and was shown to be a characteristic feature of all cell surfaces except in the region of junction complexes (Rambourg *et al.*, 1966).

In all cells, glycoproteins are present in lysosomes and some cell membranes; in the plasma membrane, carbohydrate-containing external portions form the "cell coat". Electron microscopic observations of this carbohydrate-rich cell coat, present at the surface of cells in rats, confirmed earlier light microscopic observations (Rambourg and Leblond, 1967). These glycoprotein molecules were usually constructed of a nonpolar or hydrophobic portion embedded within a lipid bilayer with a polar or hydrophobic portion extending outward from the exterior surface. This polar portion has been shown to contain glycosylated side chains composed of amino sugars, neutral sugars including fucose and sialic acid residues (Spiro, 1970; Winzler, 1970).

The intracellular pathway of glycoprotein biosynthesis has been examined over the years by experiments in which tissues have been pulsed, *in vitro* or *in vivo*, using various radioactive precursors of glycoprotein material. The label is then examined by means of radioautography of tissue

sections or by liquid scintillation spectrophotometric analysis. Radioactively labelled L-fucose, a monosaccharide precursor shown to be largely incorporated as a fucose residue into secretory and membrane glycoproteins, and to a lesser extent, into glycolipids (Bekesi and Winzler, 1967; Kaufman and Ginsburg,1968; Herscovics, 1970), has been used to study the rate of glycoprotein degradation and turnover for the plasma membrane of eukaryotic cells (Bennet and Leblond, 1970; Atkinson and Summer, 1971; Bennet *et al.*, 1974, Riordan *et al.*, 1974; Atkinson, 1975). The turnover of cell coat glycoproteins was shown to be associated with (i) the purposeful secretion of these molecules, and (ii) the loss of material resulting from the participation of glycoproteins on surface activities such as intercellular adhesion, uptake of antigens, interactions with viruses and other noxious agents, active transport across the membrane, pinocytosis and phagocytosis (Winzler, 1970; Hughes, 1973).

In the synthesis of proteins and glycoproteins, the polypeptide chain is first elaborated on the ribosomes of the rough endoplasmic reticulum (RER). In the case of many glycoprotein molecules destined for secretion or insertion into membranes, the sugars of the "core" of the complex side chains are added within the RER, whereas "peripheral" sugars are added one by one within the Golgi apparatus (Bennet and Leblond, 1977; Leblond and Bennet, 1979). Newly synthesized glycoproteins have been shown to migrate from these Golgi saccules to plasma membranes and lysosomes, further suggesting that there is a renewal of glycoprotein structure of these two organelles (Bennet *et al.*, 1974; Haddad *et al.*, 1977).

[D] RECEPTORS FOR IMMUNOGLOBULINS

The binding of immunoglobulin (Ig) complexes to lymphocytes was described

a number of years ago (Uhr and Phillips, 1966). The phenomenon was further characterized when the interaction was shown to be a function of the Fc portion of Ig (Basten *et al.*, 1972a; 1972b), thereby leading to the concept of Fc receptors (FcR) (Paraskevas *et al.*, 1972). FcR is an entity which has been operationally defined as a site on the plasma membrane which is capable of binding the Fc portion of an immunoglobulin. Since then, immunoglobulin receptors have been reported on the surface membranes of a number of different cell populations. The presence of Fc-binding receptors has been reported on surface membranes of macrophages and monocytes, neutrophils, and B lymphocytes as well as other non-lymphoid cell types, including placental cells and fetal yolk sac membranes, virus infected fibroblasts and various neoplastic tissues (Zuckerman and Douglas, 1978).

1. Fcγ Receptors on Lymphocytes and Macrophages:

Monomeric immunoglobulins of the IgG class usually bind weakly to cells, therefore, most investigations have been directed toward studying the interaction of multivalent complexes of IgG with various types of cells. Among the several approaches taken, most involve the labelling of receptor-bearing cells with heat denatured or chemically aggregated immunoglobulin, antigen-antibody complexes or antibody-coated erythrocytes (Dickler, 1976). These techniques have been effective in the identification of cells possessing IgG receptors, however, they give little quantitative information about affinities and densities of these receptors.

Within the multitude of FcR systems, the lymphoid and macrophage cell populations of the murine immune system have served as prototype models for FcR of the IgG class, and as such have been subjected to extensive investigation. These studies have revealed primarily two different populations of receptors: (i) FcR with m.w. of 45,000 to 70,000 daltons comp-

rising the major cell surface protein components which are frequently accompanied by smaller components believed to be degradation products (Rask *et al.*, 1975; Wernet, 1976; Cooper and Sambray, 1976; Loube *et al.*, 1978); and (ii) FcR with a larger m.w. of 100,000 to 130,000 daltons (Premkmar-Reddy *et al.*, 1976; Molenaar *et al.*, 1977; Frade and Kourlisky, 1977; Bourgois *et al.*, 1977).

The FcR binding proteins which have shown specificity for IgG have largely been identified using two experimental procedures; (i) affinity chromatography and (ii) co-precipitation of proteins with the use of immune complexes. With regard to the chemical nature of murine FcR, Rask *et al.* (1975) demonstrated that FcR proteins, with m.w. of 65,000, 18,000 and 15,000 daltons, as estimated by SDS-PAGE, could be isolated from murine spleen cell extracts by chromatography on heat-aggregated human IgG coupled to Sepharose. Frade and Kourlisky (1977) characterized an FcR, found in detergent extracts of a T cell lymphoma, as a glycoprotein with an apparent m.w. of 110,000 daltons; subsequent reduction of this FcR gave five bands when analyzed on SDS-PAGE with apparent m.w. of 56,000, 36,000, 25,000, 18,000 and 15,000 daltons. Mouse FcR precipitated from lysates of surface-labelled lymphocytes, macrophages and fibroblasts were shown to be molecules with an apparent m.w. of 120,000 daltons; however, this molecule was shown to be extremely sensitive to proteolysis (Bourgois *et al.*, 1977). Cooper and Sambray (1976) were able to isolate a protein with a m.w. of 45,000 daltons shed from a murine leukemia cell line (L1210) following surface-crosslinking with aggregated human IgG and anti-human IgG. However, IgG-binding proteins of m.w. of 65,000, 45,000 and 28,000 daltons were recovered from glycoprotein extracts of ^{125}I-labelled L1210 plasma membranes isolated by chromatography on human IgG-Sepharose (Cooper and Sambray, 1977). Fc-binding activity in culture supernatants of several

FcR positive B and T cell lines have been reported by Molenaar *et al.* (1977). Material released from these cells was isolated on non-aggregated human IgG-Sepharose and found to bind IgG and IgM. This FcR-like material appeared to be bivalent and had a m.w. of 100,000 daltons, as estimated by gel filtration (Molenaar *et al.*, 1977).

A murine tumor cell line ($P388D_1$) (Koren *et al.*, 1975) with macrophage-like characteristics has been extensively studied with respect to Fcγ-receptor. Binding of monomeric radioiodinated mouse IgG2a with an association constant of $10^8 M^{-1}$ was shown to be trypsin-sensitive by Unkeless and Eisen (1975). In an analysis of cell lysates and spent culture fluid for $P388D_1$, Anderson and Grey (1977) demonstrated FcR activity which behaved as a high-density lipoprotein in buoyant-density ultracentrifugation analysis. Loube *et al.* (1978) also investigated $P388D_1$ cells and, using lactoperoxidase catalyzed surface-iodination and detergent lysis with NP-40, found that a protein with an apparent m.w. of 57,000 could be isolated by affinity chromatography on human IgG1 and mouse IgG2a. Haeffner-Cavaillon *et al.* (1979), using a Scatchard analysis of the binding of murine IgG2a and IgG2b to $P388D_1$, confirmed the existence of at least two classes of Fc receptors earlier suggested by cross-inhibition studies (Unkeless and Eisen, 1975; Heusser *et al.*, 1977; Anderson and Grey, 1978).

The isolation of FcR on B cells, T cells, macrophages, thymus cells and fibroblasts with similar molecular weights suggested two alternatives: (i) there exists a family of structurally related distinct molecules which have arisen from a common precursor during the course of cellular evolution; or (ii) there exists one unique FcR structure common to many cell types, but local environments produced by different cell surfaces moderated the properties of the molecule (Bourgois *et al.*, 1977). A comparison of the data available on the macrophage receptors for homologous and heterologous

IgG in a number of different species led Haeffner-Cavaillon *et al.* (1979) to suggest that cytophilic structures diverged in parallel with cell surface receptors and that in the mouse, rabbit and human macrophage, FcR could be considered to be structurally related. There are a number of proposals available attempting to provide insight into the nature of FcR on different types of cells and the structural relationship which may exist; however, it will require more clearly defined data before these theories can be verified.

The data on Fcγ binding proteins at most are still very confusing. The studies discussed above differ chiefly in species, cell type and with the type of solubilizing agents employed, as well as the methodology by which the identification and isolation were made. Among the cell types used, there appears to be no consistency in the cell maturity, defined either on the basis of the cell lineage, functional significance, the stage of the cell cycle, or the state of activation. These parameters as well as the possibility of non-specific interaction of other components during the isolation schemes make the comparisons of the FcR among cell populations difficult. However, there is some consistency in the investigations, whereby FcR with molecular weights in the region of 50,000 - 60,000 daltons and, in addition, several FcR around the 110,000 - 120,000 dalton region, have been suggested. The variability in the estimates of the molecular weights of FcR in these regions may simply reflect differences in cell types from various species, whether the cells are obtained either from animals or from tissue culture, and/or the variable molecular weights may represent technical artifacts in the isolation and analytical procedures. Alternatively, it can be suggested that there is an actual heterogeneity of FcR's described for the different cells investigated.

2. Fc Receptors for IgM (FcμR):

The receptors for the Fc portion of IgM (FcμR) have only recently been described on human lymphocytes and has prompted a considerable amount of interest.

Such receptors were first described on normal human peripheral T cells following a period of *in vitro* incubation in IgM-free media containing fetal calf serum (Moretta *et al.*, 1975; McConnell and Hurd, 1976). Using a rosette assay, these authors described a receptor for IgM, which could be removed by treatment with pronase, required protein synthesis and demonstrated specificity, whereby the inhibition of IgM-rosette forming cells was obtained with human IgM, but not by IgG molecules.

Ferrarini *et al.* (1977) were able to detect a FcμR on B cell enriched populations of cells from the tonsils and in the peripheral blood of normal individuals. Inhibition of IgM-forming rosettes with IgM fragments showed that the FcμR had an affinity for the Fc portion of the IgM molecule. However, like the T cells described by Moretta *et al.* (1975), an overnight cultivation of IgM-free media was required, suggesting that like T cells, the FcμR on normal B cells was blocked *in vivo* by serum IgM (Ferrarini *et al.*, 1976; Moretta *et al.*, 1975).

During the course of investigations on normal human peripheral blood lymphocytes, Pilcher and Knapp (1977) reported the presence of IgM receptors on pathological B cells from patients with chronic lymphocytic leukemia (CLL). In contrast to the studies on normal patients' blood lymphocytes, the FcμR could be demonstrated before cultivation in serum-free media. The FcμR continued to be sensitive to proteolytic treatment, but could be shown to re-express receptor activity following overnight cultivation in serum-free media.

Burns *et al.* (1977) presented results which confirmed the findings of

Pilcher and Knapp (1977) in which CLL patients had FcμR on their lymphocytes. Subsequently, Burns *et al.* (1979) introduced a second pathological B cell source in patients with hairy cell leukemia (HCL), which simultaneously expressed a different and distinct receptor for the Fc of IgG. The specificity of the FcμR was demonstrated by inhibition experiments using IgM and Fcμ, but not by $F(ab')_2\mu$ fragments or by IgG, either in its monomeric or aggregated forms.

Further investigations of enriched populations of peripheral human B lymphocytes showed that these cells expressed both FcγR and FcμR simultaneously (Pilcher and Broder, 1978). The FcγR values decreased in culture at 37°C, whereas cultivation of normal B cells for 24, 48 and 72 hours enhanced the FcμR expression. Furthermore, FcμR could be removed by trypsinization, but were shown to be re-expressed following cultivation at 37°C, whereas the FcγR were insensitive to trypsinization.

3. Other FcR:

Blood platelets have also been suggested to have a membrane receptor for the Fc fragment of immunoglobulin G (Pfueller and Luscher, 1972; Henson and Speigelberg, 1973). Cheng and Hawiger (1979) described a procedure for the isolation from human platelets of a glycoprotein with specific binding properties toward the Fc fragment of IgG. By utilizing the immobilized Fc fragment of IgG on Sepharose 4B and solubilized material from human platelets, a KBr eluate of insolubilized material was isolated. Characterization of the eluted material by SDS-PAGE on 5% gels showed a single band with an apparent weight of 255,000 daltons which, upon reduction with 2-mercaptoethanol, dissociated into a major band with an apparent molecular weight of 55,000 daltons. It was identified as a glycoprotein by periodic acid-Schiff-positive staining. Experiments in which the forma-

ation of an *in vitro* complex between the isolated glycoprotein and aggregated IgG documented the binding specificity of the isolated molecule.

Infection of cultured human fibroblasts with cytomegalovirus (CMV) induced the formation of an IgG receptor, which could be shown to react with IgG by direct and indirect fluorescent antibody tests (Keller *et al.*, 1976). To gain further insight into the chemical and biological functions of human CMV-induced formation of IgG receptors, Sakuma *et al.* (1977) isolated and characterized this receptor by immunoprecipitation techniques and polyacrylamide gel electrophoresis in SDS. The IgG receptor was shown to migrate as a single peak in 7.5% gels with an apparent molecular weight of 42,000 daltons and it was further characterized as a glycoprotein by incorporation studies using (^{14}C)-glucosamine and ^{3}H-amino acids.

Secretory component (SC) is known to be a glycoprotein constituting an integral part of the s-IgA molecule. Recent results have validated the concept that SC functions as an immunoglobulin receptor (Crago *et al.*, 1978; Socken *et al.*, 1979; Orlans *et al.*, 1979). SC was identified as the receptor for polymeric immunoglobulins of the IgA and IgM classes on a colonic carcinoma cell line (HT-29) and epithelial cells of human fetal intestines (Crago *et al.*, 1978). SC-containing secretory IgA failed to bind to the surface of HT-29 cells and trypsinization abrogated surface binding of polymeric immunoglobulins. These experiments and the inhibition of immunoglobulin binding by the addition of anti-SC reagents confirmed SC as a receptor on HT-29 cells and epithelial cells of the human fetal intestine.

Rat hepatocytes grown in short-term monolayer culture have also been shown to bind radiolabelled polymeric IgA, but not IgG (Orlans *et al.*, 1979). Binding was again shown to be inhibited by an antiserum to rat SC as well as by unlabelled polymeric IgA. Similar results were obtained by

Socken *et al.* (1979) using isolated rat hepatocytes and cultured hepatocytes, which were found to be synthesizing SC. The binding of polymeric immunoglobulins could be abrogated by trypsinization.

On the whole, the data on the molecular nature of the Fc receptor still lack a unifying concept. Differences observed may be intrinsic ones, characteristic of different cell sources and species; they may also reflect variation in the methodology used for receptor isolation and characterization and, finally, they could be due to the presence of various enzymes in cell lysates or on cell membranes.

[E] THE IgE RECEPTOR

One of the best characterized FcR to date is that for IgE. This receptor plays a role in immediate type hypersensitivity reactions, in which the interactions of a specific antigen with a specific ligand-receptor complex on target cells initiates the release of pharmacological agents into surrounding tissues. The concept of a receptor which plays a role in anaphylactic reactions was first proposed by Dale (1913) and Weil (1913), long before anyone recognized the role of mast cells in this reaction, or the nature of the immunoglobulins which were involved. By definition, anaphylaxis is dependent upon humoral antibody. Anaphylaxis is an immunological reaction in which previous sensitization with a specific antigen has elicited homocytotropic antibodies, which home onto target cells such as mast cells and basophils. Renewed presentation of the same specific antigen and binding of the same by cell-bound antibody causes sudden release of short-lived pharmacologically active agents.

The selective binding of IgE to basophils and mast cells suggested the presence of a specific receptor for IgE on these cells, and prompted the search for the molecule(s) responsible for this binding. Indeed, the

isolation and characterization of the receptors for IgE should open the way for an understanding, at the molecular level, of the triggering mechanisms of allergic reactions.

1. Quantitative Estimates:

Ishizaka *et al.* (1973), using the C1 fixation and transfer test, investigated the binding parameters of the interaction between human IgE and human basophils. The results enabled them to determine the number of cell-bound IgE molecules and receptor sites, which further allowed them to estimate the affinity of the IgE molecule for the receptor. The conversion of IgE sites on the membrane of the cells to C1 fixation sites was achieved by the addition of excess anti-IgE and complement. The number of C1 sites was then estimated by measuring the hemolysis of sensitized sheep red blood cells. The number of IgE sites was directly proportional to the number of C1 fixation sites and revealed 10,000 to 40,000 molecules of IgE per basophil granulocyte. However, when the basophil-rich fraction was incubated with 100 μg to 1 mg per ml of E myeloma protein to saturate the receptor sites, the total number of IgE receptor sites on the basophils was shown to be 30,000 to 100,000 (Ishizaka *et al.*, 1973). Because the basophils were present *in vivo*, it was assumed that the cell-bound IgE and serum IgE would be in equilibrium as represented by the equation:

$$K = \frac{(\text{receptors combined})}{(\text{serum IgE})\ (\text{free receptor})} = \frac{a \times r}{(\text{IgE})\ a(1-r)} = \frac{r}{(\text{IgE})\ (1-r)}$$

where a = number of receptors per cell
r = proportion of receptors occupied by IgE
(IgE) = the IgE concentrations in the serum

Thus, they calculated an association constant of 10^8 to $10^9 M^{-1}$ (Ishizaka *et al.*, 1973). The major obstacle in this indirect method of receptor site number enumeration is the requirement for a complement-fixing anti-

IgE. However, by using a high affinity antibody preparation, demonstrating a specificity for a limited region of IgE, the chance that the number of molecules of anti-IgE bound was the same as the number of IgE molecules bound on the surface of the basophils was maximized.

Electron microscopy has also been used as an indirect method for the visualization of the number of IgE molecules bound to target cells. Using human basophils, human IgE, burro anti-IgE and hybrid rabbit anti-burro IgG-anti-horse ferritin antibodies, Sullivan *et al.* (1971) tentatively estimated that there were 4×10^5 receptor molecules per basophil.

The simplest and most direct method of studying binding parameters of specific ligands to receptors makes use of a radiolabelled ligand. The direct binding of ^{125}I-IgE to RBL cells and RMC has been used by various investigators (Ishizaka *et al.*, 1972; Kulczycki *et al.*, 1974; Conrad *et al.*, 1975; Ishizaka *et al.*, 1975; Ishizaka *et al.*, 1976; Mendoza and Metzger, 1976), because such IgE preparations can be made to meet several requirements needed for such experiments, i.e. (i) IgE preparations must be free of "unbound" radioactivity; (ii) the specific activity must be accurately known and (iii) the percentage of radioactivity capable of binding to cells must be high and must be known.

The binding of IgE to RBL-1 cells (Kulczycki and Metzger, 1974) and to cell-free particles (Metzger *et al.*, 1976) suggested that the binding is governed by a simple reversible bimolecular reaction (Rossi *et al.*, 1977):

$$\text{IgE} + \text{Receptor} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Receptor-IgE complex}$$

in which the equilibrium constant, K_a, can be defined as follows:

$$K_a = \frac{k_1}{k_{-1}}$$

where k_1 is the forward rate constant and k_{-1} is the reverse rate constant.

Ishizaka and Ishizaka (1973) were able to demonstrate the reversibility of IgE-receptor interaction by using competitive inhibition experiments. Unlabelled IgE, 50 - 100 fold in excess to radiolabelled IgE, was shown to displace membrane-bound ^{125}I-IgE myeloma protein. This assumes, of course, that the large excess of unlabelled IgE will prevent the re-binding of ^{125}I-IgE to vacated receptors. The reversibility of the IgE-binding by receptor was also demonstrated when the pH was reduced to 4.0; IgE was shown to dissociate from the cell surface, however, upon raising the pH to neutrality, IgE was found to reassociate with its receptor on the surface of the cell (Ishizaka and Ishizaka, 1974). However, when these considerations are taken into account and the proper measures taken, the direct binding of radiolabelled ligands to specific receptors can prove to be highly beneficial for the identification of cellular receptors.

Kulczycki *et al.* (1974), using purified IgE from rat reaginic serum induced in rats by the injection of *Nippostrongylus brasiliensis* demonstrated that RBL-1 cells were able to specifically bind this radiolabelled IgE protein. Kulczycki and Metzger (1974) also demonstrated that the binding properties of the IgE myeloma protein (IR162) were indistinguishable from those observed for reaginic antibody preparations used previously. The number of receptors or binding sites per cell varied between 3×10^5 to over 1×10^6 in these studies. The forward rate constant (k_1) was calculated to be $9.6 \times 10^4 M^{-1} sec^{-1}$ and the average value of k_{-1}, the rate constant of dissociation, was $\geq 1.6 \times 10^{-5} sec^{-1}$. Ka calculated from $\frac{k_1}{k_{-1}}$ had a minimum value of approximately $6 \times 10^9 M^{-1}$. The binding was further shown to be insensitive to pH's between 6 to 8, but at pH 3.0, complete dissociation of bound IgE occurred in approximately one minute at 0°C.

Conrad *et al.* (1975) studying rat peritoneal mast cells (RMC) and RBL cells determined by direct binding assays using radiolabelled rat mono-

clonal IgE, that both cell types had a similar affinity for rat IgE. The IgE bound to RMC or RBL cells was corrected for non-specific binding using P815 mouse mastocytoma cells as non-binding controls, and the data plotted according to the Scatchard equation (Scatchard, 1949):

$$r/c = nK - rK$$

where

r = number of IgE molecules bound per cell

c = total "fixable" IgE minus the bound IgE expressed in terms of molecules/ml

n = total number of binding sites per cell

K = equilibrium constant

The number of receptors per cell showed RBL cells to have a substantially higher number of receptor sites per cell than those of RMC — approximately 6×10^5 versus 3×10^5, respectively. However, the equilibrium constant, which was determined from the slope of the straight line, was of the order of $10^9 M^{-1}$ for both cell types (Conrad *et al.*, 1975).

The number of receptor sites per cell on RMC reported by Conrad *et al.* (1975) is very similar to those reported by Mendoza and Metzger (1976) and Ishizaka *et al.* (1975). Mendoza and Metzger (1976) calculated 2 to 5×10^5 sites per cell for RMC and 5×10^5 sites per cell for mouse mast cells. Normal rat mast cells and RBL cells and a mouse mastocytoma cell line all showed similar properties, i.e. $k_1 = 10^5$, $k_{-1} = 10^{-5}$ and, therefore, a $K_a = 10^{10}$. It should perhaps be pointed out that the mouse mastocytoma later proved to be an RBL cell line (Siraganian and Metzger, 1978). Ishizaka *et al.* (1975), in a study on the properties of rat mast cell form rats infected with *N. brasiliensis*, found the average number of receptors on normal RMC to be 3 to 8×10^5 per cell, whereas on mast cells taken from infected animals, the number of available receptors was only 10% of that found on normal cells. These results indicated that IgE receptors on mast cells of infected

animals were occupied by IgE.

Carson and Metzger (1974), studying the redistribution phenomenon of RBL-1 cells, demonstrated that the receptors for IgE were diffusely distributed over the surface of these cells and that they could be capped. This study, in relation to those carried out previously, demonstrated the redistribution of receptor-IgE complexes into "patching" and "capping" using anti-IgE (Sullivan *et al.*, 1971; Ishizaka *et al.*, 1974; Becker *et al.*, 1973) and clearly established that the receptor was mobile in the plane of the plasma membrane. In conjunction with the reversibility of IgE binding, especially the dissociation at low pH values and the relative stability at or near neutrality (Ishizaka and Ishizaka, 1974), these results suggest an association of IgE with its receptor on the ectomembrane surface.

2. Isolation of the Receptor(s) for IgE:

In order to isolate the receptor for IgE from either RMC or RBL cells, these cells are first surface iodinated by the lactoperoxidase catalyzed reaction. Following solubilization of the cells with the appropriate non-ionic detergent, several methods for receptor isolation can be employed. Initial attempts made use of a sandwich preparation technique, which involved rat myeloma IgE (IR162), ε-specific rabbit anti-IgE and goat anti-rabbit Ig (Conrad and Froese, 1976). More recently, only precipitates with IgE and rabbit anti-IgE were used or, alternatively, insolubilization of receptor-IgE - rabbit anti-IgE complexes was achieved with protein-A-Sepharose (Conrad and Froese, 1978a).

Isolation of IgE-receptor complexes free of other immunoglobulins was accomplished by the use of IgE, to which either dinitrophenol (DNP) residues (Conrad and Froese, 1978b) or arsanilic acid molecules (Kanellopoulos *et al.*, 1979) were coupled. After formation of IgE-receptor complexes with

the haptenated IgE, the complexes were adsorbed onto conjugates of Sepharose with the appropriate anti-hapten antibody. Complexes were then eluted with the addition of the corresponding free hapten.

Isolation of receptors free of IgE was achieved by a number of investigators with IgE-Sepharose (Conrad and Froese, 1978a; Rossi *et al.*, 1977; Kulczycki and Parker, 1979). Elution was accomplished with either 3M KSCN (Conrad and Froese, 1978a) or 0.5N acetic acid or 6M guanidine HCl (Kulczycki and Parker, 1979).

3. Nature of the Receptors for IgE:

To determine the molecular weights of the solubilized receptors, initial attempts in several laboratories involved either gel filtration or density gradient centrifugation in the presence of non-ionic detergents. Gel filtration on Agarose A- 1.5 of IgE-receptor complexes, prepared by the addition of ^{125}I-IgE to whole RMC or RBL cell extracts, yielded an approximate apparent molecular weight of 3.5 - 5.5 x 10^5 daltons (Conrad *et al.*, 1976). Subtracting the molecular weight of IgE left a molecular weight of the order of 2 - 4 x 10^5 daltons for the receptor. An apparent molecular weight of 410,000 daltons for IgE-receptor complexes was obtained by Rossi *et al.* (1977) using gel filtration on Sepharose 6B, while the molecular weight of the free receptor of RBL cells was estimated to be 250,000 daltons.

By density gradient centrifugation, Newman *et al.* (1977) arrived at a molecular weight of 130,000 daltons for the receptor in the presence of NP-40 and estimated that the receptor would be about 77,000 daltons in a detergent-free solution. Conrad and Froese (1978a) reported a molecular weight of 150,000 daltons using the same technique.

When the molecular weights of receptors were determined by SDS-PAGE,

generally lower values were observed. Using 5% gels and a receptor preparation isolated by the sandwich precipitation technique, Conrad and Froese (1976) observed a single cell surface component (in the case of both RMC and RBL) with an apparent molecular weight of 62,000 daltons. Kulczycki *et al.* (1976) confirmed these results and showed that, in addition, on 10-12% gels the molecular weight was of the order of 45,000 to 50,000 daltons. Isersky *et al.* (1978) using 10% gels also reported a somewhat higher molecular weight of 58,000 daltons for a receptor molecule isolated by means of IgE and anti-IgE. The dependence of the apparent molecular weight on the porosity of the gel suggested that the receptor was a glycoprotein (Weber and Osborn, 1975).

Recently, Conrad and Froese (1978a) observed that when receptors of RBL cells were isolated by means of IgE-Sepharose and eluted with 3.0M KSCN, two surface molecules, having molecular weights (determined on 10% SDS-PAGE) of 55,000 and 45,000 daltons, were present in the receptor preparations. Both molecules were shown to have specificity for IgE (Conrad and Froese, 1978a) and they were designated H and R, respectively. Only R was isolated by means of IgE and anti-IgE (Conrad and Froese, 1978a). Also, IgE-receptor complexes isolated by the use of DNP-IgE and anti-DNP-Sepharose were associated primarily with R (Conrad and Froese, 1978b). These results suggest that binding of an antiserum to the IgE molecule results in a dissociation of H, but not of R.

However, there are still differences in observations made in various laboratories. Thus, Isersky *et al.* (1978) and Kanellopoulos *et al.* (1979), using IgE and antibodies directed against the IgE molecule, isolated a receptor molecule of 53,000 to 58,000 daltons from RBL cells, while Conrad and Froese (1976), using the same procedures, isolated a receptor (R) of 45,000 daltons. Also, Kulczycki *et al.* (1976) isolated a molecule of

45,000 to 50,000 daltons by means of IgE-Sepharose. Using the same technique, Conrad and Froese (1978a), as pointed out earlier, isolated two molecules having molecular weights of 45,000 and 55,000 daltons. The reasons for these differences are presently under investigation through the collaborative efforts and there are indications that they are consequences of differences among RBL cell lines maintained in the various laboratories.

A comparison of the molecular weights of the receptor molecules analyzed by these various techniques suggests that it is generally higher in the presence of NP-40 than in the presence of SDS. Several explanations have been offered: (i) in NP-40, the receptor may exist in the form of aggregates of limited sizes (Conrad and Froese, 1978a); (ii) in the form of dimers; (iii) in association with an unrelated surface molecule; and (iv) as a single molecule to which micelles of NP-40 are bound (Conrad and Froese, 1978a; Newman *et al.*, 1977). Although no direct evidence for any of these possibilities exists, it should be pointed out that extrinsic binding of non-ionic detergents to membrane molecules has been reported if these detergents are used above the critical micelle concentration (Tanford and Reynolds, 1976).

The fact that the molecular weight of the receptors for IgE as determined by SDS-PAGE, varies depending on the porosity of the gel, suggests that these molecules are glycoproteins (Weber and Osborn, 1975). This was confirmed by the fact that both radiolabelled amino acids and sugars can be incorporated into the receptors (Kulczycki *et al.*, 1976; see also Chapter IV of this study). Moreover, isolated receptors were shown to be degraded by proteolytic enzymes and they can, of course, be labelled with ^{125}I. However, results published so far show incorporation of radiolabelled precursors only into one major membrane component of RBL cells. It will be shown in the present study that both amino acids and sugars

can be incorporated into the two receptors identified by Conrad and Froese (1978a), which are present on RBL cells.

4. Antisera to the Receptor for IgE:

Several attempts have been made to prepare antisera to either intact cells, membranes or partially purified receptors (Yiu and Froese, 1976; Ishizaka *et al.*, 1977b; Conrad *et al.*, 1978; Isersky *et al.*, 1978; Conrad *et al.*, 1979). Yiu and Froese (1976) prepared an antiserum to RMC that was shown to inhibit the binding of IgE to RMC and to precipitate a surface component, among others, that when analyzed by SDS-PAGE on 5% gels had a mobility similar to that of the receptor for IgE. Isersky *et al.* (1978) were able to demonstrate that an antiserum prepared against RBL cells would react with solubilized IgE-receptor complexes as well as with free receptor.

The bridging of receptor-bound IgE antibody molecules by multivalent antigen or anti-IgE has been shown to induce the release of chemical mediators from target cells (Ishizaka *et al.*, 1977b; Conrad *et al.*, 1978; Ishizaka and Ishizaka, 1978; Ishizaka *et al.*, 1978). Since the bridging of cell-bound IgE molecules was believed to bring target cell receptor molecules into close proximity, it was suggested that local changes in membrane structure or the interaction of adjacent receptor molecules might possibly be responsible for the activation of enzymatic changes resulting in the release of histamine and other mediators (Ishizaka *et al.*, 1978).

Ishizaka *et al.* (1977b) observed that antibodies prepared against immune precipitates composed of IgE-receptor complexes and anti-IgE induced non-cytotoxic histamine release both *in vivo* and *in vitro*. The antibody preparation was specifically purified by adsorption onto and elution from RBL cells and rendered specific for RMC by adsorption with RMC-

depleted peritoneal cells. It was subsequently found that the $F(ab')_2$ fragments of the anti-RBL serum were capable of inducing histamine release from RMC (Ishizaka *et al.*, 1978). This antiserum was clearly shown to interact with the receptor for IgE, i.e. the R receptor isolated by means of IgE-Sepharose (Conrad and Froese, 1978a). Independently, an antiserum (RAR), prepared by injecting rabbits with a KSCN eluate from RBL material bound to IgE-Sepharose, was capable of reacting with RBL cell surface components having molecular weights of 55,000 and 45,000 daltons (Conrad *et al.*, 1979). This antiserum preparation also reacted with IgE receptor complexes, however, it failed to initiate histamine release *in vivo* or *in vitro* (Conrad *et al.*, 1979).

The aggregation of receptor molecules rather than cross-linkage of cell-bound IgE has also been proposed as being responsible for the transduction of a ligand-membrane interaction into intracellular signals leading to histamine release (Ishizaka and Ishizaka, 1978; Isersky *et al.*, 1978).

Evidence that mast cell alloantigens participate in anaphylactic alloantibody-induced mast cell degranulation by allowing the bridging of the Fc receptor with H-2 molecules has also been suggested (Daeron and Voisin, 1979). This evidence confirmed and extended findings that direct allogenic anaphylactic degranulation (DAAD) results from the double interaction of antibody molecules with two separate structures on the same cell membrane. Thus, alloantibodies have been suggested to recognize specific antigens by their Fab portions on the same cell they activate (Daeron and Voisin, 1978) for histamine release by interaction of their Fc portion with the mast cell Fc receptor (Daeron and Voisin, 1979). These results suggest that molecules other than receptors for IgE may be involved in triggering RMC to release vasoactive amines.

Rat alloantibodies recognizing classical transplantation antigens (CTA)

have been shown to compete effectively with monomeric IgE or IgG-coated sheep erythrocytes for receptor sites on rat mast cell surfaces (Mossmann *et al.*, 1979). The inhibitory capacity was shown to be confined to anti-CTA antibodies of the IgG2a class, as it was concluded that receptor sites recognizing the Fc portion of the anti-CTA molecule were involved in the inhibition process. An explanation put forth was that the firm binding of the alloantibody molecule to antigenic determinants on the RMC surface amplified the interaction between its Fc portion and the appropriate receptor site. Similarly, an anti-rat β_2 microglobulin antibody was able to interfere with IgE binding, causing greater than 40% inhibition of histamine release which is consistent with the findings of Isersky *et al.* (1977) obtained in the RBL cell system. However, the latter authors demonstrated that anti-β_2m antibody did not interact directly with the receptor for IgE.

[F] RECEPTORS FOR IgE ON OTHER CELLS

1. Macrophages:

Although the involvement of IgE in immediate hypersensitivity with specific target cells has clearly indicated that a highly specific cellular receptor exists for the Fc portion of this molecule, several other cell types have been shown to possess membrane-binding structures for IgE. IgE has been shown to be involved in the activation of macrophage cytotoxicity against *Schistosoma mansoni* (Capron *et al.*, 1975; Capron *et al.*, 1977). Using ε-monospecific rabbit anti-rat IgE, IgE could be identified on the surface of peritoneal macrophages from rats infected with *S. mansoni* (Dessaint *et al.*, 1979). However, the binding kinetics of IgE to macrophages differed markedly from those reported for mast cells. The macro-

phage-IgE interaction was shown to be much faster as well as more transient than the IgE interaction with mast cells (Dessaint *et al.*, 1979).

2. Lymphocytes and Lymphoid Type Cells:

Human lymphoblastoid cells and normal peripheral B cells have also been shown to possess a receptor for the Fc fragment of human IgE myeloma proteins (Lawrence *et al.*, 1975; Gonzalez-Molina and Spiegelberg, 1976; Gonzalez-Molina and Spiegelberg, 1977). Two radioactively labelled proteins (biosynthetically labelled with ^{14}C-leucine or enzymatically with ^{125}I) with molecular weights of 86,000 and 47,000 daltons were consistently found in eluates from IgE-Sepharose columns previously mixed with solubilized Wil-2WT B lymphocytes (Meinke *et al.*, 1978). These proteins were suggested to be single polypeptide chains, as reduction failed to alter their molecular weights. However, they were extremely sensitive to proteolysis and recovery of intact molecules could be achieved only when enzyme inhibitors were added at the time of cell solubilization. Furthermore, IgE binding could be abolished by trypsinization of the cells (Gonzalez-Molina and Spiegelberg, 1976). Fritsche and Spiegelberg (1978) have also shown that rat B cells possess an FcR for IgE.

The majority of normal (Gonzalez-Molina and Spiegelberg, 1977; Hellstrom and Spiegelberg, 1979) and cultured lymphocytes (Gonzalez-Molina and Spiegelberg, 1976), as well as chronic lymphatic leukemia lymphocytes (Spiegelberg and Danier, 1979), which have FcεR, also have cell surface bound immunoglobulin, further supporting the suggestion that the cells were B lymphocytes. The low affinity for monomeric IgE and the failure of an anti-lymphocyte FcεR preparation to release histamine from basophils suggested that the FcεR on lymphocytes differ in structure from those demonstrated on basophils and mast cells (Meinke *et al.*, 1978).

Data presented by Yodoi and Ishizaka (1979a) confirmed that subsets

of human and rat lymphocytes have FcεR on their surfaces. FcεR-bearing T and B cells were demonstrated in the blood of some patients and in rats infected with *N. brasiliensis*, suggesting that there may have been an increase in the proportion of FcεR-bearing cells. Indeed, O'Conner and Spiegelberg (1979) have shown that the proportion of FcεR-bearing cells increased in patients whose serum IgE level was high. The results of these experiments implied that there was a limited population of FcεR-bearing T cells and B cells in the normal lymphocyte population, and that this population was expanded under pathologic conditions which enhance IgE production.

In vitro investigations of FcεR-bearing rat lymphocytes carried out by Yodoi *et al.* (1979) showed that FcεR-bearing cells were induced by isologous IgE but not by human IgE, rat IgG nor rabbit IgG. Furthermore, the number of IgE-receptors per mast cell did not change after *N. brasiliensis* infection, which enhanced IgE synthesis; however, it markedly affected the proportion of lymphocytes bearing receptors for IgE. The observations that FcεR-bearing lymphocytes increased in *N. brasiliensis* infected rats without a change in the proportion of mast cells in the peritoneal cell population, or in the number of IgE-receptors per mast cell, indicated that expression of the two receptors, which had specificity for the same immunoglobulin, was regulated through different mechanisms.

A kinetic investigation into the induction of FcεR(+) - bearing cells showed that the majority of FcεR(+) cells induced *in vitro* had previously carried FcγR (Yodoi and Ishizaka, 1979b). Results obtained in previous experiments demonstrated that both RNA synthesis and protein synthesis, but not DNA synthesis, were required for the induction and maintenance of FcεR(+)-bearing cells (Yodoi *et al.*,

1979). From data obtained during the culture of normal rat lymphocytes with IgE, a subset of lymphocytes was suggested to express receptors for both IgG and IgE immunoglobulins at different differentiation stages. Collectively, these results suggest that the binding of IgE with either FcγR or FcεR stimulate lymphocytes to form FcεR.

SCOPE OF THE PRESENT INVESTIGATION

The aims of this study were to characterize the receptors for IgE on RBL cells in terms of their glycoprotein nature, to establish differences between the H and R receptor observed on these cells, and to test for the presence of receptor molecules or receptor-associated molecules not labelled by the lactoperoxidase catalyzed surface-iodination procedure.

In Chapter II, the isolation and characterization of surface-iodinated receptors is described. These experiments represent a repetition of work already described by others, and they were performed in order to standardize techniques and to provide reference standards for the subsequent experiments.

In Chapter III, the binding of surface-iodinated receptors to various lectins is described, and these lectins are evaluated for their usefulness in separating the H and R receptors on RBL cells.

Chapter IV deals with the incorporation of both tritiated amino acids and sugars into the receptors for IgE. While this study was in progress, other laboratories had also reported similar incorporation studies. However, in some instances, different precursors were used and, moreover, RBL cells maintained in other laboratories appeared to carry receptors which differ somewhat from those observed in this laboratory. Thus, the results in Chapter IV are the only ones which deal with the incorporation of precursors into both H and R molecules.

Finally, the results obtained in this study are discussed in detail in a section entitled "GENERAL DISCUSSION".

CHAPTER II

INTRODUCTION

The study of the interaction between rat monoclonal IgE and isolated rat peritoneal mast cells (RMC) or rat basophilic leukemia (RBL) cells has been performed using surface-iodinated cell preparations. Methods have been presented which indicate that receptor preparations for IgE on RBL cells can be isolated by means of immunoprecipitation (Conrad and Froese, 1976), IgE-Sepharose (Conrad and Froese, 1978a), and by anti-DNP-Sepharose (Conrad and Froese, 1978b). The analysis, by SDS-PAGE, of these receptor preparations has provided the basis for significant progress toward the understanding of the nature and function of the receptors for IgE.

Although the receptor preparations, using enzymatically surface-labelled RBL cells, suggested that the receptors for IgE were glycoproteins, investigations were initiated to determine the carbohydrate nature of the receptors using lectin-affinity chromatography. Inasmuch as the isolated receptors were shown to be relatively pure, investigations using biosynthetically labelled RBL cell preparations were also initiated to monitor the purity of these receptors and to assess the possibility that membrane components attached to the receptor and/or molecules which failed to be labelled by iodination might also be present.

However, before these investigations could be carried out, it was essential to define the systems to be used for the isolation and identification of the receptors for IgE on RBL cells. In this Chapter, the procedures used for the isolation of surface-labelled receptors have been repeated and are presented to establish reference standards which will be

used to determine the identity of receptor material in subsequent chapters.

MATERIALS AND METHODS

Buffers:

The following buffers were used where indicated:

(a) Phosphate buffered saline (PBS) (0.14 M NaCl, 0.01 M PO_4, pH 7.4) with 0.05% BSA (PBS/BSA).

(b) Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.5).

(c) Borate-buffered saline (BBS) (0.16 M NaCl, 0.20 M boric acid, pH 8.0) with 0.05% BSA (BBS/BSA).

Buffers routinely contained 0.1% or 0.5% NP-40 (Particle Data Laboratories Ltd., Elmhurst, Ill.) for cell disruption and experimental procedures.

Rat Basophilic Leukemia (RBL) Cells:

A litter of Wistar rats bearing the basophilic leukemia described by Eccleston *et al.* (1973) was kindly provided by Dr. B.J. Leonard of Imperial Chemical Industries, Ltd., Aderley Park, England. Sufficient adult breeding animals were also obtained to initiate and maintain a colony at the University of Manitoba for propagation of the tumour *in vivo*. The tumour was maintained in the *in vivo* state as described by Eccleston *et al.* (1973) and Kulczycki *et al.* (1974) by injecting RBL cells into newborn rats. Basophilic tumors were aseptically removed from 2- to 3-week-old rats and minced in cold MEM with Spinner's salts. Single cell suspensions were obtained by trypsinization [0.05% trypsin (Grand Island Biological Co., Burlington, Ontario) in MEM] at 0°C and filtration through glass wool. *In vivo* maintenance was by subcutaneous inoculation into the nuchal

region of newborn to 48-hour-old ICI Wistar rats of 0.05 - 0.10 ml of suspended cells at concentrations of 0.5 - 1.0 x 10^6/ml. Tumours routinely developed within 10-15 days in about 70-80% of the inoculated animals. The leukemic cells were adopted to cell culture and propagated as described by Kulczycki *et al.* (1974) using Eagle's minimal essential medium (MEM) with Earle's salts (Difco Laboratories, Detroit, Michigan). This medium was supplemented with non-essential amino acids, vitamins, 100 units/ml of penicillin, 100 µcg/ml of Streptomycin and 15% heat inactivated fetal calf serum. The latter ingredients of the culture medium were purchased from Grand Island Biological Co., Burlington, Ontario. Tumour cells destined for *in vitro* passaging were handled aseptically as described above. After filtration through glass wool into MEM with 15% FCS, the cells were aliquoted at 5-10 x 10^5 cells/ml in either 30 or 50 ml Falcon flasks. Cultures were then incubated in a 5% CO_2 atmosphere and maintained in a 37°C humidified incubator with constant air flow. Cell densities were normally maintained at 2-3 x 10^6 cells/ml. At intervals, cells maintained *in vitro* were passaged through animals as described above.

RBL Cell Iodination and Disruption:

RBL cells used in the course of this study were obtained from tissue culture. Cells at a concentration of 2 - 3 x 10^7/ml were washed in PBS and labelled by the lactoperoxidase procedure previously described (Conrad and Froese, 1976). Briefly, 2 - 3 x 10^7 RBL cells were suspended in 0.5 ml of PBS, agitated and 0.5 mCi of carrier-free $Na^{125}I$ (Amersham Corporation, Oakville, Ontario) was added. Three aliquots of lactoperoxidase (Calbiochem, San Diego, California) (10 µl of 166 IU/ml) and hydrogen peroxide (Fisher Scientific Co., Canada) (15 µl of 0.03% H_2O_2) spaced one minute apart were then added with intermittent agitation. The reaction was quenched by adding the cells to 10 ml of cold PBS and the

cells were collected by centrifugation. In order to reduce the presence of ^{125}I bound to low molecular weight lipids, intact cells were incubated in PBS/BSA for 60-90 minutes at 37°C with intermittent agitation. Subsequently, the iodinated RBL cells were centrifuged through a 1.5 ml cushion of FCS, washed 2x in PBS and solubilized. Solubilization was achieved by mixing pelleted cells with 1.0 ml of 0.5% NP-40 in PBS for 30 minutes at 4°C. Particulate material was removed by centrifugation in an Eppendorf microcentrifuge (Model 3200) at 12,000 g and the supernatant material used for experimental procedures. Whenever RBL cells were solubilized directly after iodination, supernatants were dialyzed overnight against one liter of 0.1% NP-40 in PBS at 4°C.

Preparation of Rat Monoclonal IgE:

Rat ascitic fluid obtained from LOU/M/Ws1 rats bearing the IgE-secreting IR-162 immunocytoma (Bazin *et al.*, 1974) was originally a gift from Dr. H. Bazin. Subsequently, ascitic fluid was harvested from intraperitoneally grafted members of a LOU/M/Ws1 rat colony maintained in this Department. The IgE was routinely purified in Dr. Froese's laboratory as follows. The ascitic fluid was adjusted to 50% saturation of $(NH_4)_2SO_4$, stirred for 2 hours at room temperature and the precipitate was removed by centrifugation, washed with 50% saturated $(NH_4)_2SO_4$, dissolved, and dialyzed against 0.2 M Tris-HCl, .15 M NaCl, 0.5% NaN_3, pH 8.0, overnight at 4°C. Gel filtration chromatography of ≃500 mg of IR-162 was then carried out on either BioGel P-300 (Bio-Rad Laboratories, Richmond, California) or Ultra-gel Ac34A (LKB Produktor, Broma, Sweden). Proteins comprising the second peak were collected and adjusted to 40% saturation of $(NH_4)_2SO_4$ and the supernatants from this precipitation step were dialyzed against 0.01 M glycine HCl, pH 7.6, overnight at 4°C. Dialyzed samples were

then subjected to isoelectric focusing using 1% carrier ampholytes, pH 4.0 - 6.5 (LKB) in a sucrose gradient of 0 - 40%. Samples were inserted into the centre of the preformed ampholine gradient and run at 600V for 48 hours. Material focusing at pH 5.9 was collected and dialyzed against PBS to remove both the sucrose and ampholytes. The IgE concentration was determined by using an extinction coefficient of 13.6 ($E^{1\%}_{280\ nm}$) (Carson and Metzger, 1974) and the preparations stored at -70°C until used. IgE to be coupled to Sepharose Cl-4B for use in affinity chromatography was subjected to only the preliminary purification steps of ammonium sulfate precipitation and gel filtration chromatography; all other IgE preparations were purified by isoelectric focusing.

Preparation of DNP-IgE:

This was routinely available in the laboratory and had been prepared as follows. The IgE peak from Ultragel AcA 34 was precipitated in a final concentration of 40% $(NH_4)_2SO_4$ and solubilized in PBS. Approximately 10^{OD}/ml (7.3 mg/ml) was then mixed with an equal amount of 2,4-dinitrophenylsulfonate in 0.5 ml of 0.2 M Na_2CO_3 (Eisen, 1964). The final coupling reaction mixture was adjusted to 0.2 M Na_2CO_3 using 2.0 M Na_2CO_3 and gently stirred in a 23°C water bath for 5 hours. Small aliquots were removed at intervals, passed through a Sephadex G25 column equilibrated with PBS and optical densities measured in a Zeiss PM QII spectrophotometer at 280 nm and 360 nm. The number of DNP groups coupled to IgE was calculated as follows:

$$\text{IgE (mg/ml)} = \frac{\text{optical density at 280} - \frac{\text{optical density at 360}}{2.89^{(a)}}}{1.36^{(b)}}$$

$$\text{Moles of IgE} = \frac{\text{mg/ml of IgE}}{1.8 \times 10^{5(c)}}$$

$$\text{Moles of DNP} = \frac{\text{optical density at 360}}{1.753 \times 10^{4}(d)} = \text{DNP/IgE}$$

(a) $\frac{OD_{360}\ \text{dinitrophenyl residue}}{OD_{280}\ \text{dinitrophenyl residue}} = 2.89$

(b) $E^{0.1\%}_{1\ cm}$ of IgE at 280 nm

(c) molecular weight of IgE

(d) molar extinction coefficient of DNP-lysine at 360 nm

Using these formulae, an average of seven DNP molecules was bound per molecule of IgE. Following dialysis against PBS, DNP-IgE preparations were de-aggregated in the Beckman L2-65 Ultracentrifuge with a SW 50L rotor at 40,000 rpm for one hour. The preparations were then aliquoted into 300-400 µg quantities and stored at -70°C until used.

Antisera:

Commercially prepared goat anti-rabbit IgG (H & L) was purchased from Miles Laboratories (Kankakee, Ill.). Monospecific rabbit anti-rat IgE and goat anti-rat IgE were prepared as described by Bazin *et al.* (1974). Normal rabbit serum (NRS) was obtained from pre-immunized rabbits. All sera were subjected to 50% ammonium sulfate precipitation at room temperature with constant stirring for two hours. The precipitated material was centrifuged at 27,000 g for 20 minutes and re-dissolved in a reduced volume of PBS and dialyzed against PBS overnight at 4°C. The dialysate was subsequently adjusted to a protein concentration of 8^{OD}/ml (5.3 mg/ml), using an extinction coefficient of 15 ($E^{1\%}_{280\ nm}$).

Monospecific sheep anti-rat IgE was kindly supplied by Dr. K. Kelly of the Department of Immunology, University of Manitoba. Briefly, this antiserum had been precipitated at 40% $(NH_4)_2SO_4$ with constant stirring at room temperature for 2 hours. The precipitate was pelleted in a Sorvall RC2-B centrifuge at 27,000 g for 20 minutes and subsequently dis-

solved in a reduced volume of 0.05 M Tris/0.15 M NaCl. Euglobulins were precipitated by dialysis against two liters of 0.01 M KH_2PO_4, pH 4.75, with two buffer changes. The final dialysate was centrifuged at 48,000 g for 30 minutes in the Sorvall to collect the euglobulins and the pellet dissolved in 0.05 M Tris/0.15 M NaCl, pH 7.5, and stored in frozen aliquots at -70°C.

Bovine anti-DNP antibodies, a generous gift from Dr. S.I. Wie, had been isolated and purified as described previously (Wie *et al.*, 1976).

Immunoprecipitation:

For the indirect or "sandwich" precipitation system, conditions which yielded precipitation of rabbit immunoglobulins by goat anti-rabbit Ig were established using a standard antibody titration curve. Rabbit anti-IgE at 7.4 ^{OD}/ml (4.9 mg/ml) and normal rabbit serum at 8.0 ^{OD}/ml (5.3 mg/ml) were titrated against goat anti-rabbit immunoglobulins and the optimal concentrations for complete precipitation were established following a 60-minute incubation at 37°C and 30 minutes at 4°C. The precipitates were then washed four times with 2-3 ml of PBS and dissolved in 0.05 N NaOH and the optical density read at 280 nm in the Zeiss PMQII spectrophotometer. The ratio of antigen to antibody giving the highest optical density reading was considered to represent optimal conditions: in the case of rabbit anti-IgE to goat anti-rabbit immunoglobulin (30 µl versus 100 µl proved optimal); for normal rabbit serum versus goat anti-rabbit immunoglobulin (33 µl versus 100 µl was optimal).

Conditions for direct precipitation of IR-162 IgE were determined in a similar fashion. Under these conditions, 112 µl of IgE (2.22 ^{OD}/ml, 1.6 mg/ml) were optimally precipitated by 200 µl of goat anti-IgE (8.0 ^{OD}/ml, 5.3 mg/ml), or 20 µl of IgE (2.65 ^{OD}/ml, 1.9 mg/ml) could be optimally precipitated with 50 µl of sheep anti-rat IgE (61.2 ^{OD}/ml, 40 mg/

ml). Antibody titration curves were routinely run throughout the course of the experimental period and adjustments were made as required.

Immunoabsorbent Preparation:

(a) IgE-Sepharose Cl-4B: Sepharose Cl-4B (Pharmacia, Uppsala, Sweden) was activated using the cyanogen bromide procedure by mixing 150-200 mg of cyanogen bromide per ml of washed Sepharose beads (Cuatrecasas and Anfinsen, 1971). The reaction was carried out in distilled H_2O with the pH being maintained between 9 and 11 for 20 minutes or until the pH failed to drop below 9. The activated Sepharose was then washed with 0.1 M $NaHCO_3$, pH 8.0. Routinely, 50 - 100 mg of IgE in PBS, purified by $(NH_4)_2SO_4$ precipitation and gel filtration were reacted with 5.0 ml of activated Sepharose Cl-4B for 16-18 hours at 4^oC with gentle agitation. The gel mixture was then washed to remove non-coupled protein and any remaining unreacted groups were quenched with 5.0 ml of 0.5 M ethanolamine (pH 8.0) for an additional 3-4 hours. The coupling efficiency was determined by spectrophotometric measurement of the supernatant using an extinction coefficient ($E^{1\%}_{280\ nm}$) for IgE of 13.6 (Carson and Metzger, 1974) and was in the range of 80-95%. Control immunoabsorbents were similarly prepared using BSA or CNBr-activated ethanolamine quenched Sepharose Cl-4B. All gels were washed with PBS containing 0.1% sodium azide and stored at 4^oC until used.

(b) Anti-DNP-Sepharose Cl-4B: Sepharose Cl-4B was activated and coupling was achieved as described above. Approximately 50 mg of the anti-DNP antibody preparation in PBS were reacted with 5.0 ml of activated packed Sepharose Cl-4B with a coupling efficiency of 80-90%. All immunoabsorbents were washed with buffer containing 0.1% NP-40 before use.

Affinity Chromatography with IgE-Sepharose:

Normally, 100 - 200 µl aliquots of labelled cell extracts from

$2 - 3 \times 10^7$ cells solubilized in 1.0 ml of 0.5% NP-40 buffer were added to 0.1 - 0.2 ml of IgE-Sepharose immunoabsorbent (or controls) in plastic tubes at $4^{\circ}C$ and rotated on a Fisher Roto Rack (Fisher Scientific Co. Ltd., Canada) for 60 - 90 minutes. The immunoabsorbent beads were then washed in 2 - 3 ml of 0.1% NP-40/PBS four times and once in 0.0625 M Tris, pH 6.8. Elution was achieved either with 3.0 M KSCN in 0.1% NP-40 and the eluate dialyzed against one liter of 0.1% NP-40 buffer overnight at $4^{\circ}C$ to remove the KSCN, or with sample buffer containing sodium dodecyl sulfate (SDS) at the same concentration (2%) used in polyacrylamide gel electrophoresis.

Isolation of IgE-Receptor Complexes:

RBL cells (3×10^7), which were labelled with ^{125}I using the lactoperoxidase technique, were incubated for 60 minutes at $37^{\circ}C$ with 400 µg of DNP-IgE in PBS/BSA (Conrad and Froese, 1978b). The cells were subsequently washed through 2.0 ml of FCS, followed by two washes in PBS/BSA. Solubilization of cells was achieved by adding 0.5 ml of 0.5% NP-40/PBS and allowing the mixture to stand for 30 minutes at $4^{\circ}C$. After removal of insoluble material by centrifugation, the supernatant was added to 0.2 - 0.3 ml of anti-DNP-Sepharose and the mixture was agitated at $4^{\circ}C$ for 60 - 90 minutes. After transferring the reaction mixture to a Pasteur pipette column, the anti-DNP-Sepharose was washed with 15 - 20 ml of 0.1% NP-40/PBS and the IgE-receptor complexes eluted at room temperature with 0.1 M 2,4-dinitrophenylate (DNP-Na) in 0.1 M phosphate buffer, pH 7.4, containing 0.1% NP-40. Eluted samples could then be stored at $4^{\circ}C$ or were dialyzed against 0.0625 M Tris, pH 6.8, containing Dowex 1 X 8 resin to remove the DNP-Na.

Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE):

(a) Fairbanks' System: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Fairbanks *et al.* (1971). ^{125}I-labelled marker proteins consisting of IR-162 IgE, heavy and light chains of IR-162 IgE, mouse IgG1 (MOPC-21A), heavy and light chains of mouse IgG1 (MOPC-21A) and ovalbumin (Nutritional Biochemical Corp., Cleveland, Ohio) were used as molecular weight markers to calibrate the Fairbanks' gel system. Immune precipitates containing receptors were collected by centrifugation, washed four times with PBS, transferred to a fresh tube and then solubilized by the addition of 0.1 - 0.2 ml of sample buffer (1% SDS, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Before electrophoresis, all samples were dialyzed overnight at 4^{o}C against electrode buffer (00.1 M Tris, 0.2 M sodium acetate, 0.2 M EDTA, pH 7.4, containing 0.1% SDS). Reduced and alkylated samples were dialyzed against the same buffer with the addition of 0.2 M 2-mercaptoethanol. 5.6% gels (90 mm) were prepared in electrode buffer and electrophoresis carried out at 10mA/gel. Electrophoresis was stopped when the tracking dye reached the bottom of the gel and each gel was then sliced in a Gilson Model B200 gel fractionator (Gilson Medical Electronics, Inc., Madison, Wisconsin) into 2 mm fractions and counted in a Beckman Model 300 gamma counter (Beckman Instrument Co., Fullerton, California).

(b) Laemmli System: To increase the resolving power of polyacrylamide gel electrophoresis, SDS-PAGE analysis was performed using the Tris-buffered system described by Laemmli (1970). Ten percent separation gels of 140 mm with a 10 mm 3% stacking gels were used.

For analysis of radiolabelled components specifically bound to affinity gels, elution was carried out by the addition of 0.2 ml of 0.0625 M Tris buffer, pH 6.8, containing 2% SDS. Eluates from affinity gels by other

means were routinely dialyzed overnight at 4°C against 0.0625 M Tris buffer, pH 6.8. Subsequently, aliquots were adjusted to 2% SDS and in all cases heated at 100°C for 90 seconds. Whenever indicated, samples were also reduced by the addition of sufficient 2-mercaptoethanol to make a 5% solution. After cooling, glycerol (50 µl) and 3 µl of bromophenol blue tracking dye were added. Electrophoresis was carried out at 1mA/gel for stacking and 3 - 4 mA/gel for separation. Following electrophoresis, the gels were treated as described for the Fairbanks' gel system using 5.6% gels. The Laemmli gel system was calibrated using proteins of known molecular weights as standards: β-galactosidase, m.w. 130,000 daltons (Sigma Chemical Co., St. Louis, Missouri); phosphorylase a, m.w. 100,000 daltons (Sigma Chemical Co., St. Louis, Missouri); lactoperoxidase, m.w. 75,000 daltons (Calbiochem, San Diego, California); ε-chain of IR-162 IgE, m.w. 75,000 daltons; bovine serum albumin, fraction V., m.w. 68,000 daltons (ICN Pharmaceuticals, Cleveland, Ohio); catalase, m.w. 58,000 daltons (Sigma Chemical Co., St. Louis, Missouri); ovalbumin 3X crystallized, m.w. 43,000 daltons (Nutritional Biochemicals Corp., Cleveland, Ohio); pepsin, m.w. 35,000 daltons (Worthington Biochemical Corp., Freehold, New Jersey); α-chymotrypsinogen, m.w. 25,700 daltons (Sigma Chemical Co., St. Louis, Missouri); trypsin 2X crystallized, m.w. 23,000 daltons (Mann Research Laboratories, New York, New York); light chain of IR-162 IgE, m.w. 23,000 daltons; myoglobin, m.w. 17,200 (Miles Laboratories, Kankakee, Ill.); and cytochrome c, m.w. 11,700 (Sigma Chemical Co., St. Louis, Missouri). In all cases, 20 - 40 µg of each protein standard was applied with a maximum of 120 µg per gel. After electrophoresis, the tracking dye was marked with a thin wire and the gels were stained with a modified procedure described by Fairbanks *et al.* (1971). Briefly, the gels were stained and fixed simultaneously in 0.05% Coomassie blue, 25% isopropyl

alcohol and 10% acetic acid for 18-24 hours. This solution was then replaced with 0.025% Coomassie blue, 10% isopropyl alcohol and 10% acetic acid for an additional 18-24 hours. The gels were then destained to clearness with 10% acetic acid, leaving the protein bands stained blue.

Relative mobilities of the protein standards were then calculated by dividing the distance of protein migration from the origin by the distance of the tracking dye migration from the origin. Mobilities (R_f) were then plotted against molecular weights on semilogarithmic graph paper. Alternatively, protein standards were radiolabelled by the Chloramine T procedure (McConahey and Dixon, 1966) and reduced and alkylated as described by Maizel (1971). β-galactosidase, lactoperoxidase, bovine serum albumin, ovalbumin, catalase, pepsin, ε-chain and light chain of IR-162 IgE served as marker proteins for this standardization. Following electrophoresis, the gels were sectioned into 2 mm fractions by the Gilson fractionator and the fraction number with the peak radioactivity regarded as the distance of protein migration. The distance of radiolabelled protein migration divided by the migration of the tracking dye again served as measurement of the R_f. R_f values from the standard curves using these two techniques were routinely used to determine the relative molecular weights of the unknown samples throughout the course of the study.

RESULTS

Immune Precipitation of NP-40 Solubilized Surface-Labelled RBL Cells:

The flow diagram (Fig. 1) represents the experimental conditions used in this investigation for the demonstration of the receptor for IgE on RBL cells by immunoprecipitation. Protocol A outlines the indirect or sandwich precipitation procedure described under Materials and Methods. Routinely, surface labelled RBL cells were pre-mixed with 10 μg of IgE, solubilized by NP-40 and subjected to the respective optimal ratio of antigen (rabbit serum or rabbit anti-IgE) to antibody (goat anti-rabbit immunoglobulin), as previously determined by an antibody titration curve. Protocol B outlines the direct precipitation procedure used, in which IgE was directly precipitated by sheep anti-IgE. The specific immune precipitates obtained in these two methods contained anti-rat ε-chain antibodies and were compared to control precipitates containing normal rabbit serum precipitated by goat anti-rat immunoglobulin at optimal ratios. Alternatively, Protocol C was used; soluble RBL IgE-receptor complexes were prepared by increasing the antibody levels to assure antibody excess and the receptor complexes, then insolubilized by Protein A-Sepharose. An enhancement was obtained under each condition in the amount of radiolabel present in the specific immune precipitate versus the control precipitates, indicating surface material co-precipitating by IgE anti-IgE complexes. Table I contains the results obtained utilizing these experimental procedures for the isolation of the receptor from RBL cells by immunoprecipitation procedures. It can be easily noted that the percentages of surface material insolubilized were not identical using the three different procedures. The explanation for this, in part, is due to the fact that the results were obtained on different occasions and, thus, involved different batches of labelled cells. The specific activity of the radiolabelled RBL cells

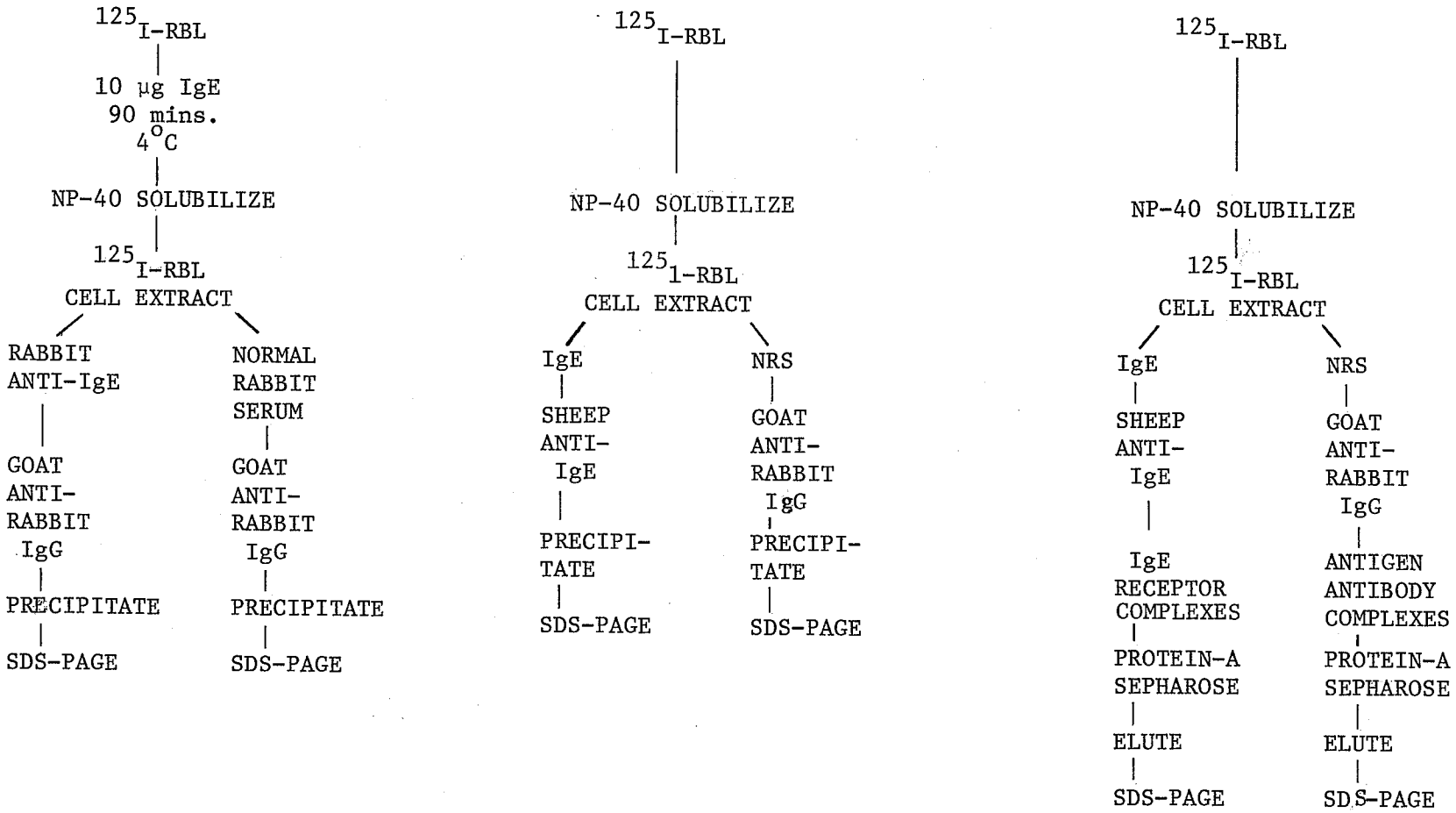


Figure 1: Isolation of the IgE receptor(s) from radiolabelled rat basophilic leukemia (RBL) cells.

Protocol "A" Indirect (Sandwich) precipitation procedure

Protocol "B" Direct precipitation procedure

Protocol "C" Protein-A Sepharose

TABLE I

ISOLATION OF SURFACE RADIOLABELLED MATERIAL FROM RBL CELLS

	EXPERIMENTAL CONDITION[a]	% SURFACE MATERIAL INSOLUBILIZED[b]
A.	IgE/RABBIT ANTI-IgE/GAR	7.8
	IgE/NRS/GAR	2.7
B.	IgE/SHEEP ANTI-IgE	3.2
	NRS/GAR	0.9
C.	IgE/SHEEP ANTI-IgE PROTEIN A-SEPHAROSE	4.9
	NRS/GAR-PROTEIN A-SEPHAROSE	1.5

(a) In all cases, $\sim 2 \times 10^6$ RBL cell equivalents from 2×10^7 surface iodinated RBL cells were used.

(b) Results obtained from a typical experiment. Results show approximately a three-fold difference between specific and control insolubilization procedures routinely seen under these experimental conditions.

and the wide range in number of receptors expected per cell would account for the ranges obtained. It was deemed sufficient, under each experimental condition, that an enhancement (about 3-fold) was found in the amount of radiolabel present in the specific versus control precipitates. Similar results were obtained by Conrad and Froese (1976).

SDS-PAGE Analysis of Immunoprecipitated Surface Components from Solubilized RBL Cells:

The calibration of the 5.6% gels used in the Fairbanks SDS-PAGE system (Fairbanks *et al.*, 1971) is shown in Fig. 2. Radiolabelled proteins, described in the Materials and Methods section, were run and the molecular weight was plotted versus the relative mobility on a semilogarithmic scale.

The distribution patterns of radiolabelled material in the immune precipitates isolated on 5.6% gels using SDS-PAGE as described by Fairbanks *et al.* (1971) is shown in Fig. 3. The receptor for IgE isolated by the indirect "sandwich" precipitation procedure is shown in Fig. 3a. It can be readily noted that the significant amount of radio-iodinated material specifically precipitated corresponds to a major radioactive band migrating with an approximate molecular weight of 60,000 - 65,000 daltons, determined by its relative mobility compared to that of proteins with known molecular weights. Similar results were seen by Conrad and Froese (1976) using the Shapiro *et al.* (1967) system with 5% gels. This single band was clearly dependent upon the presence of IgE and was not found when the control precipitates using IgG were analyzed. This band was further shown to be unaffected by reduction and alkylation (not shown). Analysis of precipitates obtained by both the precipitating systems gave similar, if not identical patterns. Shown in the Fig. 3b panel is the profile of the receptor obtained by the direct precipitating system, clearly demonstrating the single component

Figure 2: Calibration curve for the SDS-PAGE system according to Fairbanks *et al.* (1971) using 5.6% gels (90 mm). Proteins (3-5 µg), in Fairbanks sample buffer containing 1% SDS, were dialyzed overnight against Fairbanks electrode buffer and analyzed by SDS-PAGE. The following protein standards were used to calibrate the gels: rat monoclonal IgE (IR-162), 200,000 daltons; MOPC-21A (IgG_1), 150,000 daltons; ε-chain (IR-162 IgE), 75,000 daltons; ovalbumin, 43,000 daltons; and L-chain MOPC-21A (IgG_1), 22,500 daltons.
(x) radiolabelled proteins; (●) stained proteins.

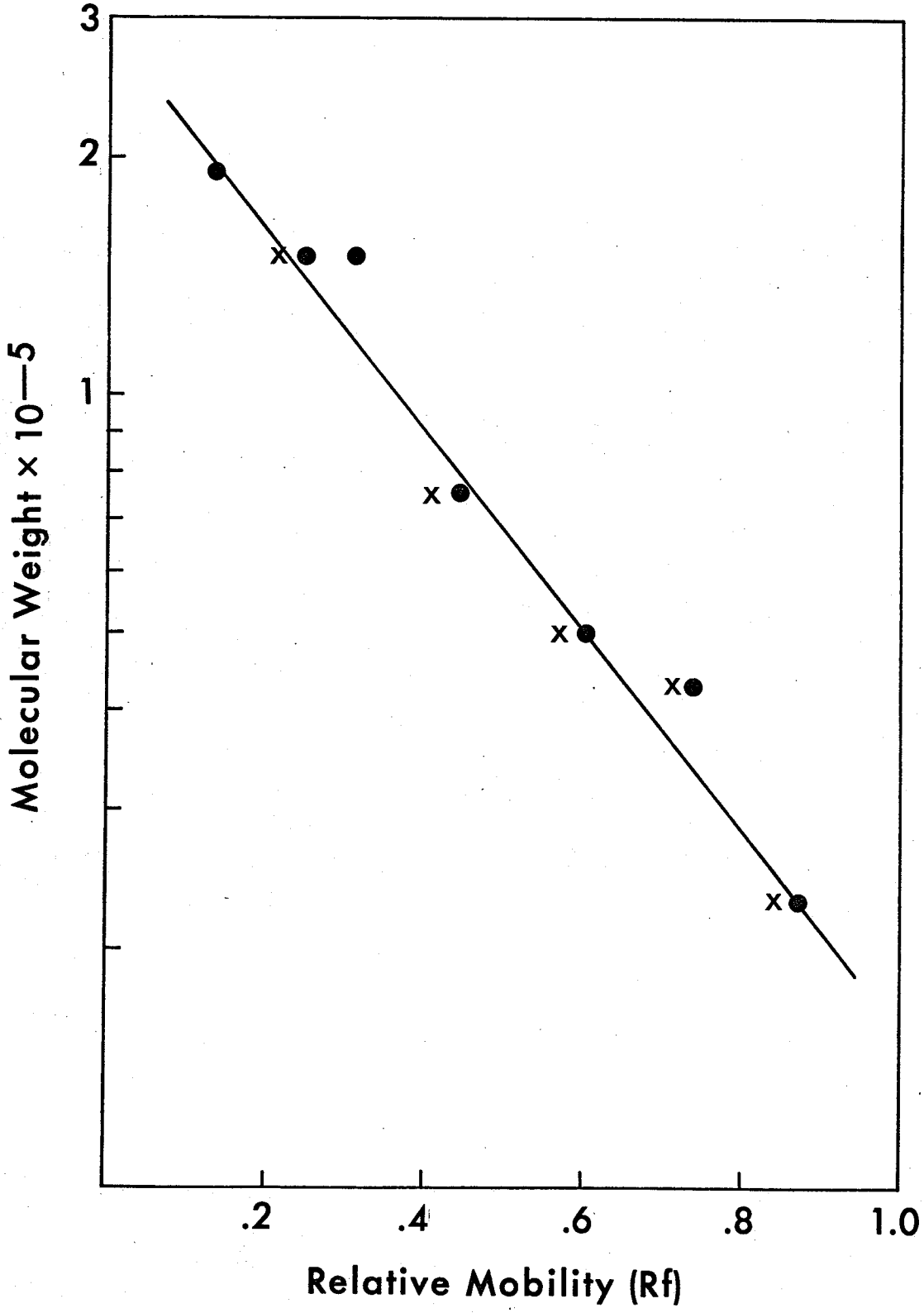
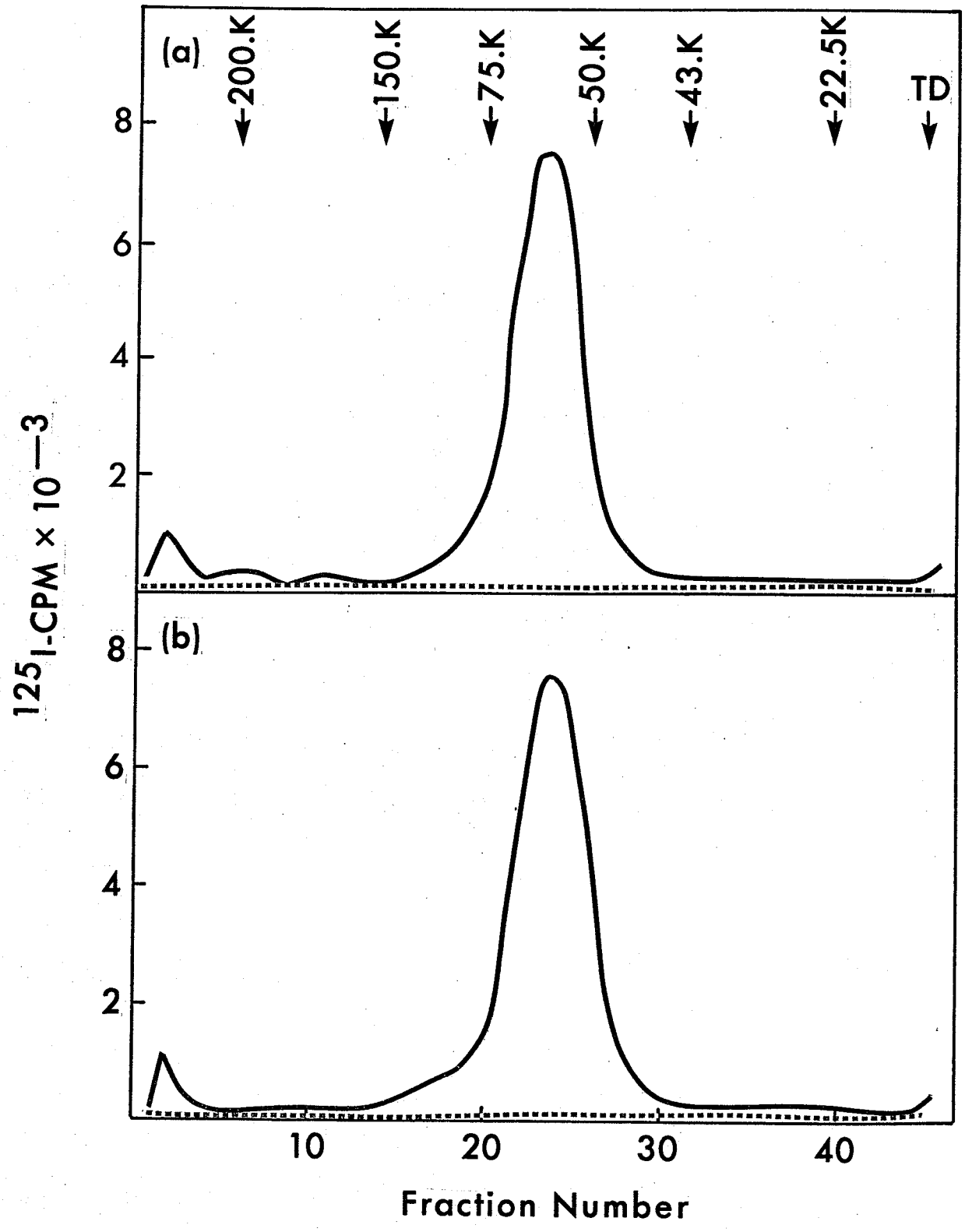

3
2
1
Molecular Weight x 10—5
.2
.4
.6
.8
1.0
Relative Mobility (Rf)

Figure 3: SDS-PAGE analysis on 5.6% gels of specific and control precipitates from surface-iodinated RBL cells. Solubilized RBL cells (2×10^6) from 2×10^7 iodinated RBL cell extracts were used for immunoprecipitations. (a) Surface material co-precipitated by the "sandwich" precipitation procedure (Protocol A, Fig. 1). (b) Surface material co-precipitated by the "direct" precipitation procedure (Protocol B, Fig. 1). The precipitates were washed in PBS and solubilized in Fairbanks sample buffer containing 1% SDS and dialyzed overnight at $4^{\circ}C$ against electrode buffer. Samples were then analyzed by SDS-PAGE. The arrows and molecular weights indicate the migration behaviour of the standards used to calibrate the gels; from left to right in decreasing molecular weight: rat monoclonal IgE (IR-162); MOPC-21A (IgG_1); ε-chain (rat IgE); γ-chain (IgG_1); ovalbumin; and L-chain (IgG_1). TD marks the position of the tracking dye. (———) specific precipitates; (-----) control precipitates.


(a)
200.K
150.K
75.K
50.K
43.K
22.5K
TD
(b)
8
6
4
2
^{125}I-CPM × 10—3
10
20
30
40
Fraction Number

identified as receptor.

To achieve a better resolution of the precipitated receptor, the Laemmli polyacrylamide gel electrophoresis system (Laemmli, 1970) was adopted. Fig. 4 shows the calibration curve for the Laemmli gel system using both stained and radiolabelled proteins on 10% separation gels. The relative mobilities (R_f) were calculated by measuring the distance of protein migration divided by the migration of the tracking dye and plotted against the known molecular weights of the standards. By determining the distance of migration of unknown molecules divided by the migration distance of the tracking dye, molecular weights could be assigned to these unknowns. The standard curve showed good linearity in the range of 20,000 to 80,000 daltons. Proteins migrating outside this range could only be assigned approximate molecular weights. Analysis of the receptor by the direct precipitation system is shown in Fig. 5. It can be seen that on 10% gels, as in the case of 5.6% gels, a single cell surface component (receptor) was detected. However, a comparison to molecular weight standards now revealed this component to migrate with an apparent molecular weight of 45,000 - 48,000 daltons. The reason for this lower molecular weight (as compared to 5.6% gels) can be attributed to the glycoprotein nature of the receptor (Weber and Osborn, 1975). A similar observation was made by Kulczycki *et al.* (1976). Both gel systems demonstrated the specificity of the receptor isolation procedure, as only one major peak was observed compared to control experiments.

The insolubilization of IgE-receptor complexes in the presence of antibody excess by protein A-Sepharose and subsequent elution of these complexes proved to be another convenient method of receptor isolation. The profile seen in Fig. 6 is that of receptor isolated by this approach

Figure 4: Calibration curve for the SDS-PAGE system according to Laemmli (1970) using 10% gels (140 mm). Proteins (3 - 5 µg) in sample buffer containing 2% SDS were heated in a boiling water bath and analyzed by SDS-PAGE. The following protein standards were used to calibrate the gels: β-galactosidase, 130,000 daltons; phosphorylase A, 100,000 daltons; ε-chain (IR-162 IgE), 75,000 daltons; bovine serum albumin, 68,000 daltons; catalase, 58,000 daltons; ovalbumin, 43,000 daltons; pepsin, 35,000 daltons; α-chymotrypsinogen, 25,700 daltons; trypsin, 23,200 daltons; L-chain (IR-162 IgE), 23,000 daltons; myoglobin, 17,200 daltons; and cytochrome c, 11,700 daltons. (x) radiolabelled and (•) stained proteins.

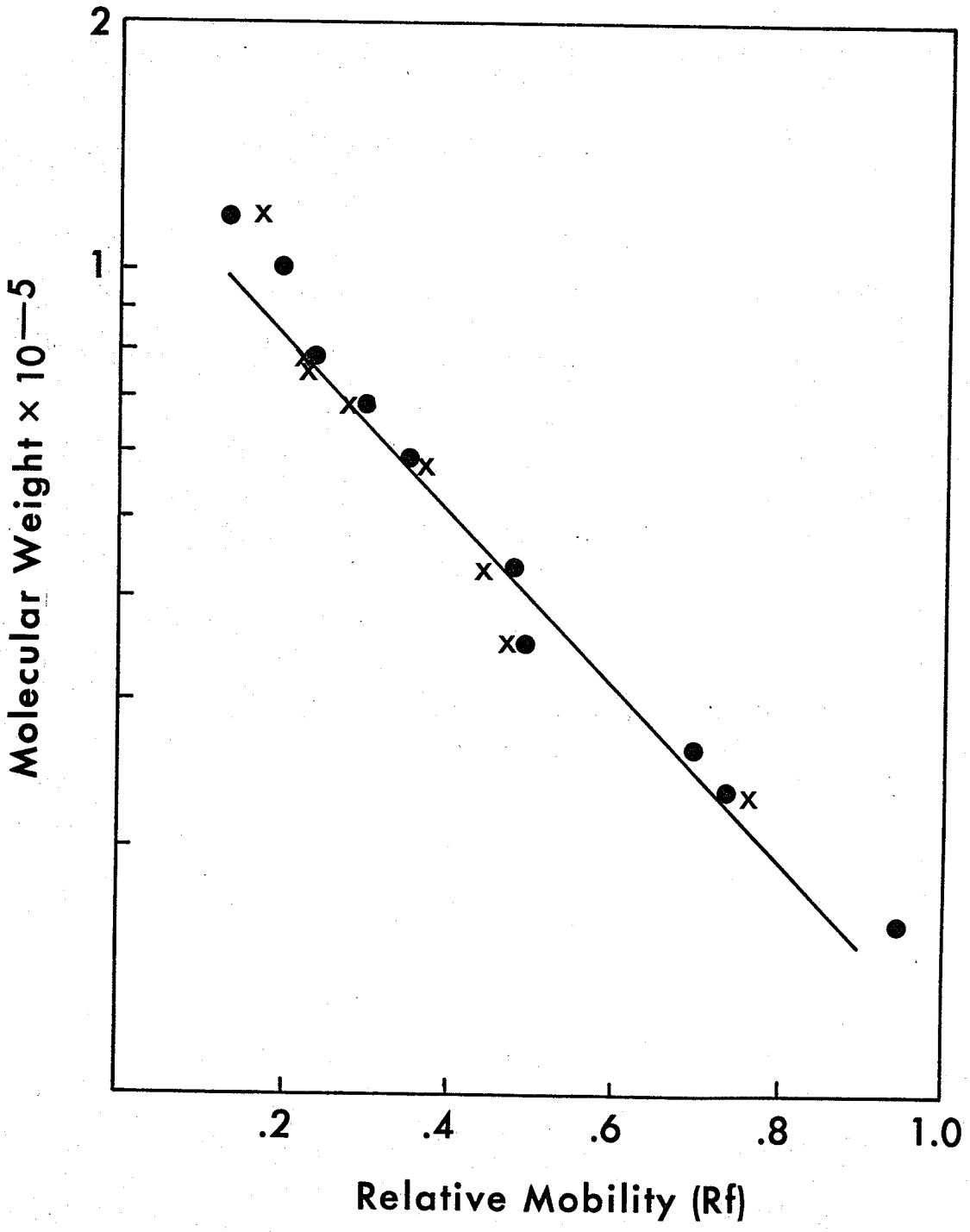

2
1
Molecular Weight x 10—5
.2
.4
.6
.8
1.0
Relative Mobility (Rf)

Figure 5: SDS-PAGE analysis on 10% Laemmli gels of specific and control precipitates from surface-iodinated RBL cells. Solubilized RBL cell equivalents (2×10^6) from 2×10^7 iodinated RBL cell extracts were used for the immunoprecipitation procedure. (a) Surface material co-precipitated by the "direct" precipitation procedure using rat IgE and ε-specific sheep anti-rat IgE. (b) Control precipitates from normal rabbit serum and goat anti-rabbit immunoglobulin (Protocol B, Fig. 1). The precipitates were washed in PBS and solubilized with Tris sample buffer containing 2% SDS. Samples were then heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE. The arrows indicate the migratory behaviour of protein standards used to calibrate the gel in order of their decreasing molecular weight from left to right: β-galactosidase, 130,000 daltons; ε-chain of IgE, 75,000 daltons; bovine serum albumin, 68,000 daltons; ovalbumin, 43,000 daltons; L-chain of IgE, 23,000 daltons; myoglobin, 11,700 daltons; TD marks the migration of the tracking dye.

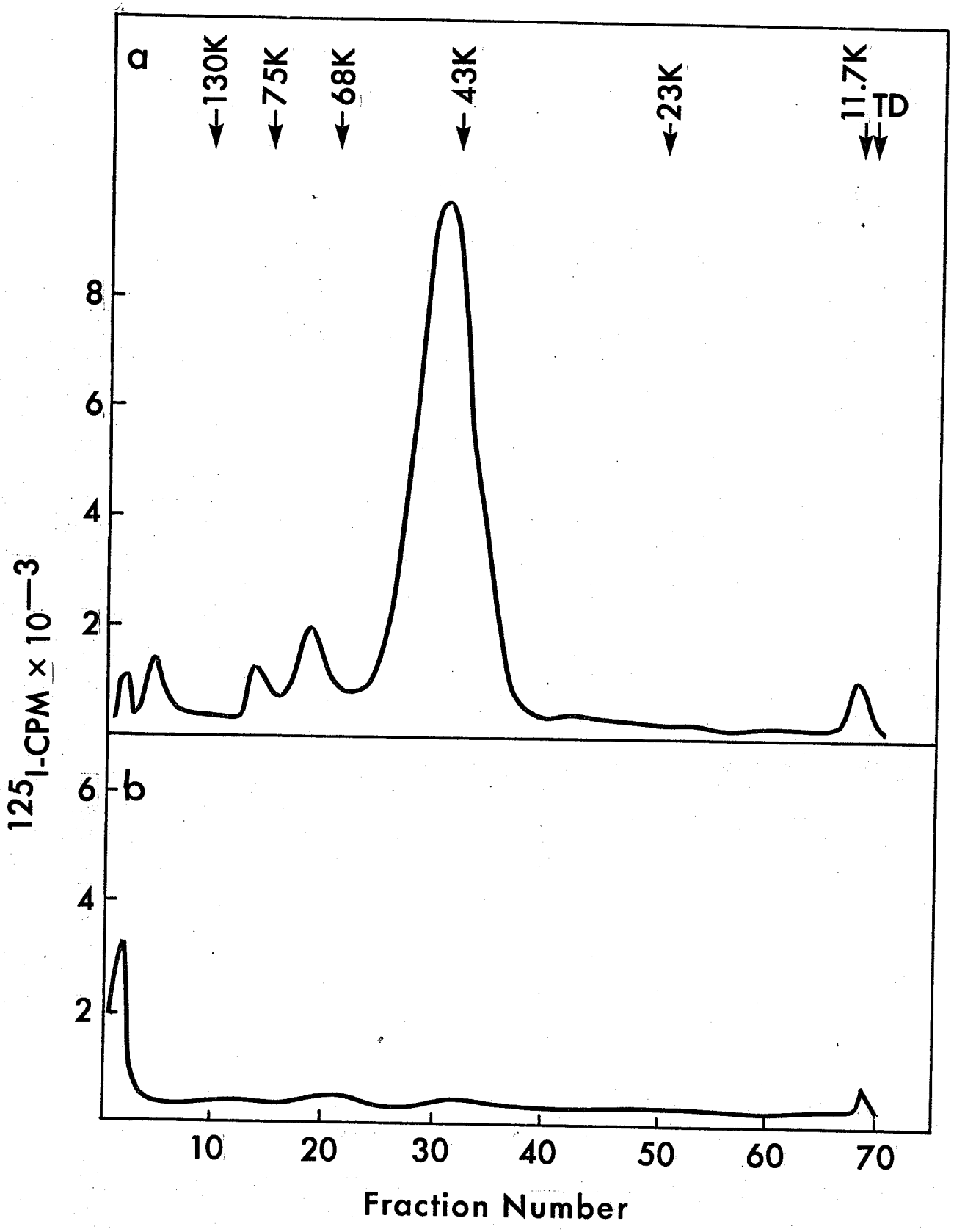
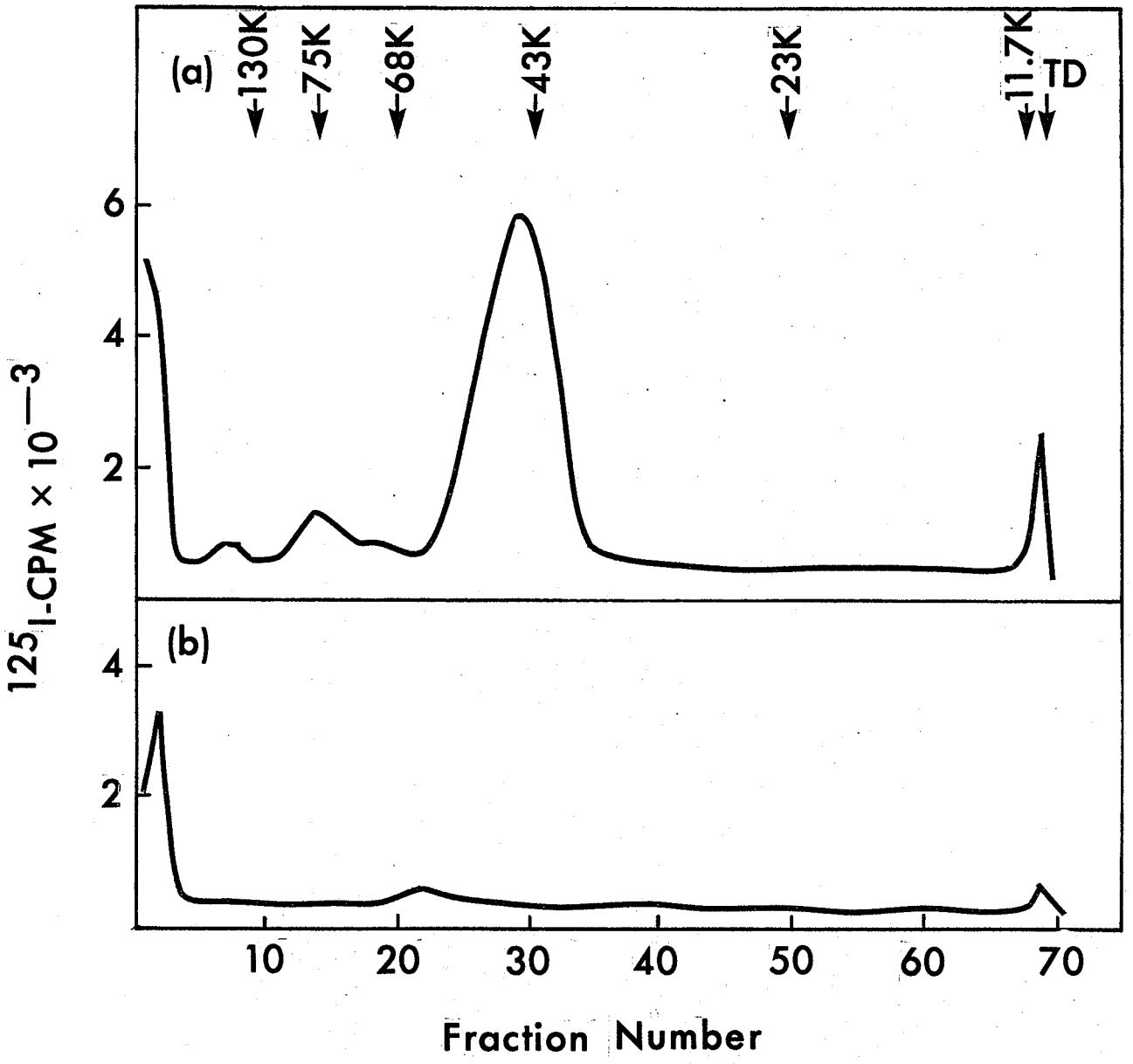

a
130K
75K
68K
43K
23K
11.7K
TD
8
6
4
2
b
6
4
2
^{125}I-CPM × 10^{-3}
10
20
30
40
50
60
70
Fraction Number

Figure 6: SDS-PAGE analysis on 10% Laemmli gels of surface iodinated RBL cell components isolated as IgE-receptor complexes insolubilized on protein A-Sepharose (Protocol C, Fig. 1). RBL cells (1×10^7) were solubilized and mixed with either IgE and ε-specific sheep anti-rat IgE (a) or normal rabbit serum and goat anti-rabbit Ig (b). Both were insolubilized on 0.2 ml protein A-Sepharose and eluted with Tris sample buffer containing 2% SDS. The samples were heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE. The molecular weight markers are as in Fig. 5.


(a)
130K
75K
68K
43K
23K
11.7K
TD
(b)
^{125}I-CPM × 10^{-3}
6
4
2
4
2
10
20
30
40
50
60
70
Fraction Number

(i.e. Fig. 1c). The results indicated that protein A-Sepharose could be used to isolate the IgE receptor of RBL cells as antigen-antibody complexes.

Isolation of the ^{125}I-Labelled Receptor for IgE from RBL Cells by IgE-Sepharose:

For isolation of the receptor from surface-labelled RBL cells by affinity chromatography, NP-40 solubilized RBL cells representing 2-3 x 10^7 RBL cell equivalents were mixed with 1.0 ml of IgE-Sepharose Cl-4B for one hour at 4^{o}C and subsequently transferred to a 1.0 ml Pasteur pipette column. The gel was then washed with 15-20 ml of a 0.1% NP-40/PBS and finally eluted with 3.0 M KSCN in 0.1% NP-40/PBS with a recovery of about 85% of the bound counts. The eluate was then dialyzed extensively against 0.0625 M Tris sample buffer adjusted to 2% SDS and a representative aliquot examined by SDS-PAGE on 10% gels. Normally, for routine use, a batchwise procedure using smaller (0.2 - 0.3 ml) amounts of affinity gel were utilized. After mixing radiolabelled cell extracts with IgE-Sepharose, the affinity gel extract mixture was washed five times with 2 - 3 ml of 0.1% NP-40/PBS in a 5.0 ml plastic tube, and once with 0.0625 M Tris sample buffer and eluted with Tris/SDS sample buffer with the same results as seen by column elution with KSCN. To demonstrate the specificity of the system, controls consisting of Sepharose Cl-4B or CNBr-activated and ethanolamine quenched Sepharose Cl-4B were used. Table II presents the percentages bound under each of these conditions. The specificity was clearly demonstrated. The IgE-Sepharose affinity gel insolubilized approximately 7.5 - 9.5%, while the controls retained only 2.0 - 3.6%. The method of elution did not affect the SDS-PAGE profile; however, the recoveries were different. Eluted material from affinity gels by the column procedure represented 80 - 85% of the bound material, while only 50 - 60% of bound material could be eluted using the batch procedure. The lower values in the latter case

TABLE II

ISOLATION OF ^{125}I-SURFACE IODINATED RBL CELLS[a]

BY AFFINITY CHROMATOGRAPHY

AFFINITY GELS	% of LABELLED MATERIAL BOUND
IgE-Sepharose Cl-4B	7.5 - 9.4
Sepharose Cl-4B[b]	3.1 - 3.6
Sepharose Cl-4B	2.0 - 3.1

(a) 2×10^6 RBL cell equivalents were mixed with 0.2 ml of the gel in a slurry for 90 minutes at $4^{\circ}C$, washed four times with buffer and counted in a Beckman gamma spectrometer. % bound refers to the amount of counts retained versus those applied. The results represent a range obtained from at least three sets of experiments.

(b) Cyanogen bromide activated and ethanolamine quenched Sepharose Cl-4B.

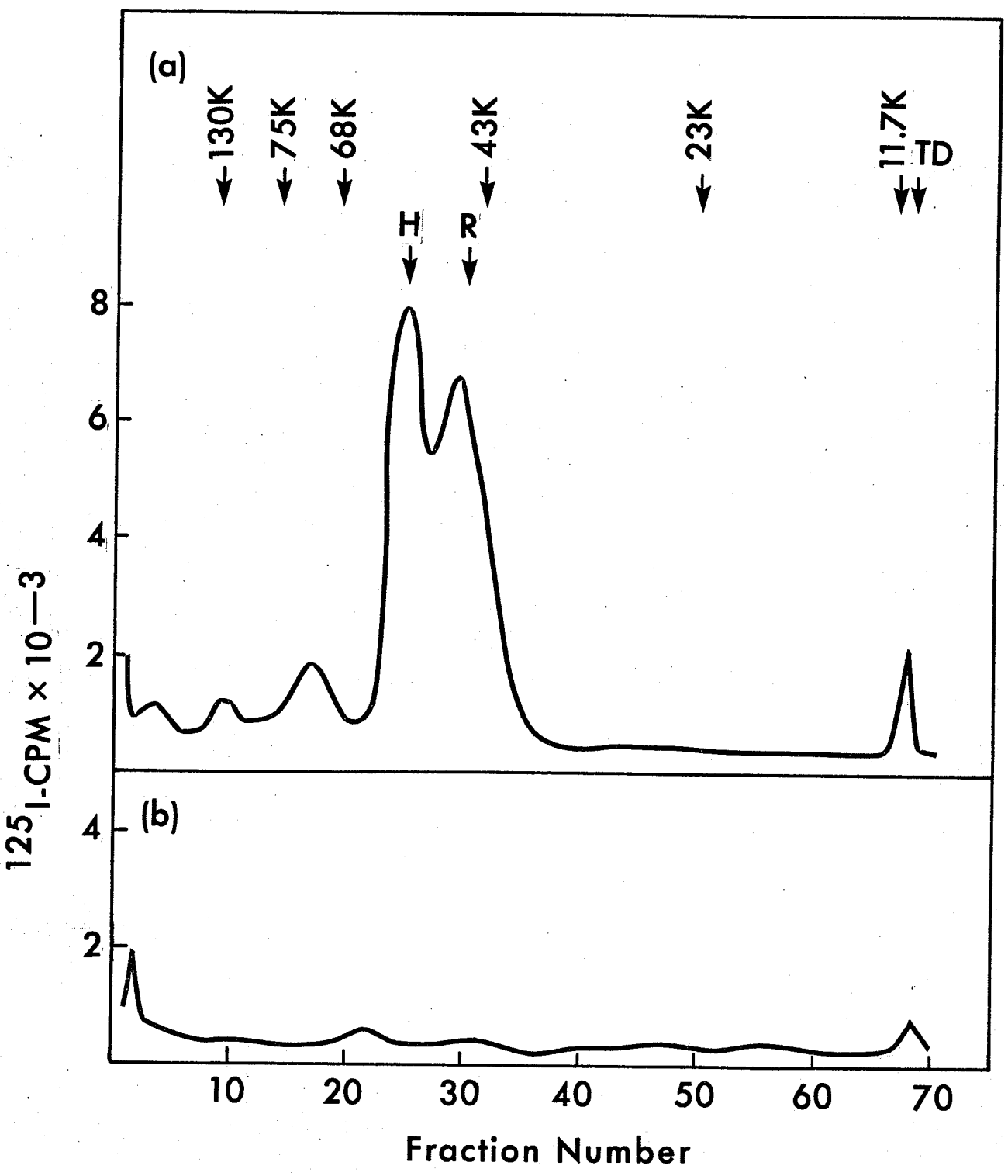
could easily be attributed to the trapping of cell surface material within the matrix of the gel.

For routine analysis of receptor components bound to affinity gel, the batchwise procedure was used. For quantitative recoveries of the receptor(s), the Pasteur pipette column elution procedure was followed. The analysis of receptor eluates by SDS-PAGE on 10% gels revealed two components with apparent molecular weights of 45,000 and 55,000 daltons, as shown in Fig. 7a. These two molecules will henceforth be designated R and H, respectively. No such peaks were seen with control eluates (Fig. 7b). A comparison of receptors isolated by this procedure and by means of IgE and anti-IgE using the same batch of labelled RBL cells is shown in Fig. 8. As can be seen, the 45,000 dalton receptor (R) was isolated by both methods, while the 55,000 dalton receptor, (H), was isolated only by IgE-Sepharose. These results are in agreement with those seen by Conrad and Froese (1978a).

Specificity of the Receptors for IgE:

To demonstrate the specificity of H and R receptors (55,000 and 45,000 daltons, respectively), inhibition experiments using free IgE were carried out. Table III demonstrates that the inhibition of receptor binding to IgE-Sepharose by free IgE was concentration-dependent. In experiment A, 1×10^7 RBL cells from a total of 3×10^7 surface-iodinated RBL cells were aliquoted into separate test tubes which contained different amounts of IgE and the mixture incubated for 60 minutes at $37^{\circ}C$, washed free of excess IgE, solubilized and added to 0.2 ml of IgE-Sepharose. As can be seen, the binding of surface counts decreased as free IgE was added to intact cells, but in repeated experiments it always levelled off near 50%, suggesting that not all receptors were sterically available to the IgE (Table IIIA). When IgE was added to the solubilized cells, more pronounced inhibition was seen (Table IIIB). The second set of experiments is in agreement with

Figure 7: SDS-PAGE analysis on 10% Laemmli gels of surface iodinated RBL cell components isolated by IgE-Sepharose. Solubilized RBL cell extracts equivalent to 5×10^6 cells from a total of 2×10^7 iodinated RBL cells were mixed with (a) 0.1 ml of IgE-Sepharose or (b) 0.1 ml of Sepharose Cl-4B for 90 minutes at $4^{\circ}C$. The gels were then washed and eluted with Tris sample buffer containing 2% SDS. The samples were heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE. H marks the position of the receptor with an apparent molecular weight of 55,000 daltons and R marks the position of the receptor with an apparent molecular weight of 45,000 daltons.


(a)
130K
75K
68K
H
R
43K
23K
11.7K
TD
8
6
4
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(b)
4
2
^{125}I-CPM × 10—3
10
20
30
40
50
60
70
Fraction Number

Figure 8: SDS-PAGE analysis on 10% Laemmli gels of surface iodinated RBL cell components isolated by IgE-Sepharose and immunoprecipitation. Solubilized RBL cell equivalents (2×10^6) from 2×10^7 iodinated RBL cells were mixed with (a) 0.1 ml of IgE-Sepharose or (b) co-precipitated by the direct precipitation procedure using IgE and ε-specific sheep anti-rat IgE. The affinity gel and precipitate were washed; elution of material bound to the affinity gel and solubilization of the precipitate was achieved with Tris sample buffer containing 2% SDS. Both samples were heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE.

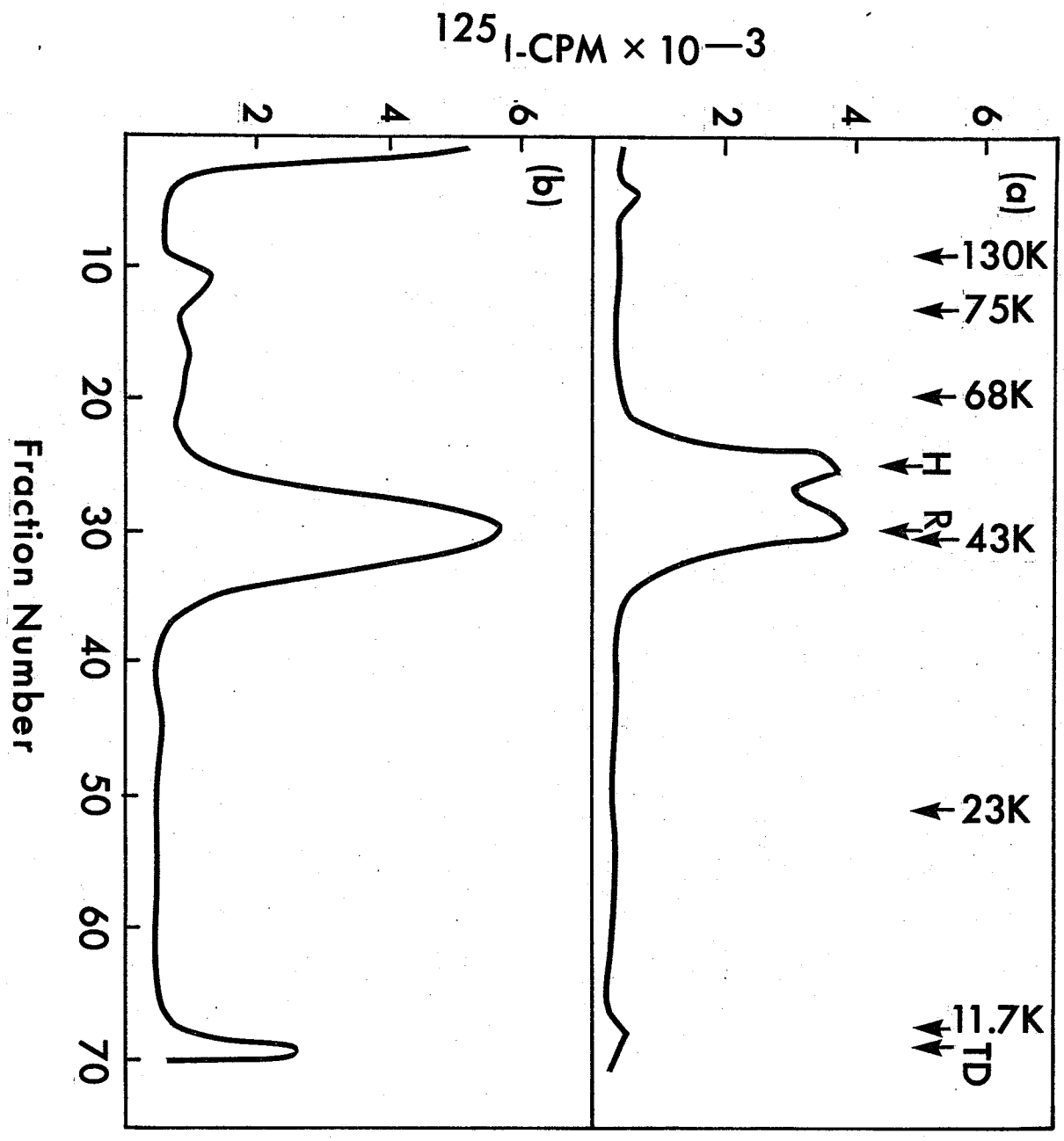

^{125}I-CPM × 10—3
2
4
6
(b)
(a)
130K
75K
68K
H
R
43K
23K
11.7K
TD
10
20
30
40
50
60
70
Fraction Number

TABLE III

INHIBITION BY IgE OF THE BINDING OF SURFACE IODINATED MOLECULES BY IgE-SEPHAROSE

EXPERIMENT A

No. Cells[a]	Conc. IgE	Bound cpm	% Bound
1×10^7	--	274,159	100
1×10^7	500 µg	125,634	45.8
1×10^7	1,000 µg	132,734	48.4

EXPERIMENT B

No. Cells[b]	Conc. IgE	Bound cpm	% Bound
3×10^6	--	53,036	100
3×10^6	250 µg	30,102	56.8
3×10^6	500 µg	24,806	46.8
3×10^6	1,000 µg	19,749	37.2

(a) 1×10^7 ^{125}I-RBL cells were incubated in PBS/BSA with the designated amounts of free IgE, washed free of excess IgE, solubilized and added to 0.1 ml of IgE-Sepharose.

(b) Solubilized ^{125}I-RBL cell equivalents (3×10^6) were incubated with the designated amounts of free IgE and added to 0.1 ml of IgE-Sepharose.

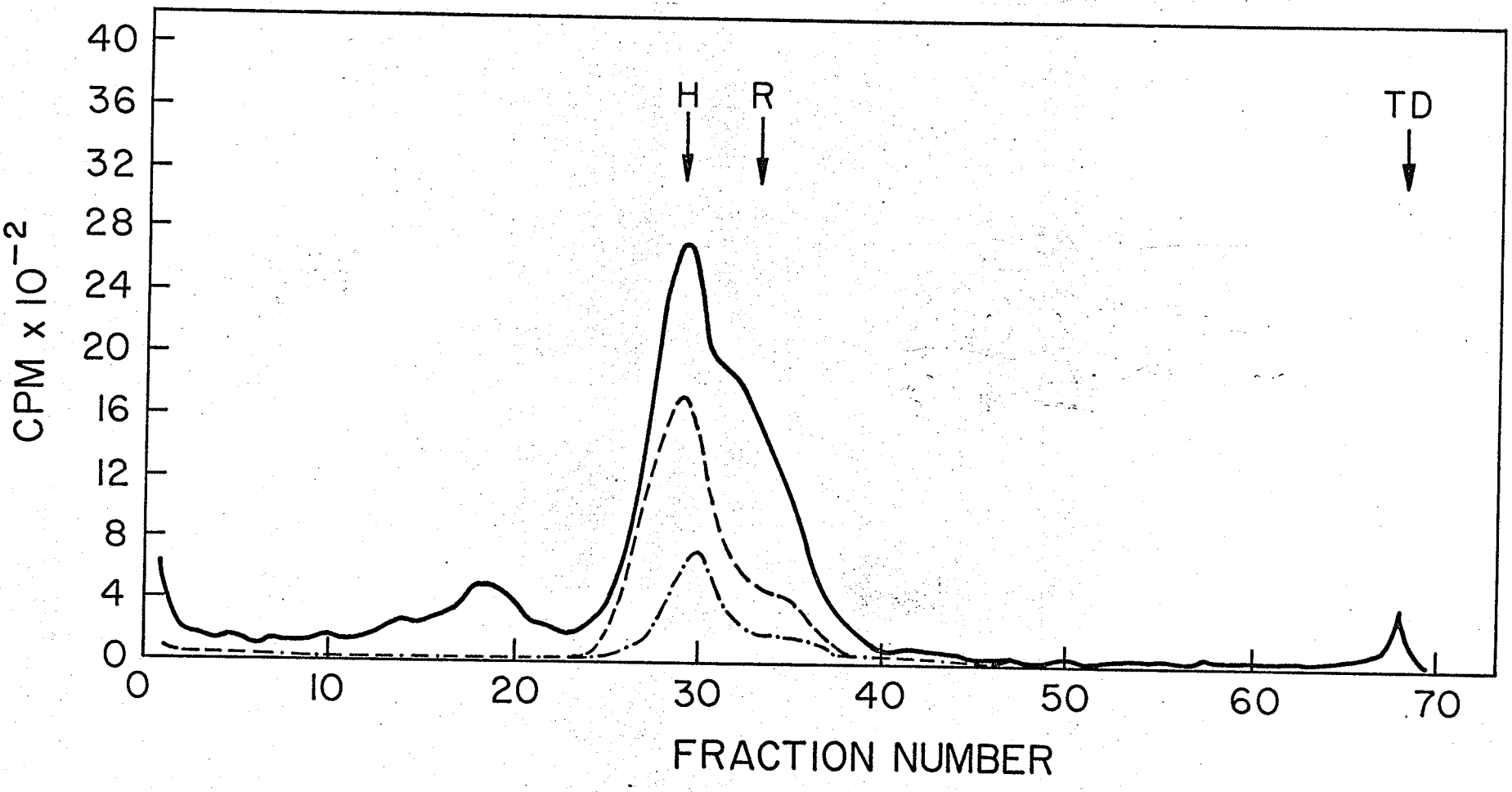
The affinity gel was washed five times with 0.1% NP-40/PBS and the samples then counted in a Beckman gamma spectrometer. The counts bound by the affinity gel in the absence of IgE represent the 100% bound value.

Representative data from a single experiment. Similar data obtained in two other experiments.

the IgE concentration dependent inhibition of RBL receptor binding demonstrated by Conrad and Froese (1978a). A comparison of the SDS-PAGE patterm of receptor isolated from cells which had been incubated with different concentrations of IgE clearly demonstrates the concentration dependence of blocking of receptor binding to IgE-Sepharose (Fig. 9). The results indicated that the inhibition was concentration-dependent and that R was relatively more susceptible to inhibition than H. A third peak, fraction 19, representing a relative molecular weight of ∿71,000 daltons, was normally present in eluates isolated from IgE-Sepharose affinity gels. The peak height of this molecule was considerably smaller than that for H and R; however, as can be seen in Fig. 9, this molecule was inhibited by the presence of IgE. Inasmuch as it can be bound by IgE-Sepharose and this binding can be inhibited by IgE, it also must be considered to be a receptor for IgE on RBL cells. Further studies are being planned to investigate this newly identified receptor more rigorously.

Analysis on 10% gels by SDS-PAGE of receptors isolated in the form of complexes using DNP-IgE and anti-DNP-Sepharose revealed a single surface component with an apparent molecular weight of 45,000 daltons (Fig. 10). When the DNP-Na eluate was compared to the receptor for IgE precipitated with rat IgE and goat anti-rat IgE, both peaks had the same mobility on either 5% or 10% gels (Conrad and Froese, 1978b). Thus, the results described provided a means of isolating the receptor in such a way that it was largely free of the second receptor (H) under chromatographically defined conditions.

Figure 9: SDS-PAGE analysis on 10% Laemmli gels of RBL surface components bound to IgE-Sepharose in the presence or absence of IgE. Solubilized RBL cell equivalents (3×10^6) from 3×10^7 surface iodinated RBL cells were mixed in the absence of (————) or presence of 250 µg (-------) or presence of 1,000 µg (-·-·-·-) of IgE for 90 minutes at 37°C before addition to 0.1 ml of IgE-Sepharose. They were allowed to mix for an additional 60 minutes at 4°C before washing five times with 2-3 ml of 0.1% NP-40/PBS by centrifugation. Elution was achieved with Tris sample buffer containing 2% SDS, the samples heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE.


CPM x 10⁻²
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36
32
28
24
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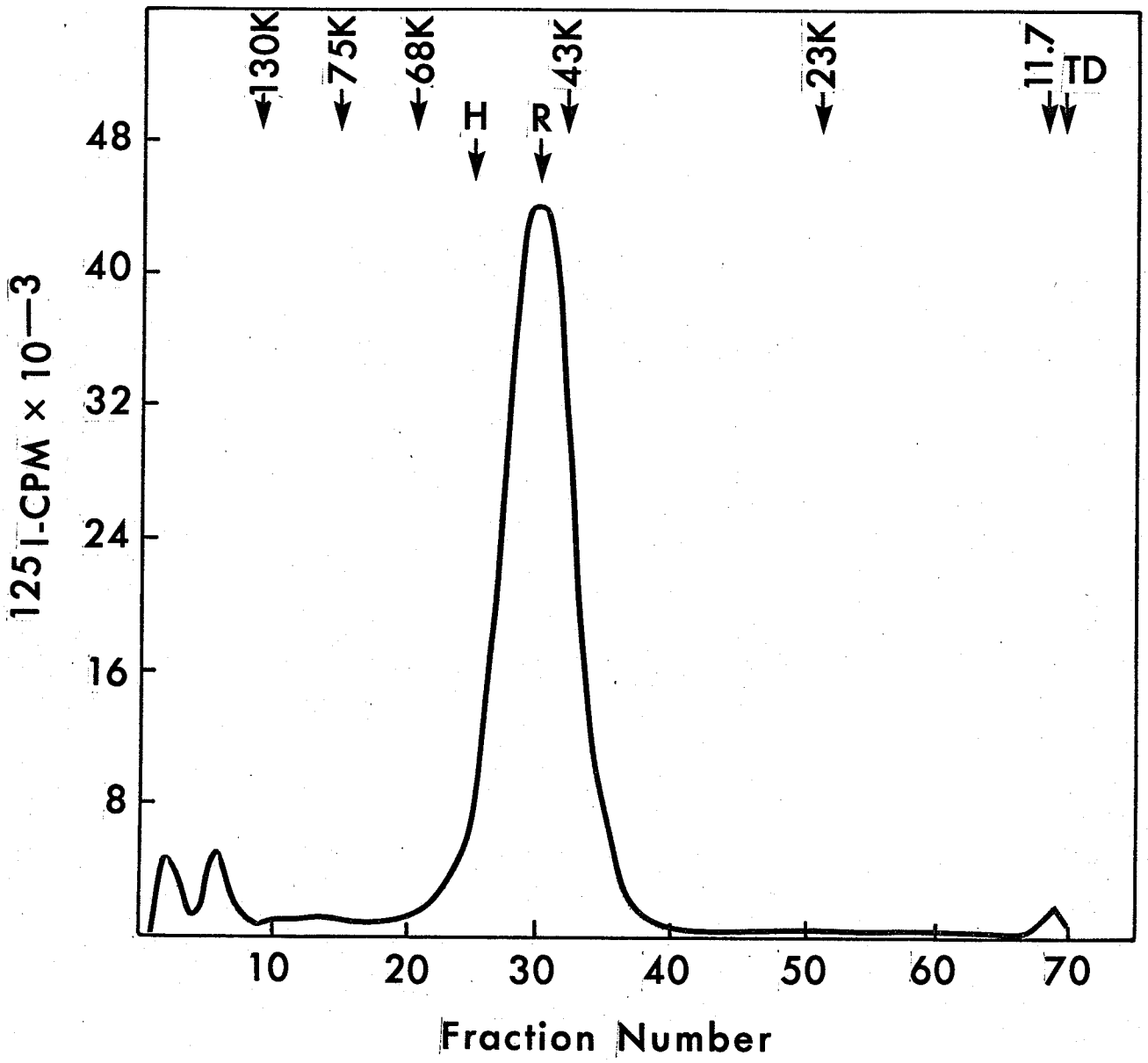
16
12
8
4
0
H
R
TD
0
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 10: SDS-PAGE analysis on 10% Laemmli gels of the receptor isolated as DNP-IgE-receptor complexes by anti-DNP-Sepharose. Surface labelled RBL cells (1×10^7) were mixed with 400 µg of DNP-IgE for 90 minutes at 37°C, washed through FCS and with PBS/BSA. The washed cell pellet was then solubilized with 0.5% NP-40/PBS and the extract mixed with 0.2 ml of anti-DNP-Sepharose for 60 minutes at 4°C. The mixture was transferred to a 1.0 ml Pasteur pipette column and washed with 15-20 ml of 0.1% NP-40/PBS. Elution was achieved with 0.1 M 2,4-dinitrophenolate in 0.1% NP-40/PBS. The eluate was dialyzed against Tris sample buffer and adjusted to 2% SDS, heated in a boiling water bath for 90 seconds and analyzed by SDS-PAGE. The arrows marked H and R mark the migration position of the two receptors isolated by means of IgE-Sepharose.


130K
75K
68K
H
R
43K
23K
11.7
TD
48
40
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24
16
8
125I-CPM × 10—3
10
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30
40
50
60
70
Fraction Number

DISCUSSION

As pointed out in the Introduction, the experiments described in this Chapter were undertaken in order to establish techniques and to provide points of reference for the two following Chapters which deal with the lectin binding and the glycoprotein nature of the receptors for IgE on RBL cells.

These experiments have confirmed that receptors isolated from NP-40 solubilized cells by means of IgE; rabbit anti-IgE and goat anti-rabbit antibody, when analyzed on 5.6% gels by SDS-PAGE, migrate as a single peak with an apparent molecular weight of about 60,000 daltons. Similar results had previously been obtained by Conrad and Froese (1976) and Kulczycki *et al.* (1976) using 5% gels, and as observed by the same two groups of investigators (Kulczycki *et al.*, 1976; Conrad and Froese, 1978a), when immunoprecipitated receptors were analyzed on 10% gels, the apparent molecular weight had dropped to 45,000 daltons. A single molecule of the same molecular weight was isolated when DNP-IgE and anti-DNP-Sepharose were used for isolation, corroborating the observations of Conrad and Froese (1978b) and those of Kanellopoulos *et al.* (1979). It should, however, be pointed out that the latter authors used arsanilic acid-IgE conjugates and the corresponding anti-arsanilic antibodies coupled to Sepharose. Moreover, the receptor isolated had a somewhat higher molecular weight (53,000 or 58,000 daltons) which most likely can be attributed to differences among the cell lines maintained in the two laboratories.

In conformity with the finding by Conrad and Froese (1978a), isolation of receptors with the aid of IgE-Sepharose yielded two surface molecules having molecular weights of 55,000 and 45,000 daltons, which have been designated H and R, could be identified. The binding of both to IgE-

Sepharose could be inhibited by free IgE. In addition to H and R, a third surface molecule was frequently observed in receptor preparations isolated by IgE-Sepharose. It migrated in fraction 17-19, which should correspond to a molecular weight of the order of 70,500 (±2,000) daltons. A corresponding molecule was occasionally seen in receptor preparations characterized previously in this laboratory (Conrad and Froese, 1978a; Conrad *et al.*, 1979). Moreover, the present study has shown for the first time that the binding of this molecule to IgE-Sepharose can be inhibited by free IgE, clearly pointing to the specificity of this surface molecule for IgE.

CHAPTER III

INTRODUCTION

Lectin Affinity Chromatography of Surface Glycoproteins and the Receptor for IgE from RBL Cells.

The study of cell surface components has achieved a prominent place in the investigations of ligand-receptor interactions. These cell surface components are frequently glycoproteins which are firmly associated with the plasma membrane, dipping into and even spanning the lipid bilayer. Being integral proteins of the plasma membrane, they are insoluble in common aqueous solvents, but are readily soluble in non-ionic and weakly ionic detergents, often with the preservation of their specific interactions with lectins, antibodies and other ligands. Investigations of cell surface glycoproteins has been facilitated by the use of lectins, which are carbohydrate-binding proteins derived mainly from plant sources (Sharon and Lis, 1972; Nicolson, 1974; Sharon, 1977; Brown and Hunt, 1978). Lectins have been shown to be oligomeric proteins with several carbohydrate-binding sites, which is to say, they are multivalent molecules. The interactions of lectins with glycoproteins of cells can be inhibited by the presence of the appropriate sugar which competes for or modifies the carbohydrate-binding site of the respective lectin. The interaction of lectins with "stabilized" glycoproteins normally involves more than one terminal sugar residue; however, it is not unlikely that internal residues bind as well, corresponding to the monomeric sugar(s) for which the lectin demonstrates specificity. Therefore, in all probability, lectins bind to a complex sequence of sugars in glycoproteins and the affinity which these lectins demonstrate for certain oligosaccharides and/or glycoproteins

is often several orders of magnitude higher than their affinity for the free monomer sugar (Young and Leon, 1974; Adair and Kornfield, 1974; Nagata and Burger, 1974). Plant lectins bound to an insoluble matrix have been used to study the glycoprotein nature of solubilized cell surface molecules from membranes of lymphocytes (Hunt and Marchalonis, 1974; Allan *et al.*, 1972; Hayman and Crumpton, 1972), erythrocytes (Adair and Kornfield, 1974; Findlay, 1974), platelets (Nachman *et al.*, 1973; Clementson *et al.*, 1977), thymocytes and splenocytes (Warr and Marchalonis, 1976), as well as many other cell types. Receptor glycoproteins isolated by affinity chromatography on lectin-Sepharose columns are then normally characterized by their mobility in SDS-PAGE.

One of the most commonly used lectins is Concanavalin A (ConA). This lectin, isolated from *Canavalia ensiformis*, was shown to be a tetramer of identical protomers, each having a molecular weight of 25,500 daltons, and containing binding sites for two metal ions, Mn^{+2} and Ca^{+2}, as well as one saccharide binding site which interacts specifically with carbohydrate structures of α-D-mannopyranoside configuration or similar configurations (Goldstein *et al.*, 1979). The phytohemagglutinin, lentil lectin, (LcH), a dimer isolated from *Lens culinaris* beans, has a subunit molecular weight of 24,500 daltons and has been shown to bind the same residues as ConA, but with a 50-fold lower affinity (Stein *et al.*, 1971). It has further been demonstrated that LcH-coupled Sepharose will give a 2-fold yield in glycoprotein as compared to ConA upon elution with α-methylmannoside (Hayman and Crumpton, 1972). Wheat germ agglutinin (WGA), a protein with a molecular weight of 36,000 daltons, is composed of two similar polypeptide chains, each possessing two binding sites for sugars (Nagata and Burger, 1974; Goldstein and Hayes, 1978). It is generally accepted that WGA binds specifically to 2-acetamido-2-deoxy-D-glucose (GlcNAc) and

its β1,4-linked oligomers (Nagata and Burger, 1974; Allen *et al.*, 1973). The Castor bean lectin (RCA), isolated from *Ricinus communis*, is a tetrameric molecule which preferentially interacts with terminal β-galactosides (Nicolson *et al.*, 1974; Olsnes *et al.*, 1975). Gorse lectin, *Ulex europaeus*, and Pea lectin, *Lotus tetragonolobus*, belong to a class of lectins which have specific affinity for L-fucose (Matsumoto and Osawa, 1969,1970).

When this study was initiated, little was known about the glycoprotein nature of the plasma membrane macromolecules of RBL cells and the receptors for IgE. The only study known at the time (Kulczycki *et al.*, 1976) had demonstrated that N-acetylglucosamine could be biosynthetically incorporated into the receptor for IgE on RBL cells. Therefore, the present study was undertaken in order to obtain information on the glycoprotein nature of the receptors for IgE on RBL cells using affinity chromatographic principles involving lectins coupled to Sepharose Cl-4B. The specificities demonstrated by each of the selected lectins should provide further insight into the carbohydrate composition of glycoprotein molecules isolated from RBL cells by IgE-Sepharose affinity chromatography. It was also hoped that such an approach would yield information on the differences or similarity of the two receptor molecules (H and R) observed on RBL cells.

MATERIALS AND METHODS

The buffers, preparation of IgE, RBL cell iodination and disruption were the same as those presented in Chapter II, Materials and Methods.

Lectin Affinity Chromatography:

Lectins from lentil, *Lens culinaris*; Wheat Germ Agglutinin, WGA, *Triticum vulgaris*; *Ricinus communis* agglutinin, RCA; Gorse, *Ulex europaeus*; and Pea, *Lotus tetragonolobus* were purchased from Sigma Chemical Co., St. Louis, Missouri. N-acetyl-D-galactosamine, N-acetyl(D) glucosamine, D(+)galactose and α-L-(-)fucose were obtained from Sigma Chemical Co., St. Louis, Missouri, and α-methyl-d-mannoside (αMM) from ICN Pharmaceuticals, Cleveland, Ohio.

Sepharose Cl-4B (Pharmacia, Uppsala, Sweden) was activated with cyanogen bromide (20 mg/ml of washed beads) and the coupling of lectins with 1 mg/ml of settled beads was performed in the presence of a 0.1 M solution consisting of the monomeric sugar for which the lectin showed specificity. Routinely, 5 to 10 ml of activated beads were reacted with 5 to 10 mg of lectin in 0.1 M $NaCO_3$, pH 8.4, at 4^oC for 18-24 hours. Lectin-coupled slurries were then stirred for an additional 3 hours in 5 mM ethanolamine and washed with 0.1 M $NaCO_3$. Lectin-coupled beads were then stored in 0.1 M $NaCO_3$ in the presence of the appropriate sugar (0.1 M) and 0.1% NaN_3 at 4^oC until further use. Absorbancy measurements at 280 nm of the washings demonstrated that 85-90% of the lectins had coupled to the Sepharose Cl-4B. ConA Sepharose (2 mg/ml) was obtained from Pharmacia. Prior to use, the lectin-coupled Sepharose beads were exposed to buffers containing 0.1% NP-40, 1 mN $CaCl_2$ and 1 mM $MnCl_2$; the latter two being required to fully activate ConA and lentil lectins. For control purposes, ethanolamine quenched Sepharose Cl-4B was prepared

in the same manner. In all cases, the beads were extensively washed with NP-40/PBS before use.

Chromatography was carried out in either a column or by a batchwise procedure. In the batchwise procedure with whole RBL cell extracts or receptors isolated from IgE-Sepharose, the interaction between lectin-coupled Sepharose and sample was carried out at $4^{o}C$ for 60 minutes, followed by 5 washes with 2-3 ml of 0.1% NP-40/PBS. Elution was achieved either with 200 mM monomeric sugar solutions in NP-40/PBS or with sample buffer containing SDS at the same concentration as that used for SDS-PAGE. During the course of the investigations, Tris-HCl or borate buffered saline were used in the place of PBS and will be indicated as such, otherwise NP-40/PBS was used routinely.

Isolation of RBL Receptor(s) for IgE:

Receptors for IgE were isolated either by means of IgE-Sepharose or immunoprecipitation using IgE and anti-IgE. Both methods were described previously (Chapter II). To obtain large amounts of receptors via IgE-Sepharose, the following scaled up procedure was used: $6 - 9 \times 10^{7}$ RBL cells, radiolabelled in batches of 3×10^{7}, were solubilized with NP-40 and allowed to interact with 1.0 ml of IgE-Sepharose for 60 minutes at $4^{o}C$. The mixture was then transferred to a 1.0 ml pasteur pipette column, washed with 15-20 ml of 0.1% NP-40/PBS and eluted with 3.0 M KSCN in 0.1% NP-40/PBS. The KSCN eluate was then dialyzed against one liter of NP-40/PBS with a buffer change to remove the KSCN and used for subsequent lectin-Sepharose chromatography.

SDS-PAGE:

This was carried out in 10% gels of 140 mm length using the Laemmli system described in Chapter II.

RESULTS

Lectin Affinity Chromatography of Isolated Cell Surface Molecules of RBL Cells:

The fractionation of the glycoproteins of surface-labelled RBL cells was initially performed using a 5.0 ml packed column of commercially prepared ConA-Sepharose. RBL cells (2×10^7) were labelled with ^{125}I by the lactoperoxidase procedure and solubilized as described under Materials and Methods, Chapter II. As shown in the insert in Fig. 11, approximately 67% of the radiolabelled cell surface components were not bound by the ConA-Sepharose (Fraction A). Of the remaining material, 33%, which bound to the affinity gel, approximately 40% could be eluted with 500 mM αMM, representing only 12.7% of the total cpm applied (Fraction B). Only a further 2.4% of the total could be eluted using 3.0 M KSCN (Fraction C). Increasing the concentration of the monomeric free sugar or substituting it with αMM-D-glycopyranoside did not enhance the specific elution. Since less than half of the bound material could be eluted from ConA-Sepharose, it was decided to use LcH instead. This lectin has the same sugar-binding specificity as ConA, but has a much lower binding affinity for the sugar residues.

As shown in Fig. 12, approximately 30% of the radiolabelled components were bound to a 10.0 ml column of LcH-Sepharose, of which nearly 75% could then be eluted with 200 mM αMM, representing 23% of the total cpm applied. An additional 3.0% of the total was eluted using 3 M KSCN, which represented a total recovery of 95% or more under these conditions. The specificity of the reaction between LcH-Sepharose and the RBL cell surface molecules were shown by the fact that when initial binding was carried out in the presence of αMM, virtually all of the

Figure 11: Affinity chromatography of 0.5% NP-40/PBS solubilized extracts of surface iodinated RBL cells (2×10^7) on a Concanavalin A-Sepharose column (5.0 ml packed volume). The arrows indicate the stage of chromatography at which the eluting agents were applied. Each fraction consisted of a 1.0 ml volume. The insert shows the percentage of counts collected in A - unretarded fraction; B - α-MM eluate; C - KSCN eluate; and D - non-eluted fraction.

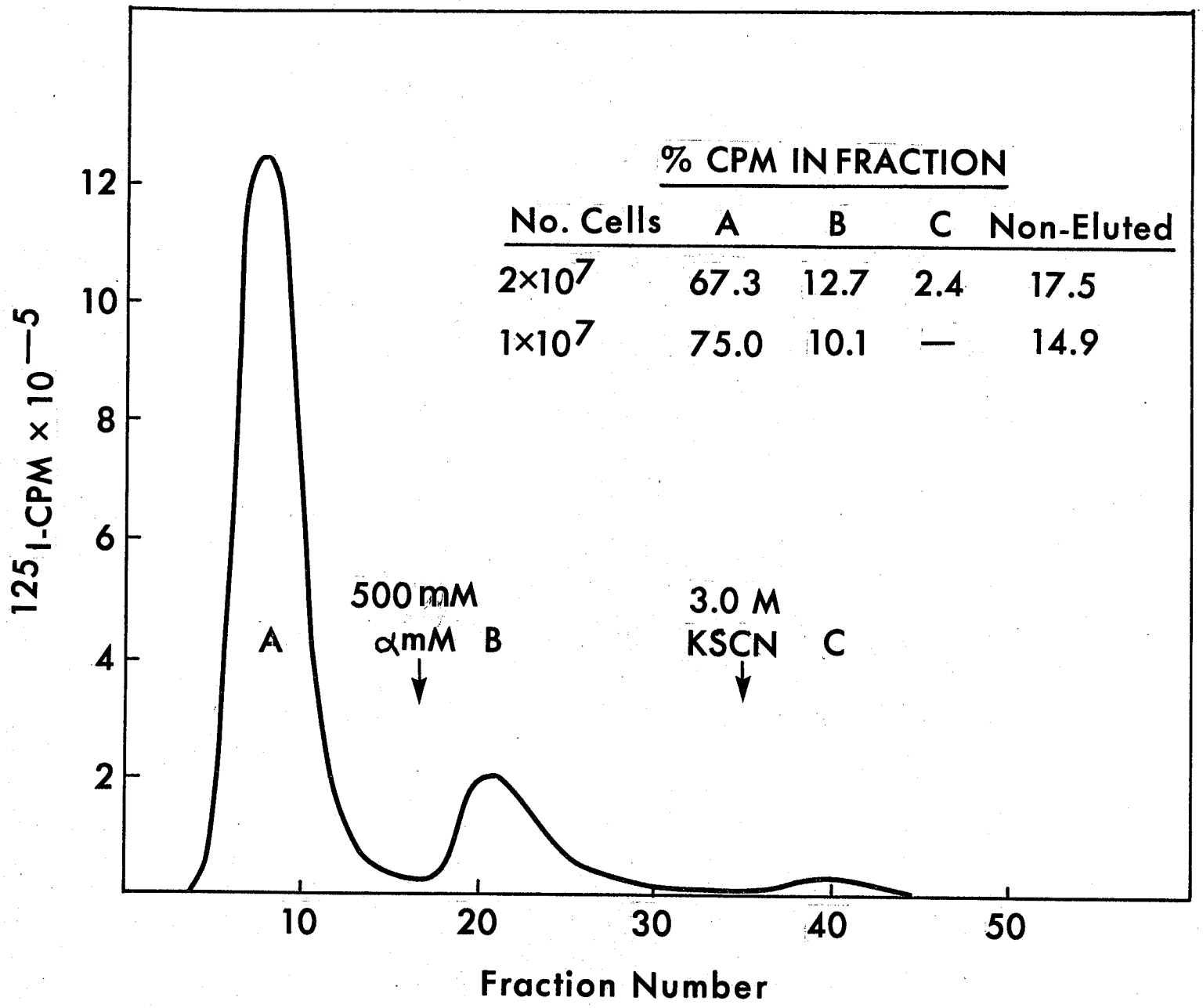
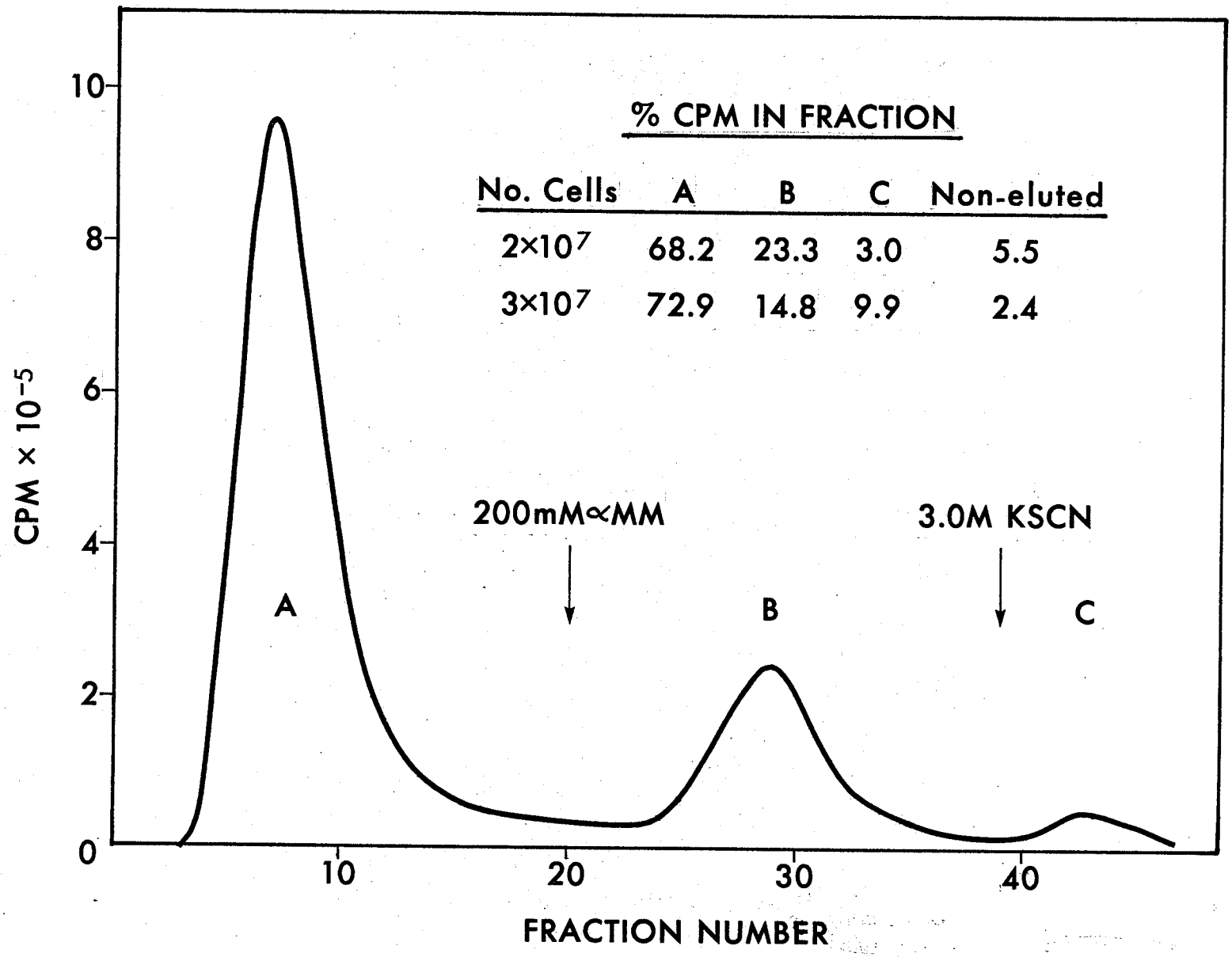

% CPM IN FRACTION
No. Cells A B C Non-Eluted
2×10^7 67.3 12.7 2.4 17.5
1×10^7 75.0 10.1 — 14.9
125I-CPM × 10—5
12
10
8
6
4
2
A
500 mM
αmM B
3.0 M
KSCN C
10
20
30
40
50
Fraction Number

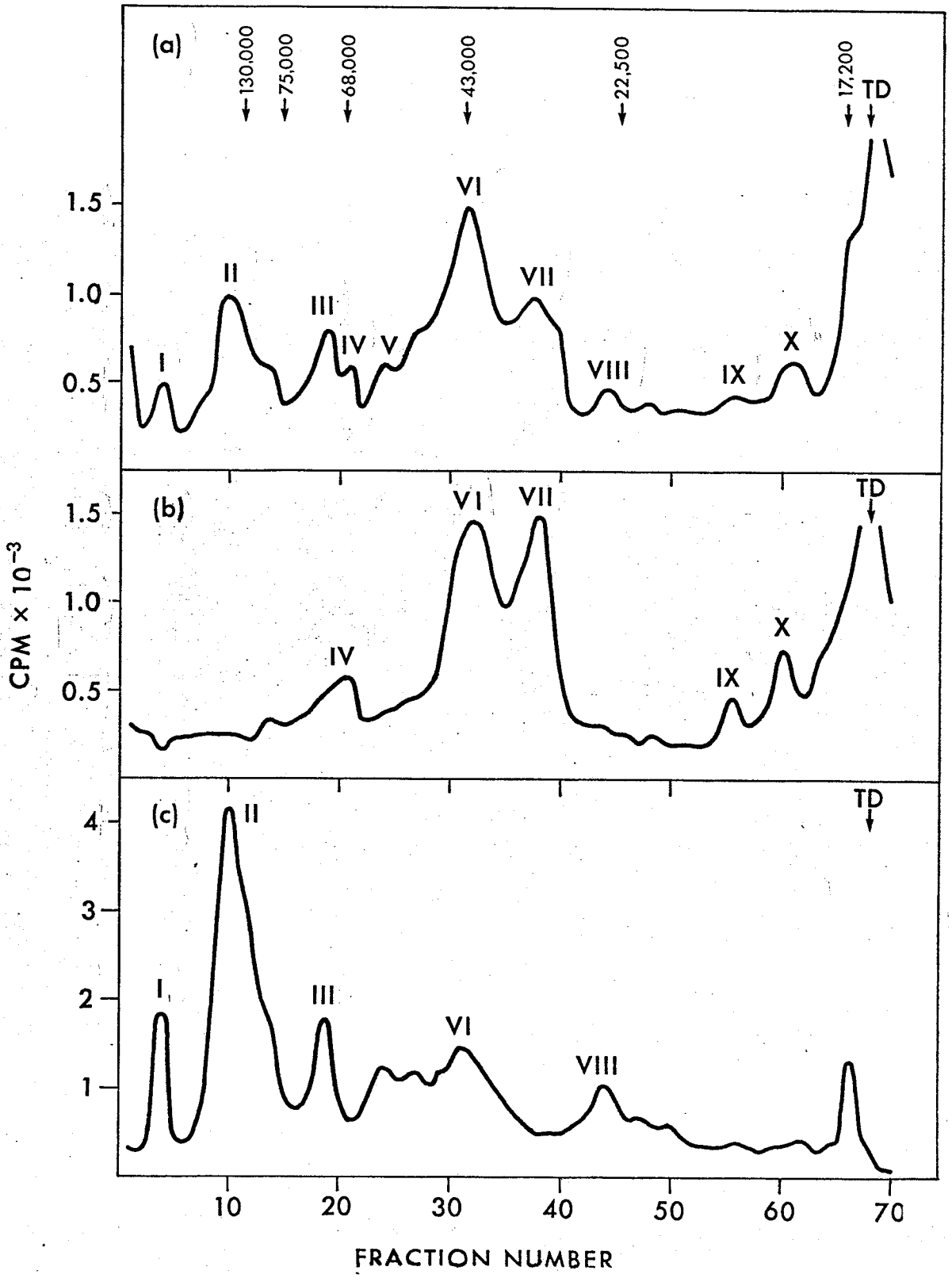
Figure 12: Affinity chromatography of 0.5% NP-40/PBS solubilized extract of surface iodinated RBL cells (2×10^7) on a LcH-Sepharose column (10 ml packed volume). The arrows indicate the stage of chromatography at which the eluting agents were applied. Each fraction consisted of 1.0 ml volume. The insert shows the percentage of counts collected in A - unretarded fraction; B - α-MM eluate; C - KSCN eluate; and D - material which remained bound to the affinity gel.


% CPM IN FRACTION
No. Cells A B C Non-eluted
2×10^7 68.2 23.3 3.0 5.5
3×10^7 72.9 14.8 9.9 2.4
200mM∝MM
3.0M KSCN
A
B
C
CPM × 10^-5
FRACTION NUMBER
0
2
4
6
8
10
10
20
30
40

binding was eliminated. Addition of radiolabelled RBL cell extracts to unconjugated Sepharose columns demonstrated that only 3-5% of the surface counts were retained, thereby demonstrating the binding to LcH-Sepharose to be of a specific nature. Fractions from the 10.0 ml column shown in Fig.12 were pooled, concentrated by Amicon pressure filtration, dialyzed against Tris sample buffer and analyzed by SDS-PAGE under reducing conditions. The use of scaled-down experiments using the batch-wise procedure for binding, washing and elution gave results approaching those seen with the 10.0 ml column.

Examination of the total cell extract and the different fractions from LcH-Sepharose by electrophoresis revealed the patterns shown in Fig. 13. Very similar profiles were noted using the same fractions taken from experimental conditions following the batch-wise procedure. Fig. 13a shows the total cell extract which yielded about ten major peaks which are numbered I - X. A major portion of the radiolabelled components migrated in fractions 25-40, representing apparent molecular weights of 30,000 - 60,000 daltons. A number of other smaller peaks appeared elsewhere throughout the gel. Analysis of the unbound fraction from LcH-Sepharose (Fig. 13b) revealed the majority of counts could be recovered in the molecular weight range represented by peaks VI (45,000 daltons) and VII (35,000 daltons). Smaller, yet distinct peaks were seen in IV (66,000 daltons), IX (20,000 daltons) and X (>20,000 daltons). Fig. 13c shows the αMM eluted material in the heavier molecular weight ranges as seen in peaks I (>200,000 daltons), II (>130,000 daltons) and III (72,000 daltons) and with smaller but significant amounts in VI (45,000 daltons) and VIII (28,000 daltons). Examination of KSCN eluted material revealed a broad peak starting in fraction 20 and ending in fraction 34 with no significant peaks (not shown).

Figure 13: SDS-PAGE analysis on 10% Laemmli gels of surface iodinated RBL cell components fractionated by LcH-Sepharose. 2×10^7 RBL cells solubilized with 0.5% NP-40/PBS were added to a 10 ml LcH-Sepharose column. Aliquots of cell surface material applied to the SDS-PAGE gels were from (a) total cell surface material, (b) cell surface components not bound by LcH-Sepharose, (c) cell surface components bound by LcH-Sepharose and eluted with α-methylmannoside. Each sample was dialyzed against Tris sample buffer overnight and adjusted to 2% SDS (v/v) and 5% 2-mercaptoethanol (v/v), heated in a boiling water bath for 90 seconds.


(a)
130,000
75,000
68,000
43,000
22,500
17,200
TD
I
II
III
IV
V
VI
VII
VIII
IX
X
(b)
(c)
CPM × 10⁻³
FRACTION NUMBER

Characterization of the Receptors for IgE by Means of Lentil-Lectin Affinity Chromatography:

Experiments performed to identify the LcH-Sepharose chromatographic fractions in which the receptors for IgE resided required relatively small quantities of LcH-Sepharose. Routinely, 2 - 3 x 10^7 RBL cell equivalents and 1.0 - 2.0 ml of LcH-Sepharose were used. Basically, two experimental protocols were followed as depicted in Fig. 14. In Protocol A, solubilized extracts from surface iodinated RBL cells were allowed to interact with LcH-Sepharose or unconjugated Sepharose. The unbound (supernatant fraction) and αMM eluates were then mixed with IgE-Sepharose. After washing with 0.1% NP-40/PBS, the IgE-Sepharose was eluted using the Laemmli sample buffer and analyzed by SDS-PAGE on 10% gels. Fig. 15 demonstrates the results obtained using this experimental protocol. Fig. 15a demonstrates the profile seen when the supernatant (unretarded) from 1.0 ml of LcH-Sepharose was mixed with IgE-Sepharose and the specifically bound surface components were eluted by sample buffer. Two major peaks were resolved with the heavier molecular weight component being the predominant one. Fig. 15b depicts the results seen when the αMM eluted fraction from LcH-Sepharose was mixed with IgE-Sepharose and the surface molecules bound by IgE-Sepharose were eluted by sample buffer. As can be seen, the lower molecular weight component (R) is more evident than the heavier molecular weight component (H). When reconstitution experiments were performed (i.e. the supernatant and eluate from LcH-Sepharose were mixed and allowed to interact with IgE-Sepharose and the bound material eluted with sample buffer), the profile in Fig. 15c was obtained. This pattern is quite similar to the one shown in Fig. 15d, in which the supernatant from a control experiment with control Sepharose was subjected to affinity chromatography on IgE-Sepharose.

Figure 14: Flow diagram outlining the characterization of surface iodinated RBL cell components by a combination of affinity chromatography using both IgE-Sepharose and LcH-Sepharose. Samples were analyzed by SDS-PAGE following the second chromatographic step.

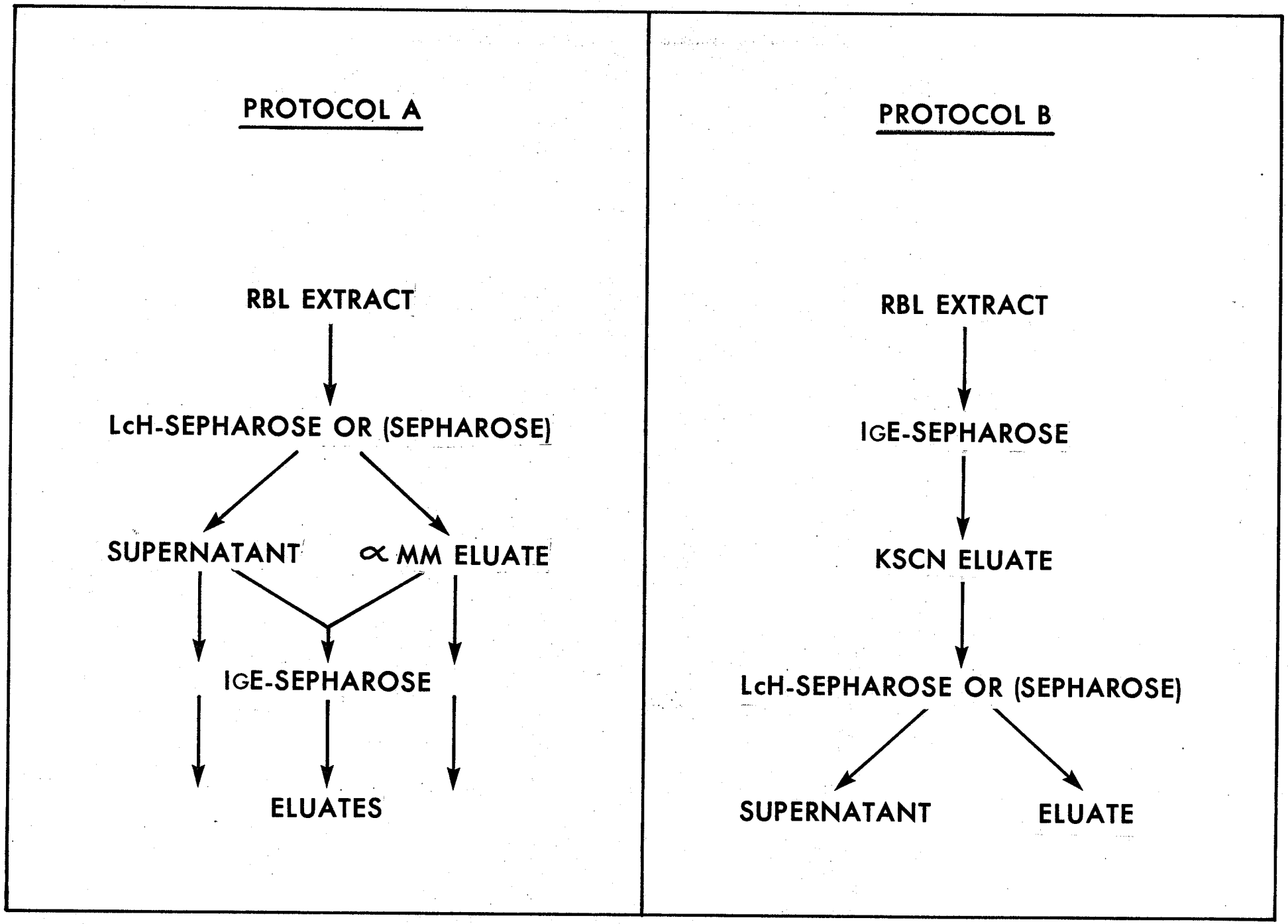
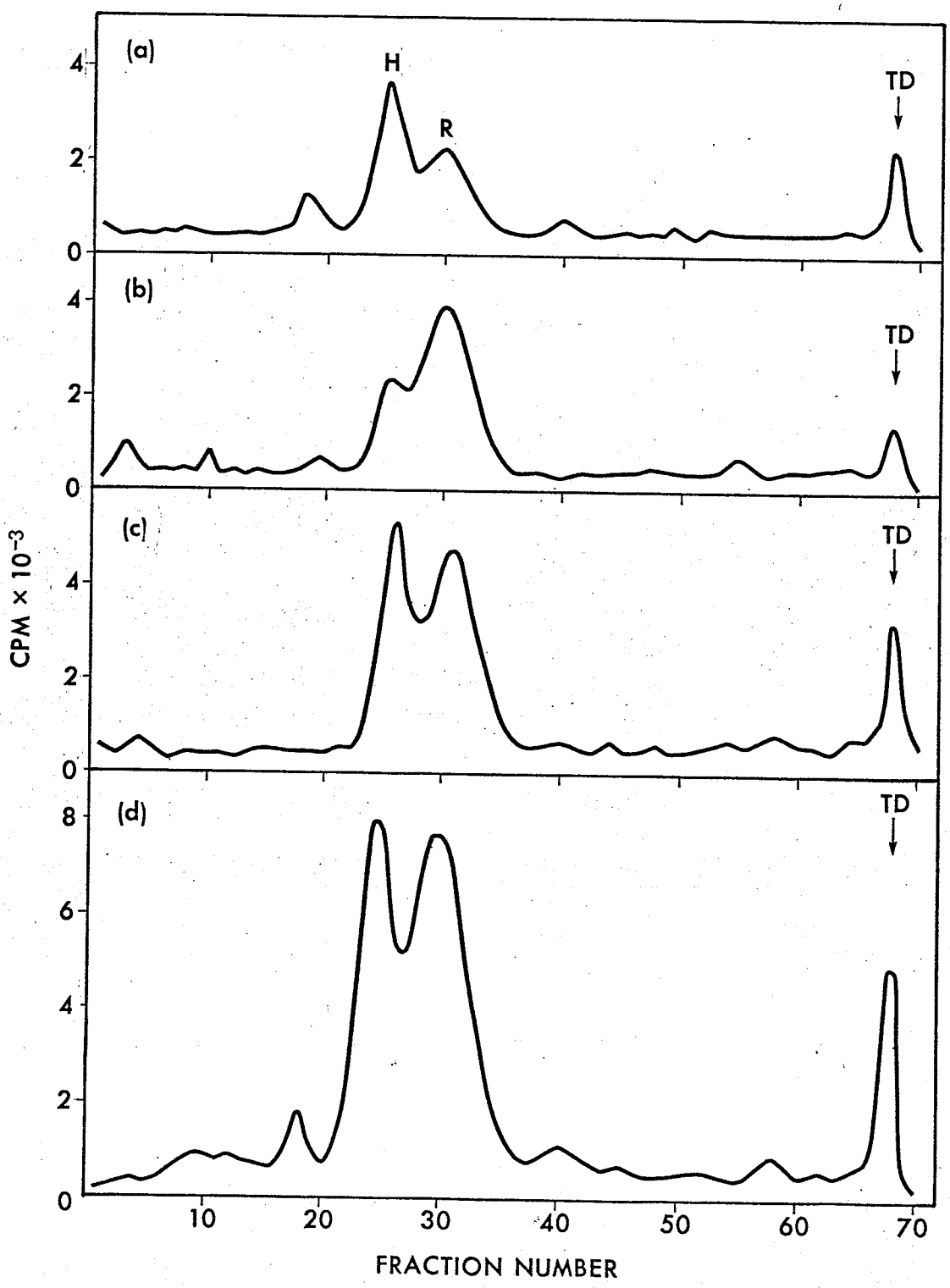

PROTOCOL A
RBL EXTRACT
LcH-SEPHAROSE OR (SEPHAROSE)
SUPERNATANT
∝ MM ELUATE
IgE-SEPHAROSE
ELUATES
PROTOCOL B
RBL EXTRACT
IgE-SEPHAROSE
KSCN ELUATE
LcH-SEPHAROSE OR (SEPHAROSE)
SUPERNATANT
ELUATE

Figure 15: SDS-PAGE analysis on 10% Laemmli gels of surface iodinated RBL cell receptors isolated by IgE-Sepharose following fractionation by either LcH-Sepharose or CNBr-activated and ethanolamine quenched Sepharose Cl-4B (Protocol A, Fig. 14). Receptors present in (a) supernatant (unbound) fraction from LcH-Sepharose, (b) α-MM eluted fraction from LcH-Sepharose, (c) mixture of the supernatant (unbound) and α-MM fractions from LcH-Sepharose and (d) the supernatant fraction from CNBr-activated and ethanolamine quenched Sepharose Cl-4B (control). Prior to receptor isolation by IgE-Sepharose, all samples were dialyzed overnight at 4°C against 0.5% NP-40/PBS. The samples were mixed with IgE-Sepharose for 60 minutes at 4°C, washed five times with 0.1% NP-40/PBS and eluted with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.


(a)
H
R
TD
(b)
TD
(c)
TD
(d)
TD
CPM × 10⁻³
FRACTION NUMBER
0
2
4
6
8
10
20
30
40
50
60
70

In order to confirm further that the receptor for IgE (R) could be bound and eluted from LcH-Sepharose, an αMM eluate was subjected to the precipitation scheme using rat IgE and ε-specific sheep anti-rat IgE. The precipitated surface components were then analyzed by SDS-PAGE and the single peak, characteristic of this receptor, was seen as depicted in Fig. 16.

In protocol B (Fig.14), a purified receptor preparation was first obtained by means of IgE-Sepharose and after removal of KSCN by dialysis, its binding to LcH-Sepharose was investigated. The surface components were once again monitored by SDS-PAGE and the results are shown in Fig. 17. In Fig. 17a the effects of treating a dialyzed KSCN eluate from IgE-Sepharose with control and LcH-Sepharose are illustrated. As can be seen, the LcH-Sepharose binds mainly the component with the faster mobility and, thus, the lower molecular weight when supernatants from these two gels are analyzed. This molecule (R) has previously been identified as the receptor for IgE (Conrad and Froese, 1976; Conrad and Froese, 1978b). An SDS-PAGE analysis of the surface material bound by LcH-Sepharose and eluted with sample buffer is shown in Fig. 17b. Only traces of the heavy molecular weight receptor (H) appeared to be bound.

Binding of the Receptors for IgE by Various Lectins:

The studies using lentil lectin had indicated that both H and R receptors were bound by this lectin. Evidence of this specificity was shown in results which pointed to α-methylpyranoside as being one of the sugar residues in the carbohydrate moiety of the receptors, R, which bound with a higher degree of avidity than H to LcH-Sepharose. This suggested that the two receptors could be separated according to their sugar content, or more specifically their carbohydrate composition.

Figure 16: SDS-PAGE analysis on 10% Laemmli gels of the receptor for IgE in the α-MM eluted fraction from LcH-Sepharose. Solubilized RBL cells (2×10^7) were applied to a 10 ml column of LcH-Sepharose, washed to background levels and eluted with α-MM. The α-MM eluate was concentrated and dialyzed overnight at 4°C against 0.5% NP-40/PBS. The sample was then mixed with rat IgE and ε-specific sheep anti-rat IgE. The co-precipitated material was washed with 0.1% NP-40/PBS, solubilized with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.

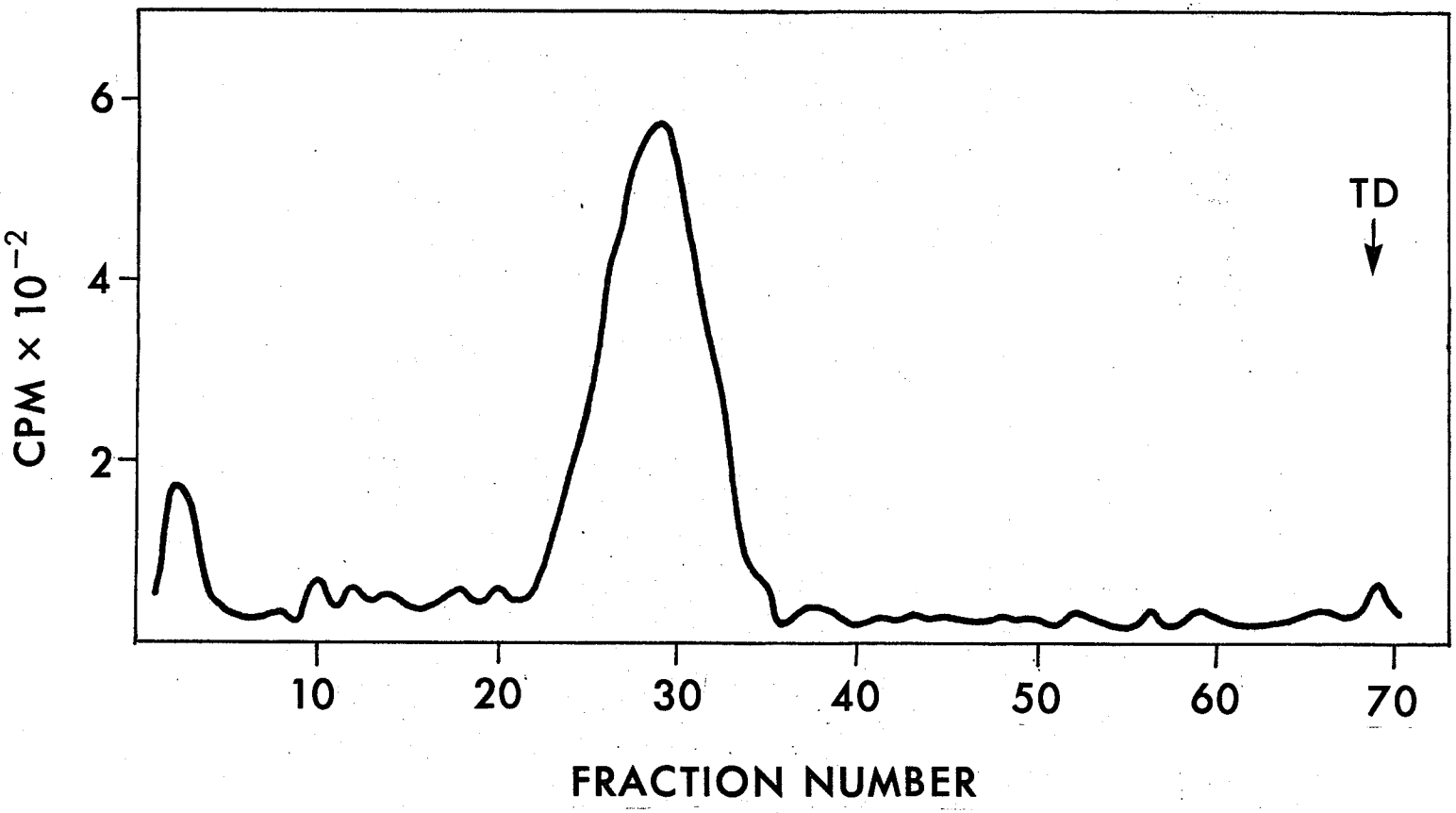
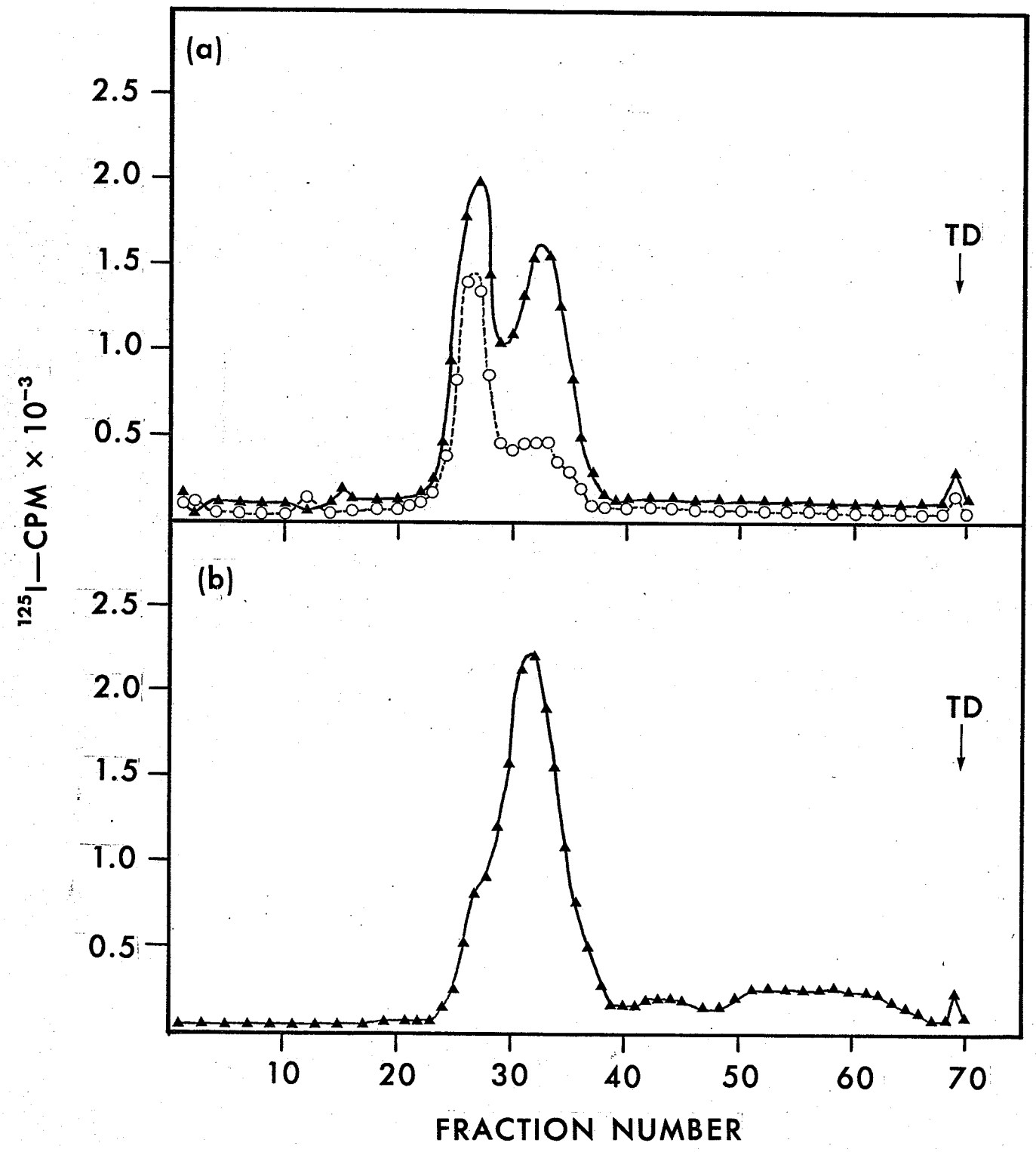

6
4
2
CPM × 10⁻²
TD
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 17: SDS-PAGE analysis on 10% Laemmli gels of isolated surface iodinated RBL cell receptors by LcH-Sepharose or CNBr-activated and ethanolamine quenched Sepharose Cl-4B (Protocol B, Fig. 14). Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C. The affinity gel was washed until washings contained background count levels. The bound material was eluted with 3.0 M KSCN in 0.5% NP-40/PBS and dialyzed overnight at 4°C against 0.5% NP-40/PBS. Aliquots of the dialyzed eluate were added to 1.0 ml LcH-Sepharose or CNBr-activated and ethanolamine quenched Sepharose Cl-4B (control). (a) Supernatant (unbound) material from control (▲——▲) and from LcH-Sepharose (o-----o). (b) Material eluted from LcH-Sepharose with Tris sample buffer containing 2% SDS. All samples were heated in a boiling water bath for 90 seconds.


(a)
(b)
TD
TD
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
FRACTION NUMBER
^{125}I—CPM × 10^{-3}

In the following study, selected lectins coupled to Sepharose Cl-4b were screened for their future application in completely separating the H and R receptors. In addition, it was hoped to gain further insight into the sugars making up the carbohydrate portion of these receptor molecules. The results of this preliminary investigation are outlined below.

Receptors were isolated from 6 - 9 x 10^7 ^{125}I-labelled RBL cells by adding solubilized extracts to 1.0 ml of IgE-Sepharose. Normally, about 85% of the specifically bound counts, representing H and R receptors, could be eluted with 3.0 M KSCN. Following the removal of KSCN by dialysis against NP-40/PBS, equal aliquots of this receptor eluate were then mixed with 0.2 - 0.3 ml of each of the lectin-Sepharose conjugates. The mixture was then shaken at 4^{o}C for 60 minutes and the gels washed five times with 2-3 ml of 0.1% NP-40/PBS. Finally, the counts bound were counted. As can be seen in Table IV, the percentage of the counts bound varied from lectin to lectin. Thus, some lectins could have differentiated between the two receptors. However, it was conceivable that both receptors were being bound by the lectins and that low affinities may have prevented the complete binding under the conditions used. Although no attempts were made at this stage to establish the capacity of the various lectin-Sepharose conjugates, it was clear from Table IV that controls consisting of protein-A Sepharose and untreated Sepharose Cl-4B bound only small amounts of the added receptor material. It should be noted that IgE-Sepharose rebound only 30-50% of the purified receptors, suggesting that some denaturation of receptors had taken place.

To define the receptor material binding to the various lectin Sepharose conjugates, the material remaining in the supernatant and sample buffer eluates of the material bound to the Sepharose conjugates were analyzed by SDS-PAGE. SDS-PAGE on 10% gels of the eluted material from

TABLE IV

BINDING OF THE RECEPTOR(S)[(a)] TO LECTIN-COUPLED SEPHAROSE C1-4B

LECTIN	% BOUND	SUGAR SPECIFICITY
Con A	85-87%	α-Methyl Mannoside
Lentil	40-45%	α-Methyl Mannoside
WGA	50-60%	N-Acetyl Glucosamine
RCA	53-60%	Galactose
Pea	40-58%	Fucose
Gorse	25-33%	Fucose
CONTROL		
Protein A-Sepharose	5-7%	
Sepharose C1-4B	3.5-5.0%	
IgE-Sepharose	30-50%	

(a) RBL cells (6-9 x 10^7) were surface-labelled with ^{125}I, solubilized and mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C. The affinity gel was transferred to a 1.0 ml Pasteur pipette column, washed with 15-20 ml of 0.1% NP-40/PBS and eluted with 3.0 M KSCN in 0.1% NP-40/PBS. Following dialysis of the eluate against 0.1% NP-40/PBS overnight, equal aliquots of the isolated receptor preparations were mixed with 0.3 ml of the respective lectin-Sepharose conjugates for 60 minutes at 4°C. The affinity gels were then washed five times with 0.1% NP-40/PBS and counted. The percentage bound is that portion of the counts remaining associated with the affinity gel following the washing procedure.

% Bound results represent a range obtained from at least three sets of experiments.

KSCN dialyzed eluates rebound and eluted from IgE-Sepharose normally showed the profile seen in Fig. 18b. A comparison with the profile in Fig. 18a, which depicts the original receptor material, demonstrates that relatively less of R was capable of re-binding to IgE-Sepharose, suggesting that, perhaps, this molecule was more easily denatured by the isolation procedure than H. Incomplete re-binding of IgE-Sepharose purified receptors had also been reported by Conrad and Froese (1978a) using IgE and anti-IgE immunoprecipitation.

ConA-Sepharose, which bound approximately 85% of the receptor eluate, was the only lectin capable of binding both receptors equally well. Although it has been shown to bind similar sugar residues as lentil lectin, ConA-Sepharose does so with a 50-fold higher affinity (Stein *et al.*, 1971). This may account for the fact that LcH-Sepharose demonstrated a significantly lower affinity for one of the receptors, H, than for R, as can be seen in Fig. 19. The profile of receptors bound to and eluted from ConA-Sepharose (Fig. 19b) was quite similar to the total eluate from the IgE-Sepharose used (Fig. 19a), whereas LcH-Sepharose, as clearly demonstrated in a previous section of this Chapter and in Fig. 19c, demonstrated preference for R receptor. It should be pointed out that both lectins appeared to bind the surface molecule migrating near fraction 17-19, indicating that it was also a glycoprotein. The ability of ConA-Sepharose to bind both receptors, H and R, equally well does point to the presence of mannosyl residues in these two receptors; however, the preferential binding of R by LcH-Sepharose suggested that the carbohydrate moieties of the two are not identical. One can only speculate that the differences in these molecules may involve structural differences in the carbohydrate moiety of the two receptors. It must not be forgotten that ConA-Sepharose demonstrates a 50-fold higher affinity for mannosyl residues than does

Figure 18: SDS-PAGE analysis on 10% Laemmli gels of isolated receptors eluted by KSCN from IgE-Sepharose bound by fresh IgE-Sepharose. Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose, washed to background levels with 0.1% NP-40/PBS and eluted with 3.0 M KSCN in 0.1% NP-40/PBS. The eluate was dialyzed overnight at 4°C against 0.1% NP-40/PBS and an aliquot analyzed (a) directly by adjusting the SDS concentration to 2% (v/v) and heating in a boiling water bath for 90 seconds, or (b) by mixing an aliquot to fresh IgE-Sepharose for 60 minutes at 4°C, washing five times with 0.1% NP-40/PBS and eluting with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.

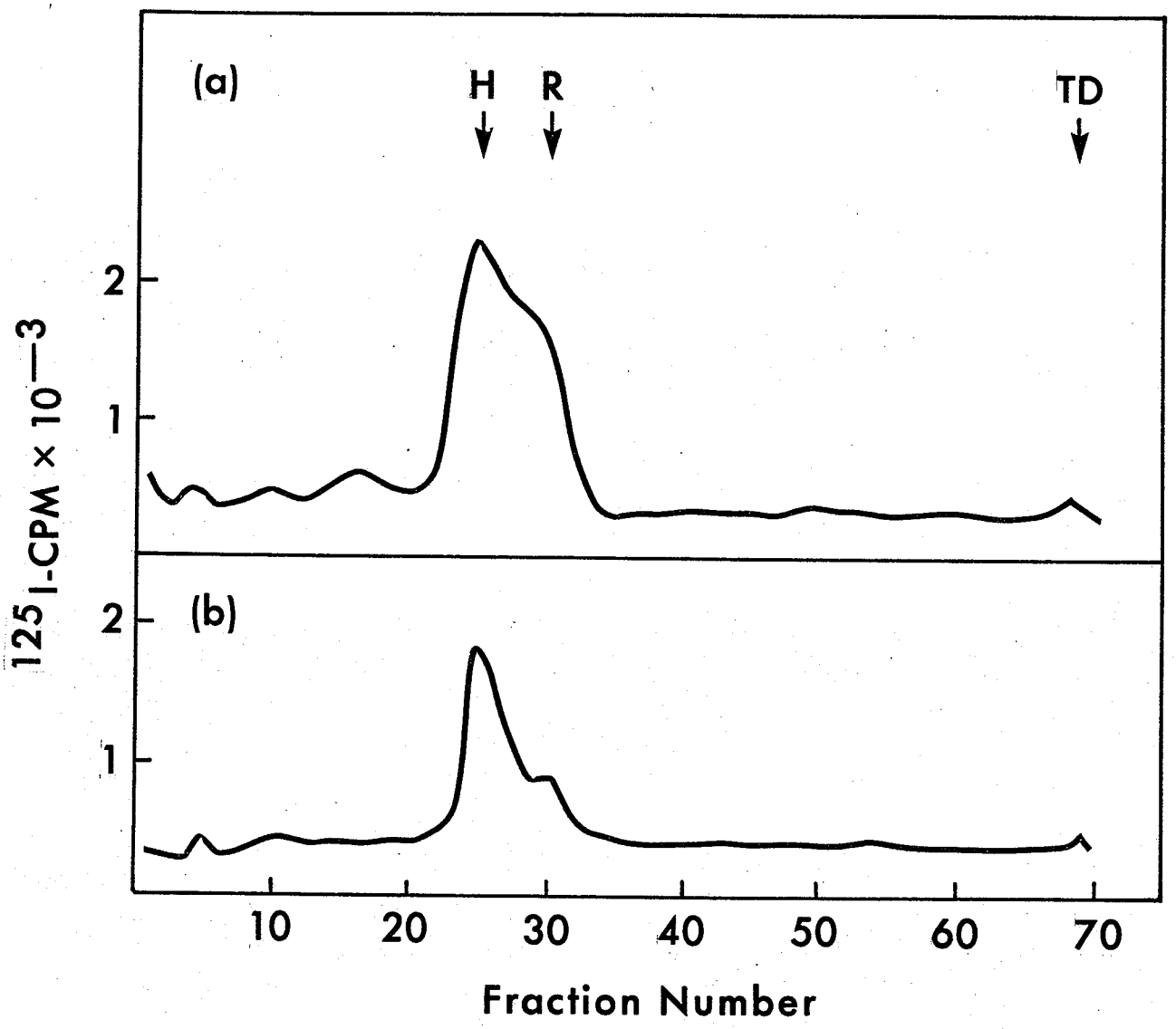
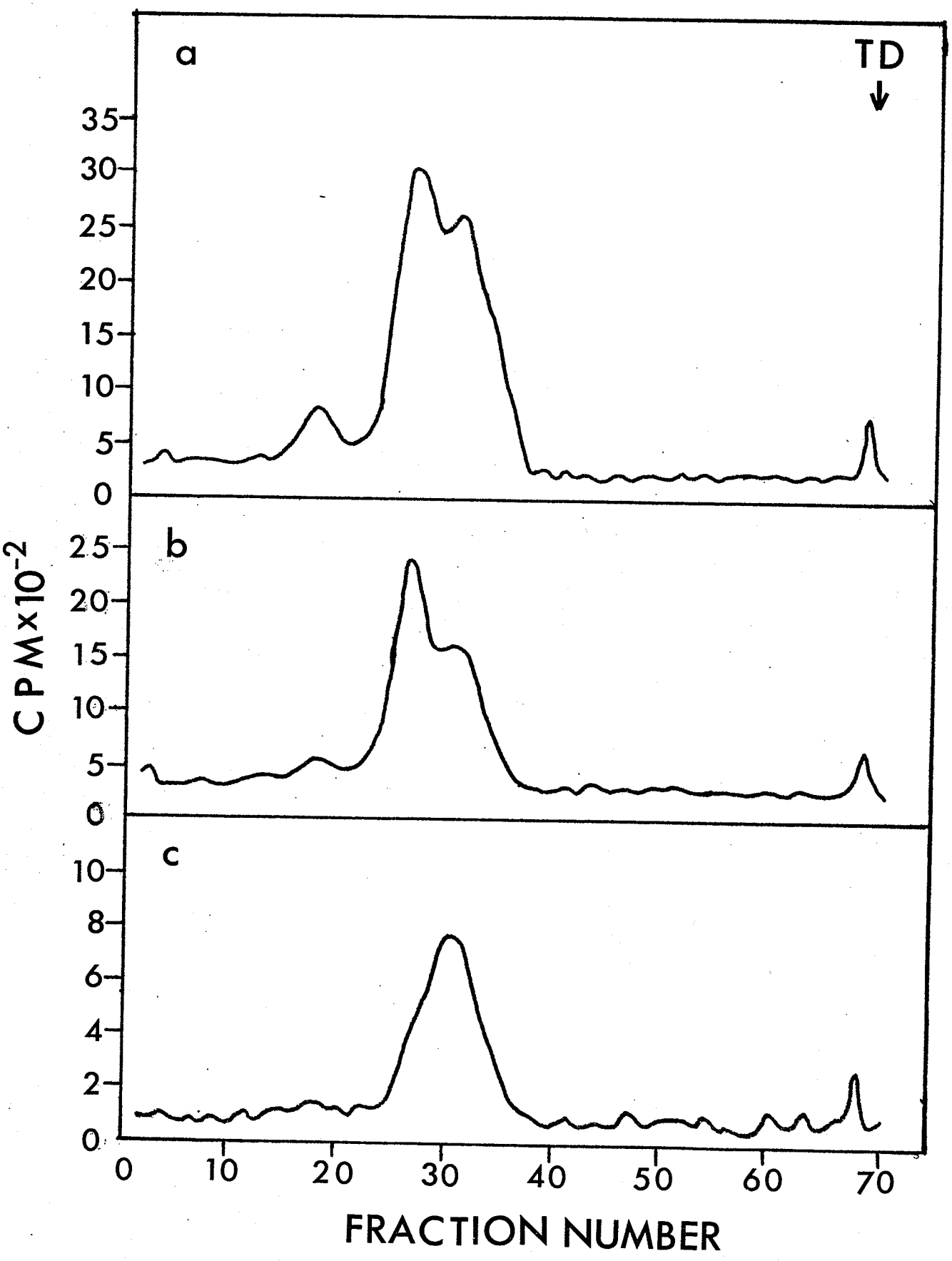

(a)
H
R
TD
2
1
(b)
2
1
^{125}I-CPM × 10^{-3}
10
20
30
40
50
60
70
Fraction Number

Figure 19: SDS-PAGE analysis on 10% Laemmli gels of isolated receptors eluted by KSCN from IgE-Sepharose bound by ConA-Sepharose and LcH-Sepharose. Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C, washed with 0.1% NP-40/PBS and eluted with KSCN. Following the removal of KSCN by dialysis against 0.1% NP-40/PBS, analysis by SDS-PAGE was carried out either directly (a) or after binding and elution with Tris sample buffer containing 2% SDS from ConA-Sepharose (b) or LcH-Sepharose (c). Samples were eluted from the affinity gels with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.


a
TD
35
30
25
20
15
10
5
0
b
25
20
15
10
5
0
c
10
8
6
4
2
0
CPMx10-2
0
10
20
30
40
50
60
70
FRACTION NUMBER

LcH-Sepharose. Clearly, ConA-Sepharose was even a less likely prospect than LcH-Sepharose for the separation of the receptor molecules, in spite of the fact that it has a higher specific binding nature for the same sugars.

The lectins from RCA and WGA were somewhat more discriminating than ConA, but not enough to affect a good separation (Figs. 20 and 21). Fig. 20 shows the profiles of the material bound to and eluted from RCA and WGA conjugated Sepharose and Fig. 21 that of the material which can be initially removed following the 60-minute mixing period before washing of the gels. Although both RCA and WGA show a reduced binding capacity for H, they both show some degree of affinity for H and R. The presence of both receptor in the unbound fraction, as well as the bound and eluted fractions makes them unlikely candidates for the complete separation of H and R.

The fact that both receptors were bound by these lectins was not surprising. In many cases, affinity columns using different lectins have been used for the fractionation of glycoproteins from cells and the eluted material from these columns were shown to have had many glycoproteins in common (Nachbar *et al.*, 1976; Gottlieb *et al.*, 1975; Gurd and Mahler, 1974).

The lectins of Pea and Gorse, both being specific for fucosyl residues, bound R preferentially, This is illustrated in Figs. 22b and 22c. The SDS-PAGE patterns of receptors bound to and eluted from LcH-Sepharose is shown for comparison in Fig. 22a. As previously shown, this lectin also binds R, but a shoulder due to H was also normally present. This shoulder was not indicated in the profiles from Pea and Gorse, suggesting that these two lectins may even be far more suitable for the separations of H and R receptors than the lentil lectin. Both lectins show material migrating in fractions 17-19, furthering the suggestion that this molecule is a glycoprotein.

Figure 20: SDS-PAGE analysis on 10% Laemmli gels of isolated receptors eluted by KSCN from IgE-Sepharose bound by RCA-Sepharose (a) and WGA-Sepharose (b). Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C, washed with 0.1% NP-40/PBS and eluted with KSCN. Following the removal of KSCN by dialysis against 0.1% NP-40/PBS, equal amounts of isolated receptor were added to 0.3 ml of the respective lectin-Sepharose and mixed for 60 minutes at 4°C. The affinity gels were then washed five times with 0.1% NP-40/PBS and eluted with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.

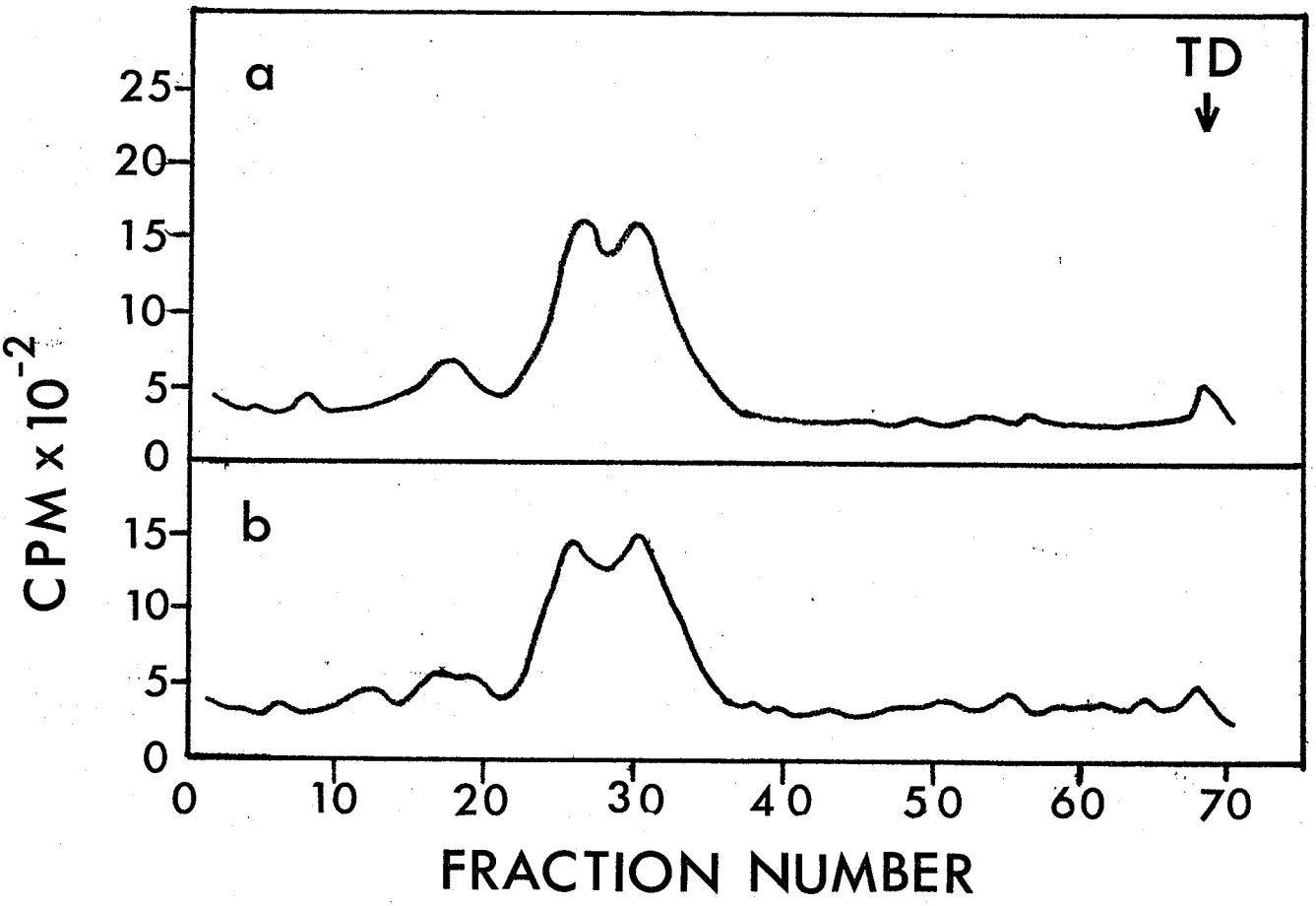

a
b
TD
25
20
15
10
5
0
15
10
5
0
0
10
20
30
40
50
60
70
CPM x 10^{-2}
FRACTION NUMBER

Figure 21: SDS-PAGE analysis on 10% Laemmli gels of receptors isolated by KSCN from IgE-Sepharose not bound by RCA-Sepharose (a) and WGA-Sepharose (b). Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C, washed with 0.1% NP-40/PBS and eluted with KSCN. Following removal of KSCN by dialysis against 0.1% NP-40/PBS, equal amounts of isolated receptors were added to 0.3 ml of the respective lectin-Sepharose and mixed for 60 minutes at 4°C. The unbound fraction was then aspirated, dialyzed against Tris sample buffer overnight, adjusted to 2% SDS (v/v) and heated in a boiling water bath for 90 seconds. (This profile represents a comparison to Fig. 20, in which the material bound to the respective lectin is depicted.)

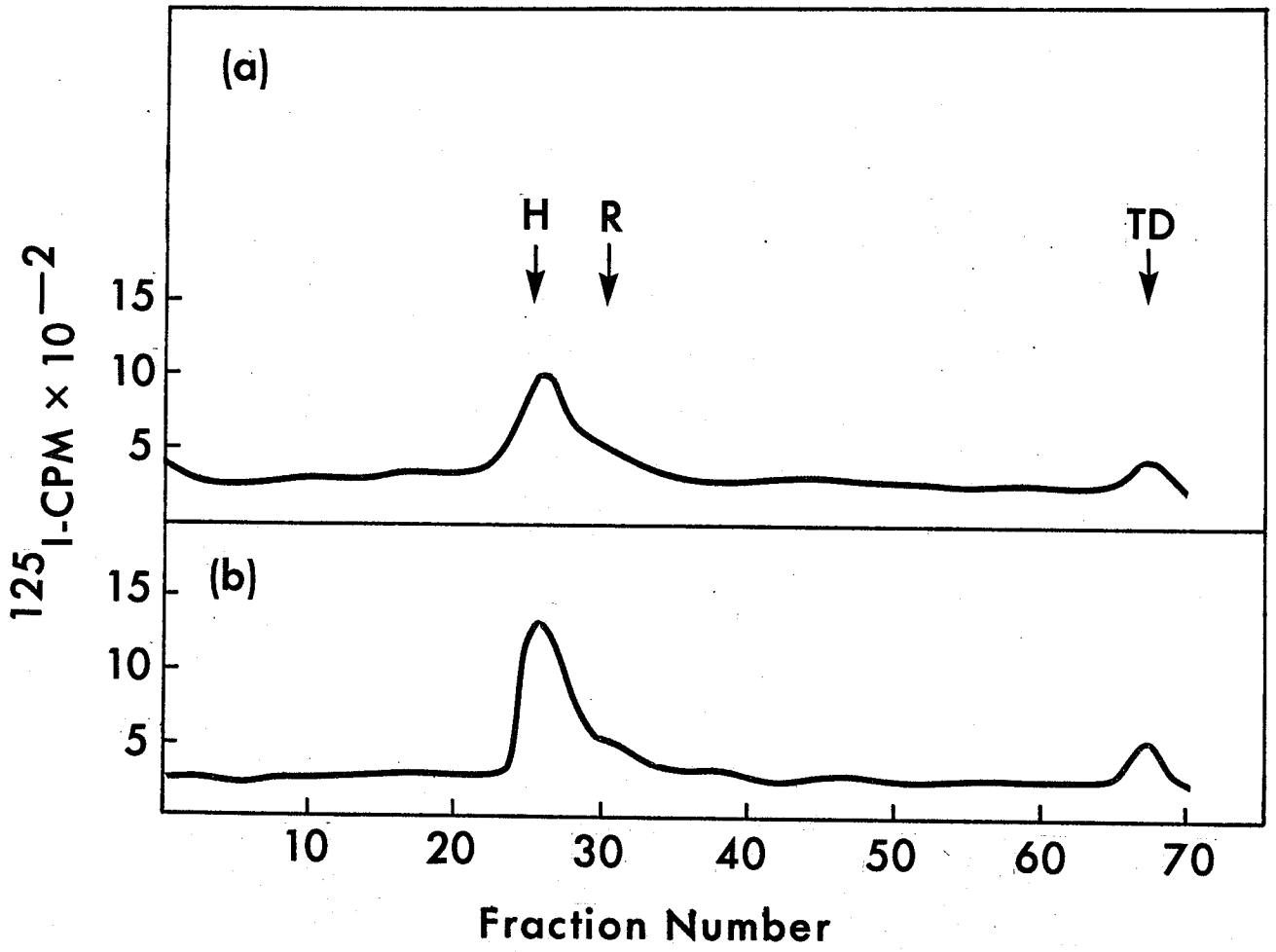
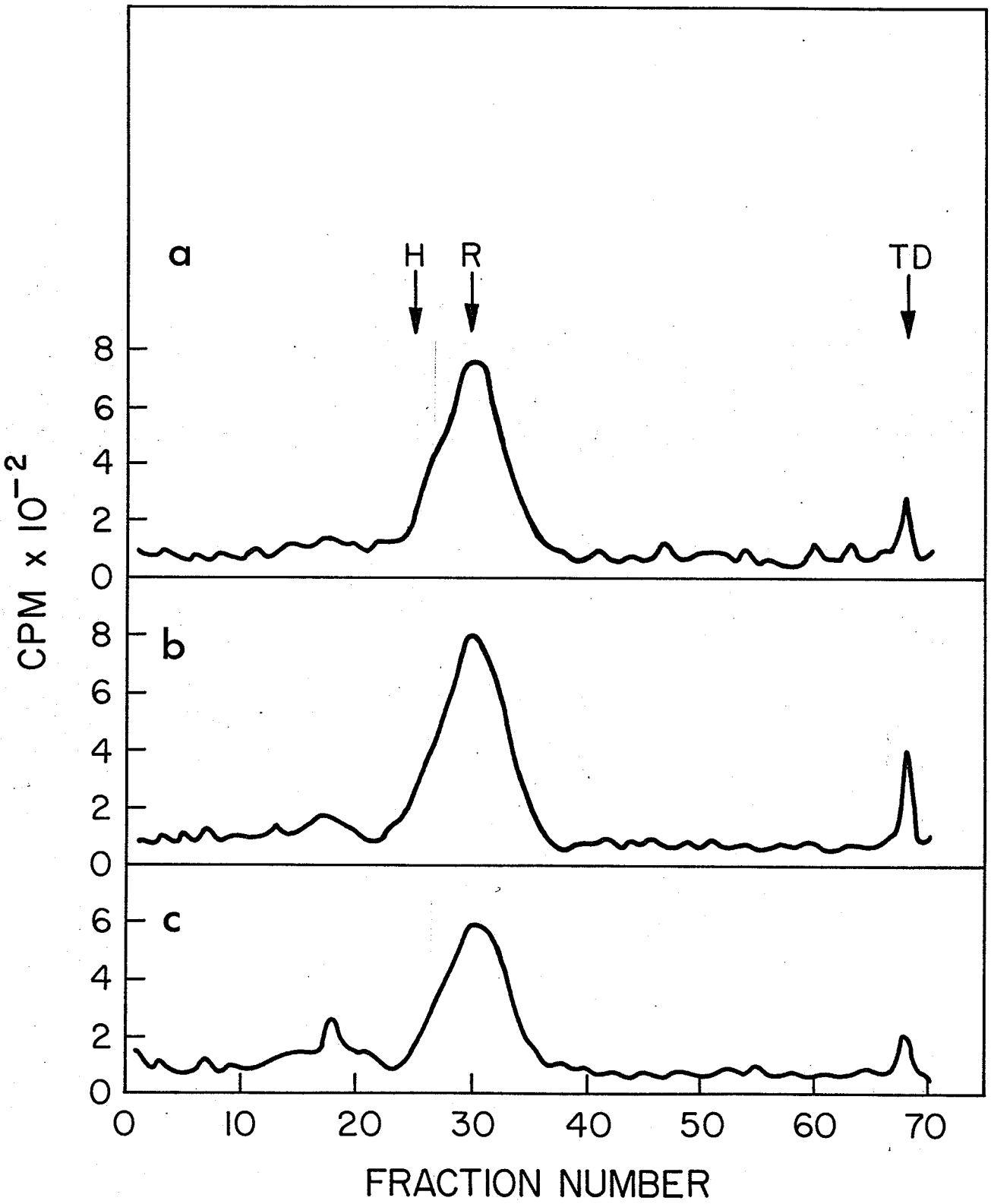

(a)
H
R
TD
15
10
5
(b)
15
10
5
^{125}I-CPM × 10^{-2}
10
20
30
40
50
60
70
Fraction Number

Figure 22: SDS-PAGE analysis on 10% Laemmli gels of receptors isolated by KSCN from IgE-Sepharose bound by LcH-Sepharose (a), Pea-Sepharose (b) and Gorse-Sepharose (c). Solubilized RBL cells (2×10^7) were mixed with 1.0 ml of IgE-Sepharose for 60 minutes at 4°C, washed with 0.1% NP-40/PBS and eluted with KSCN. Following removal of KSCN by dialysis against 0.1% NP-40/PBS, equal amounts of isolated receptors were added to 0.3 ml of the respective lectin-Sepharose and mixed for 60 minutes at 4°C. The affinity gels were then washed five times with 0.1% NP-40/PBS and eluted with Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.


a
H
R
TD
b
c
CPM x 10^{-2}
0
2
4
6
8
0
10
20
30
40
50
60
70
FRACTION NUMBER

DISCUSSION

The analysis by SDS-PAGE on 10% gels of bound and eluted receptor material from lectins conjugated to Sepharose Cl-4B demonstrated that the six lectins were capable of binding one or both of the receptors. In addition, all of the lectins tested appeared to bind the surface iodinated molecule, previously shown to have specificity for IgE, migrating near fraction 19. Even when both H and R receptors were bound to a given lectin, the extent to which they bound varied from lectin to lectin.

Two explanations can be offered for this observation: (i) The lectins which were shown to bind H with a lower affinity would require a larger excess of the lectin in order to bind all of the H receptor added. In spite of the differential binding demonstrated for the receptors, they may contain the same residues but in differing amounts. In all probability, lectins bind complex sequences of sugars and in so doing even the number of the associated polypeptides may be of great importance in the lectin-glycoprotein interaction (Kornfield and Kornfield, 1970; Young and Leon, 1974; Goldstein *et al.*, 1975); (ii) It is conceivable that the modification of some of the receptor molecules had taken place as a consequence of isolation using IgE-Sepharose and KSCN as an eluting agent. However, it is more likely that denaturation occurred in the protein moiety rather than the carbohydrates or in different structural arrangements. Loss of re-binding capacity of receptor, as measured by complex formation with IgE and anti-IgE, has previously been reported (Conrad and Froese, 1978a). It should be noted that in this case, the re-binding was higher (30-50%) as compared to the values of approximately 20% reported by Conrad and Froese (1978a). The explanation may be found in the fact that IgE-Sepharose binds both H and R receptors, while by means of IgE and anti-IgE, only R can be isolated.

The studies with the lentil lectin have clearly established that both receptors contain mannose and/or N-acetylglucosamine residues in their carbohydrate moieties. The specificity of the interaction was confirmed by the fact that receptor could be eluted from LcH-Sepharose by α-methyl mannoside, and to a lesser extent by the fact that virtually no binding of RBL cell surface molecules took place in the presence of this monosaccharide.

The binding of both H and R to WGA and RCA does point to the presence of N-acetylglucosamine and galactose, respectively. However, a possible confirmation that the binding was due to an interaction with these residues will have to await inhibition or elution studies with the appropriate sugars. However, some confirmation for the presence of these sugars comes from other sources. Thus, Kulczycki *et al.* (1976) have demonstrated the biosynthetic incorporation of N-acetylglucosamine into receptors, and in the next Chapter the incorporation of galactose into both receptors will be shown.

More direct confirmation of a specific interaction between fucose residues on R and the lectins of Pea and Gorse, as suggested by Fig. 22b and Fig. 22c, would also have to await inhibition and/or elution studies. In the case of these two lectins, it would be expected that fuco-pyranosides would be better inhibitors than L-fucose (Allen *et al.*, 1977). The studies with these two lectins would suggest that H does not contain any fucose and further suggests that pea and gorse lectins could possibly be the only two lectins which completely separate the two receptors, H and R, on RBL cells. However, the incorporation studies presented in the next Chapter negate the conclusion that fucose is absent in the H receptor.

CHAPTER IV

INTRODUCTION

Experimental studies on the metabolism of cell surface membranes indicate differences in the rate of synthesis and turnover of proteins and glycoproteins in the plasma membrane of growing and non-growing cells (Warren and Glick, 1968). Actively growing cells incorporate material into the surface membrane in order to meet cellular requirements for growth demonstrating a net increase of surface material with relatively little turnover. Non-growing cells have been shown to also incorporate membrane material, but apparently eliminate an equivalent amount with no net increase of substance, but a high rate of turnover. Two major properties of membrane glycoproteins recognized are (i) the percentage of hydrophobic residues (threonine, alanine, proline, tyrosine, valine, methionine, leucine, isoleucine, tryptophan, phenylalanine) is quite high, 30-60% and (ii) the molecular weights are relatively low (25,000-50,000) although exceptions are documented. In addition, the synthesis of glycoproteins is dependent upon a variety of complex factors such as availability of glucose donors, substrate specificities of glycosyltransferases, and the arrangement of glycosyltransferases within the cell's endomembrane system. Glycosyltransferases are known to catalyze the formation of O-glycosidic linkages between two sugars or between a sugar and a hydroxyamino acid, or they can form an N-glycosidic linkage between N-acetylglucosamine and asparagine. Two prominent types of carbohydrate-amino acid linkages found in membrane glycoproteins are (i) asparagine-N-acetylglucosamine (Asn-GlcNAc) and (ii) serine (threonine)-N-acetylgalactosamine (Ser(Thr)-GalNAc) (Schachter and Rodin, 1973;

Sturgess *et al.*, 1978). From these two backbone structures, several branching structures can be attached with sialic acid residues, mannose, fucose and galactose residues incorporated into core structures.

The use of tritiated precursors for biosynthetic labelling of polypeptide and carbohydrate portions of membrane glycoproteins is quite extensive. ^{3}H-leucine, and ^{3}H-tyrosine, two of the essential amino acids making up the hydrophobic core residues, have been used for the tracing of many secretory glycoproteins (Herscovics, 1969; Melchers, 1970) as well as surface membrane glycoproteins (Haustein *et al.*, 1975; Kulczycki and Parker, 1979).

A variety of sugars have been used as precursors for carbohydrate moieties of glycoproteins, including ^{3}H-fucose and ^{3}H-galactose. ^{3}H-fucose has been shown to be an excellent label, in that it is incorporated almost entirely into glycoproteins and is not distributed to other sugars (Yurchenco and Atkinson, 1975; Coffey *et al.*, 1964). ^{3}H-galactose has also been used; however, the label may appear elsewhere than cell surface glycoproteins (Bennet and Leblond, 1970). It has been suggested that there is a rapid passage of galactose label into glucose, hence to glycogen synthesis or transformation into amino acids for protein synthesis. There is evidence that fucose is present in the core glycoprotein structure as well as at the end of the carbohydrate side of glycoproteins with galactose occupying the penultimate position (Spiro, 1969).

The present study on the incorporation of ^{3}H-amino acids and ^{3}H-sugars was originally undertaken with three main aims in mind: (i) to obtain information about the chemical nature of the receptors, (ii) to assess the purity of receptor preparations in terms of total cellular molecules and not just the surface iodinated molecules, and (iii) to establish if any molecules which were not surface iodinated were associated

with the receptors.

While this study was in progress, others had demonstrated that the receptors were glycoproteins since the apparent molecular weight of the receptors, as determined by SDS-PAGE, decreased with increasing acrylamide concentration of the gels (Kulczycki *et al.*, 1976). The same group of authors were also able to show the incorporation of both ^{3}H-amino acids and ^{14}C-sugars into the receptor (Kulczycki *et al.*, 1976; Kulczycki and Parker, 1979). Moreover, they observed that the receptor had to be repurified several times before pure receptor preparations (in terms of ^{3}H-amino acids) could be obtained (Kulczycki and Parker, 1979). However, throughout their tracer studies, this group of authors described only a single receptor molecule using either ^{125}I or biosynthetically labelled cells. In contrast, Conrad and Froese (1978a) had shown that by means of IgE-Sepharose, two different kinds of receptor molecules could be isolated from RBL cells. Thus, it became important to assess the biosynthetic incorporation of labelled precursors into these two molecules.

MATERIALS AND METHODS

The buffers, preparation of IgE and affinity gels, and maintenance of RBL cells were described previously (Chapter II, Materials and Methods).

Biosynthetic Labelling:

For biosynthetic labelling, RBL cells were harvested from two-day-old cultures and after suspension in fresh medium, were allowed to incorporate radiolabelled protein and carbohydrate precursors. ^{3}H-leucine and ^{3}H-tyrosine were used as precursors for polypeptide synthesis; ^{3}H-fucose and ^{3}H-galactose as precursors for carbohydrates and/or glycoproteins. All radiolabelled precursors were obtained from New England Nuclear, Lachine, Quebec. Using aseptic technique, RBL cells (2×10^{7}) were washed in fresh medium, resuspended at $3 - 5 \times 10^{6}$ cells/ml with 50 uCi of one of the selected precursors. Incubation was maintained in a 5% CO_2 atmosphere for 48 hours, after which the cells were mechanically stripped and centrifuged; pelleted cells were then washed with PBS/BSA and resuspended in PBS/BSA for an additional 60 minutes at 37°C.

Isolation of Receptors:

(a) Sandwich Precipitation: Biosynthetically labelled RBL cells were solubilized with 0.5% NP-40/PBS and then mixed with either IgE/anti-IgE/GAR or NRS/GAR, as described under Materials and Methods, Chapter II. The precipitates were then washed and solubilized in the sample buffer of the SDS-PAGE system used and thereafter analyzed as described above.

(b) Direct Precipitation: During the course of the investigation, the sandwich precipitation system was dropped in favour of the direct precipitation procedure, described in Chapter II. Two methodologies were routinely employed: (i) direct precipitation at optimal antibody to antigen ratios or (ii) the insolubilization of immune complexes by either

Staphylococcus aureus bacillus carrying protein A or protein A coupled Sepharose. Twenty µl of protein A carrying *S. aureus* bacillus were suspended in 200 µl of 0.5% NP-40/PBS with 1 mg/ml of ovalbumin to make a 10% suspension. Receptor complexes consisting of IgE/rabbit anti-IgE were added to this suspension and mixed for 60 minutes at 4°C. The bacillus were then washed and sample buffer solubilized material from these preparations analyzed by SDS-PAGE. Alternatively, protein A coupled to Sepharose, protein A-Sepharose (Pharmacia AB, Uppsala, Sweden) was used and the insolubilized receptor complexes were eluted by sample buffer used for SDS-PAGE analysis.

(c) Affinity Chromatography: Insolubilization of the biosynthetically labelled receptor was also achieved by using the IgE-Sepharose affinity chromatography procedure described in Chapter II. Once again, the insolubilized material was eluted by SDS-PAGE sample buffer and analyzed by the respective SDS-PAGE system. Inhibition experiments were carried out by mixing free IgE with the biosynthetically labelled RBL cells before or after solubilization and mixing with IgE-Sepharose. Alternatively, biosynthetically labelled RBL cells were mixed with DNP-IgE and the DNP-IgE receptor complexes isolated as described in Chapter II.

For IgE inhibition or blocking of receptors for binding to affinity gels, free IgE at concentrations of 500 - 1,000 µg was routinely added to intact cells. For DNP-IgE binding, 300 µg of DNP-IgE was added to RBL cell preparations and mixed for 60 minutes at 37°C. The cells were then washed through FCS and solubilized in 1.0 ml of 0.5% NP-40 buffers.

Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE):

This was performed using the Tris-buffered system, as described by Fairbanks *et al.* (1971) or the Tris-buffered system described by Laemmli (1970). Ninety or 140 mm gels were sliced into 2 mm fractions using the

Gilson automatic gel fractionator (Gilson Medical Electronics, Inc., Middleton, Wisconsin) and evaporated overnight to dryness. Each sample was then digested in 75 µl of H_2O and 500 µl of NCS solubilizer (Amersham Corporation, Arlington Heights, Illinois) for 2 hours at 50°C, cooled and mixed with 10 ml of Econofluor (New England Nuclear, Lachine, Quebec). All samples were then dark adopted and counted in a Beckman liquid scintillation counter, Model LS-335 (Beckman Instruments Co., Fullerton, California).

RESULTS

The biosynthetic labelling of the receptors for IgE on RBL cells was initiated by investigating the incorporation of tritiated precursors into trichloracetic acid (TCA) precipitable proteins. Aliquots of cells were incubated in the presence of ^{3}H-leucine, ^{3}H-tyrosine, ^{3}H-galactose and ^{3}H-fucose for different time periods to establish an optimal incubation period for the incorporation of tritiated precursors into TCA precipitable material. For this purpose, 4.0×10^{6} RBL cells in 1.0 ml of MEM with 15% FCS were mixed individually with 10 µl of each of the tritiated amino acids or sugars. For the leucine incorporation studies, leucine-free MEM (Grand Island Biological Co., Grand Island, New York) was used. Cells were harvested at 4, 8, 12, 18 and 24 hours, washed 2 times with 2 - 3 ml of PBS/BSA and solubilized in 0.5 ml of 0.5% NP-40/PBS. Following centrifugation to remove particulate material, 150 µl of the supernatant fractions were collected and adjusted to a final TCA concentration of 10%. To determine the incorporation of radiolabelled material into TCA precipitable proteins, biosynthetically labelled cells were prepared for scintillation counting (described under Materials and Methods) at various stages of the experiment. Incorporation of radiolabelled material was determined by measuring the radioactivity of (i) the NP-40/PBS solubilized extracts and (ii) the TCA precipitate. The results of this analysis are shown in Table V. For the most part, TCA precipitatable material was obtained as early as 8 hours, however, the continued high level of incorporation of radioactive material up to 24 hours suggested that longer incubation periods could be used. It was hoped that by increasing the length of incubation periods, even slowly synthesized material would be labelled and could then be detected. Op-

TABLE V

KINETIC STUDY OF THE INCORPORATION OF RADIOLABELLED PRECURSORS INTO TCA PRECIPITABLE MATERIAL FROM RBL CELLS[(a)]

INCORPORATION TIME	^{3}H-PRECURSOR	CPM IN 25 µl OF NP-40 EXTRACT	EQUIVALENT COUNTS PRECIPITATED IN 25 µl OF NP-40 EXTRACT	% TCA PRECIPITABLE MATERIAL
4 Hour	Leucine	73,600	53,705	72.9
	Tyrosine	57,484	44,110	76.7
	Fucose	6,344	3,727	58.7
	Galactose	62,815	14,096	22.4
8 Hour	Leucine	162,053	149,394	92.1
	Tyrosine	106,065	89,394	84.2
	Fucose	15,552	10,310	66.2
	Galactose	104,065	41,429	39.8
12 Hour	Leucine	188,958	166,477	88.1
	Tyrosine	129,320	108,948	84.2
	Fucose	20,166	12,410	61.5
	Galactose	144,496	53,279	36.8
18 Hour	Leucine	170,531	159,995	93.8
	Tyrosine	124,454	99,848	80.2
	Fucose	25,443	18,052	70.9
	Galactose	55,571	21,330	38.3
24 Hour	Leucine	186,069	170,423	91.5
	Tyrosine	116,242	96,483	83.0
	Fucose	39,587	25,624	64.7
	Galactose	37,824	15,099	39.9

(a) RBL cells (4×10^6) were mixed with 10 µl of the respective tritiated precursor in 1.0 ml of 15% FCS supplemented medium. Cells were harvested at various time intervals and solubilized in 0.5% NP-40/PBS. Aliquots of 25 µl samples of total extract were counted and compared to TCA precipitates (10% final concentration) from a corresponding volume of solubilized extract.

timal physiological conditions were maintained, so that incorporation periods as long as 24-48 hours could be used. This assured a cell doubling time and the synthesis of newly synthesized receptors, as well as providing sufficient material to be used for the isolation of receptor molecules.

SDS-PAGE Analysis of the Receptors on 5.6% Gels:

Precipitation of the receptors from 8 x 10^6 RBL cell equivalents of an NP-40 extract were carried out using the sandwich precipitation procedure. Both IgE-specific and NRS control precipitates were washed and solubilized in 150 µl of Fairbanks sample buffer for 2 hours at 37°C. Ten µl aliquots were prepared for scintillation counting as described in Materials and Methods and the samples counted in the Beckman liquid scintillation spectrometer. The results are shown in Table VI. Higher counts, by at least a factor of two over controls, were precipitated by IgE/anti-IgE/GAR, suggesting some specificity for IgE with all of the precursors investigated.

However, the SDS-PAGE analysis on 5.6% gels of these precipitates did not give clear-cut results. The most encouraging results were seen with the use of the tritiated sugars (Fig. 23). Thus, Figs. 23b and 23c show the profiles of cellular material isolated from ^{3}H-galactose and ^{3}H-fucose labelled RBL cells, respectively. In Fig. 23a, the results of a parellel experiment using surface iodinated cells are shown. A molecule from surface iodinated cells migrating in fractions 22-23 of 5.6% gels, representing an apparent molecular weight of 62,000 daltons, had previously been shown to represent the receptor for IgE on 5% gels (Conrad and Froese, 1976). Therefore, it was possible to conclude that both galactose and fucose were being incorporated into the receptors for IgE, and that these molecules were indeed glycoproteins. Control precipitates with

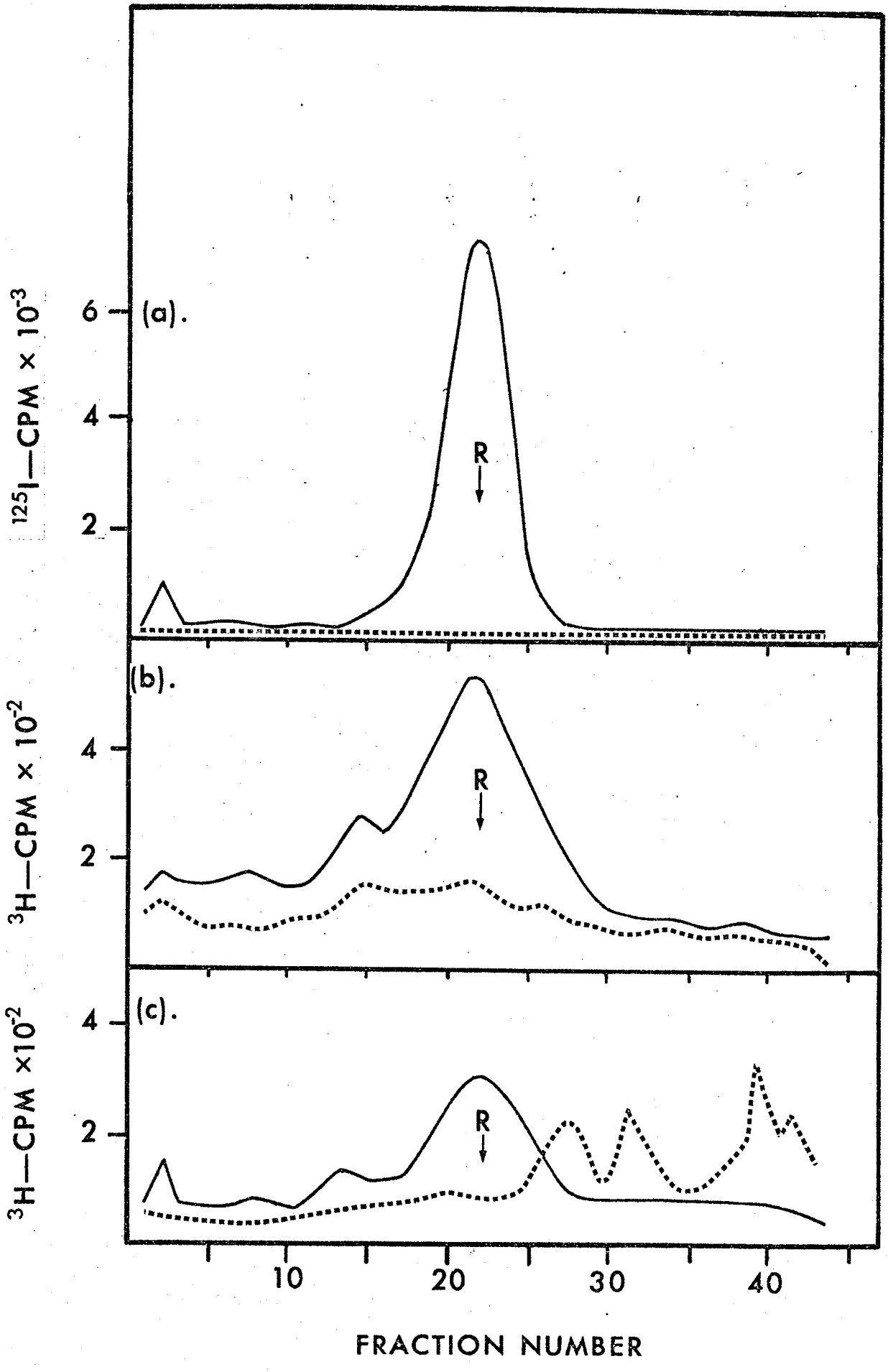
TABLE VI

IMMUNE "SANDWICH" PRECIPITATION OF INCORPORATED RADIOLABELLED PRECURSORS INTO THE RECEPTORS FOR IgE OF RBL CELLS[(a)]

	CPM PRECIPITATED			
PRECIPITATE	LEUCINE	TYROSINE	FUCOSE	GALACTOSE
IgE/Anti-IgE/GAR	47,385	25,605	3,285	7,950
NRS/GAR	17,730	12,705	1,155	3,985

(a) RBL cells (2×10^7) with the respective ^3H-precursors incorporated were solubilized and 8×10^6 cell equivalents were then precipitated. NRS: Normal rabbit serum; GAR: Goat anti-rabbit immunoglobulin.

Figure 23: SDS-PAGE analysis on 5.6% gels of receptor preparations labelled with ^{3}H-sugars and isolated by the sandwich precipitation method. RBL cells (2×10^{7}), biosynthetically labelled for 8 hours, were incubated with 10 μg of IgE in 1.0 ml of PBS/BSA for 90 minutes at 37°C, solubilized with 1.0 ml of 0.5% NP-40/PBS and precipitated. The precipitates were washed five times with 0.1% NP-40/PBS and dissolved in Fairbanks sample buffer containing 1% SDS. (a) Profile from a surface iodinated RBL cell preparation; (b) Profile from ^{3}H-galactose labelled RBL cells; and (c) Profile from ^{3}H-fucose labelled RBL cells. (———) IgE/anti-IgE/GAR and (-----) NRS/GAR.

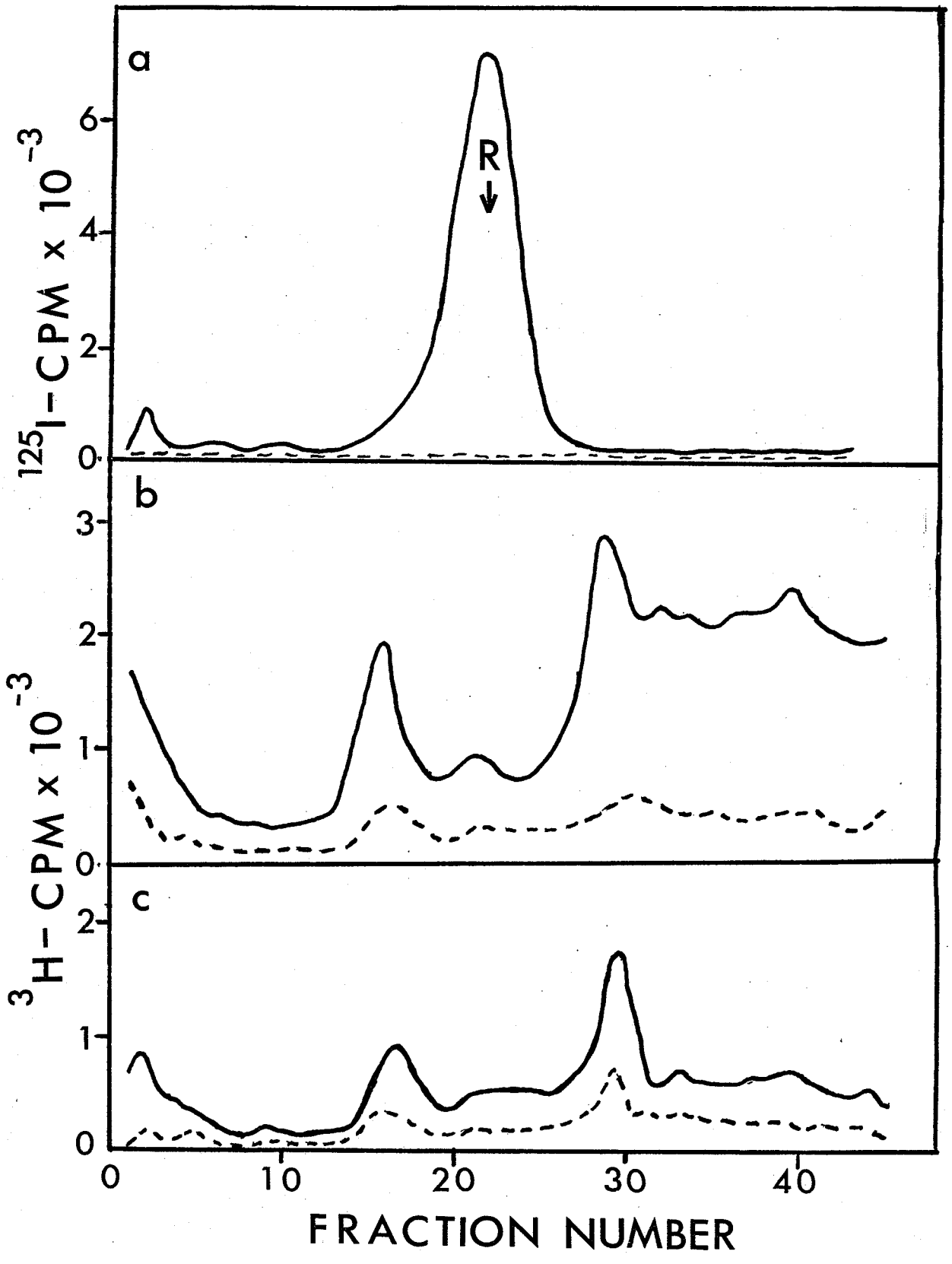

(a).
(b).
(c).
R
R
R
6
4
2
4
2
4
2
^{125}I—CPM × 10^{-3}
^{3}H—CPM × 10^{-2}
^{3}H—CPM ×10^{-2}
10
20
30
40
FRACTION NUMBER

the NRS/GAR, although they contained a substantial number of counts, showed no major peaks in the receptor area.

Experiments with the ^{3}H-amino acids as precursors were less encouraging. Even though the specific precipitates, like those with the ^{3}H-sugars, contained about a 2-fold higher number of counts than control precipitates, the SDS-PAGE gel patterns as obtained on 5.6% gels were much more complex. Fig. 24 shows the profiles normally obtained using either ^{3}H-leucine or ^{3}H-tyrosine as precursors. Even though a peak migrating in the vicinity of the receptor can be identified, the relatively high counts seen throughout the gel made it difficult to believe that all this material represented receptor, or at least receptor associated material. Results obtained with tritiated leucine (Fig. 24b) were especially difficult to interpret, as considerable material was evident in the lower molecular weight ranges; however, both patterns with leucine and tyrosine (Figs. 24b and 24c, respectively) showed radiolabelled material in fraction 23 (approximately 60,000 daltons), suggesting that the receptor was being labelled by these amino acids. Much of the remaining radiolabelled material was assumed to represent non-specifically bound cellular material, although it was not clear why so much more non-specifically binding material should have occurred with the "specific" precipitates.

In view of the fact that material isolated from cells grown in the presence of tritiated sugar precursors was found in one major peak with receptor mobility suggested that most of the cellular material seen in Fig. 24 was not glycoprotein in nature and, therefore, may not represent plasma membrane material. It was believed most likely to represent radiolabelled material of cytoplasmic origin. As suggested earlier, since both tritiated amino acid precursors gave similar patterns on 5.6% gels during the short-term incubation periods, it was decided to use longer

Figure 24: SDS-PAGE analysis on 5.6% gels of receptor preparations labelled with ^{3}H-amino acids and isolated by the sandwich precipitation method. RBL cells (2×10^{7}), biosynthetically labelled for 8 hours, were incubated with 10 µg of IgE in 1.0 ml of PBS/BSA for 90 minutes at 37°C, solubilized with 1.0 ml of 0.5% NP-40/PBS and precipitated. The precipitates were washed five times with 0.1% NP-40/PBS and dissolved in Fairbanks sample buffer containing 1% SDS. (a) Profile from a surface iodinated RBL cell preparation; (b) Profile from ^{3}H-leucine labelled RBL cell and (c) Profile from ^{3}H-tyrosine labelled RBL cell. (———) IgE/anti-IgE/GAR and (-----) NRS/GAR.


a
b
c
R
^{125}I-CPM x 10^{-3}
^{3}H-CPM x 10^{-3}
0
2
4
6
1
3
0
10
20
30
40
FRACTION NUMBER

incubation periods in order to eliminate some of the suggested cytoplasmically derived material. For this purpose, RBL cells were grown for 24-48 hours in ^{3}H-leucine to assure at least one cell doubling and to increase the number of cells harvested. The results on 5.6% gels failed to give any further significant information regarding incorporation of ^{3}H-amino acid molecules precipitated by IgE/anti-IgE/GAR.

Additional experiments were carried out with tritiated amino acid precursors using IgE-Sepharose coupled with KSCN elution of the bound material. The results of such a study in which RBL cells were cultured in the presence of ^{3}H-leucine or ^{3}H-tyrosine for 16 and 24 hours are shown in Fig. 25. Although there was labelled material in an area of the gel corresponding to receptor mobility under both culturing conditions, there remained a substantial amount of material migrating in the lower molecular weight regions of the 5.6% gels. Both this method of receptor isolation as well as precipitation yielded labelled material which was unable to penetrate the gel. One of the concerns with respect to both Figs. 24 and 25 was the lack of sharpness in the gel patterns.

SDS-PAGE Analysis of the Receptors on 10% Gels:

(a) Incorporation Studies with ^{3}H-Amino Acids: In order to get a better resolution of the components in the lower molecular weight range, SDS-PAGE on 10% gels using the Laemmli discontinuous buffer system (Laemmli, 1970) was adopted. Since receptor preparations from cells labelled with ^{3}H-amino acids gave the most complex patterns on 5.6% gels, they were used to assess the degree of purity of receptor preparations obtained by various methods. The first step was to determine the incorporation of ^{3}H-tyrosine and ^{3}H-leucine precursors into the various RBL cell proteins, as revealed by SDS-PAGE profiles on 10% gels as shown in Fig. 26. Fig. 26a

Figure 25: SDS-PAGE analysis on 5.6% gels of the incorporation of ^{3}H-amino acids into RBL cell components isolated by IgE-Sepharose. RBL cells (3×10^{7}), biosynthetically labelled for 16 hours (———) or 24 hours (-----), were washed through FCS and with PBS/BSA, solutilized with 1.0 ml of 0.5% NP-40/PBS and the extract mixed with 0.3 ml of IgE-Sepharose for 60 minutes at 4°C. The mixtures were transferred to 1.0 ml Pasteur pipette columns, washed with 15-20 ml of 0.1% NP-40/PBS and eluted with 3.0 M KSCN in 0.1% NP-40/PBS. The KSCN eluates were dialyzed against Fairbanks electrode buffer overnight at 4°C. (a) Profiles of eluates from ^{3}H-leucine and (b) Profiles of eluates from ^{3}H-tyrosine.

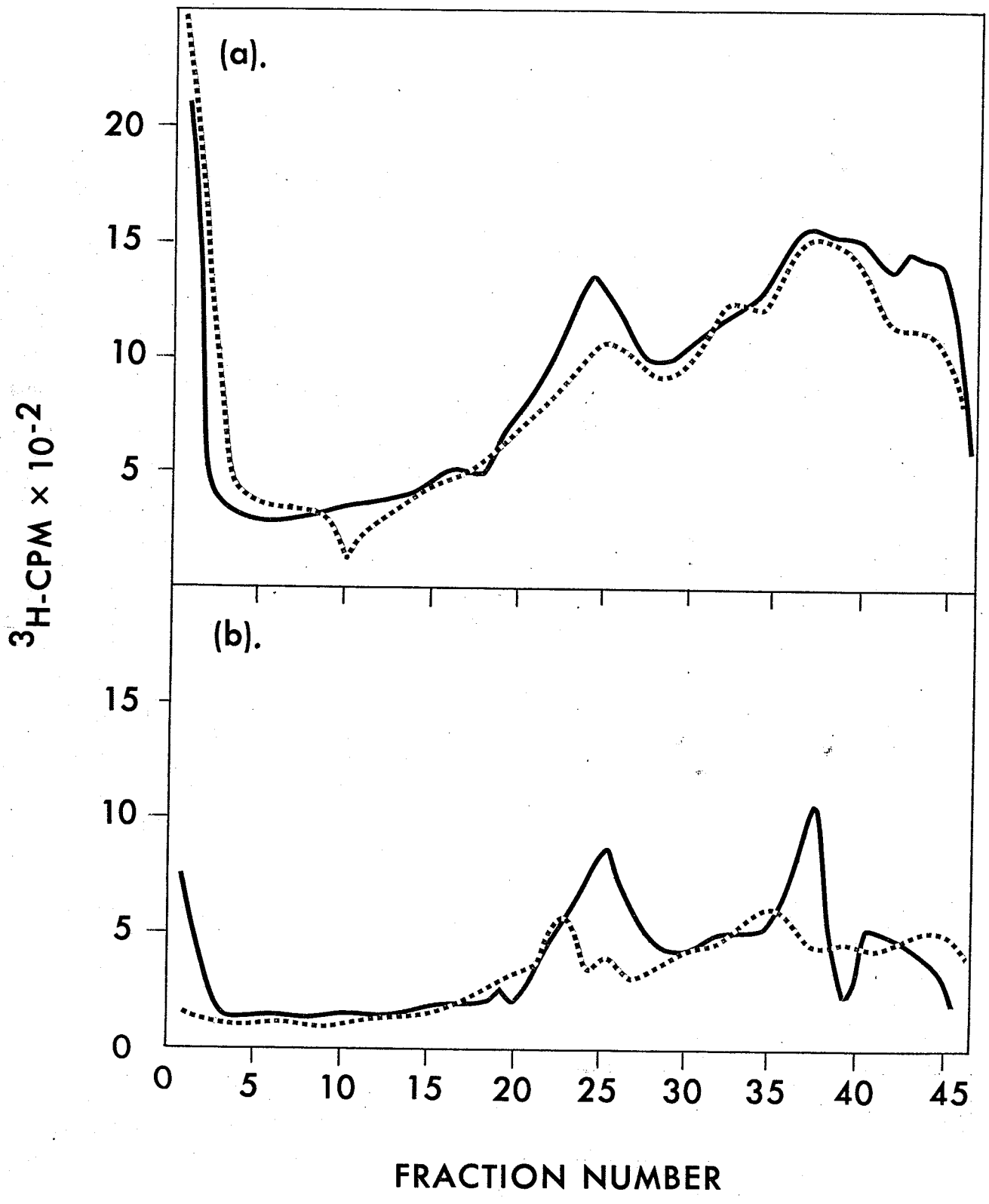
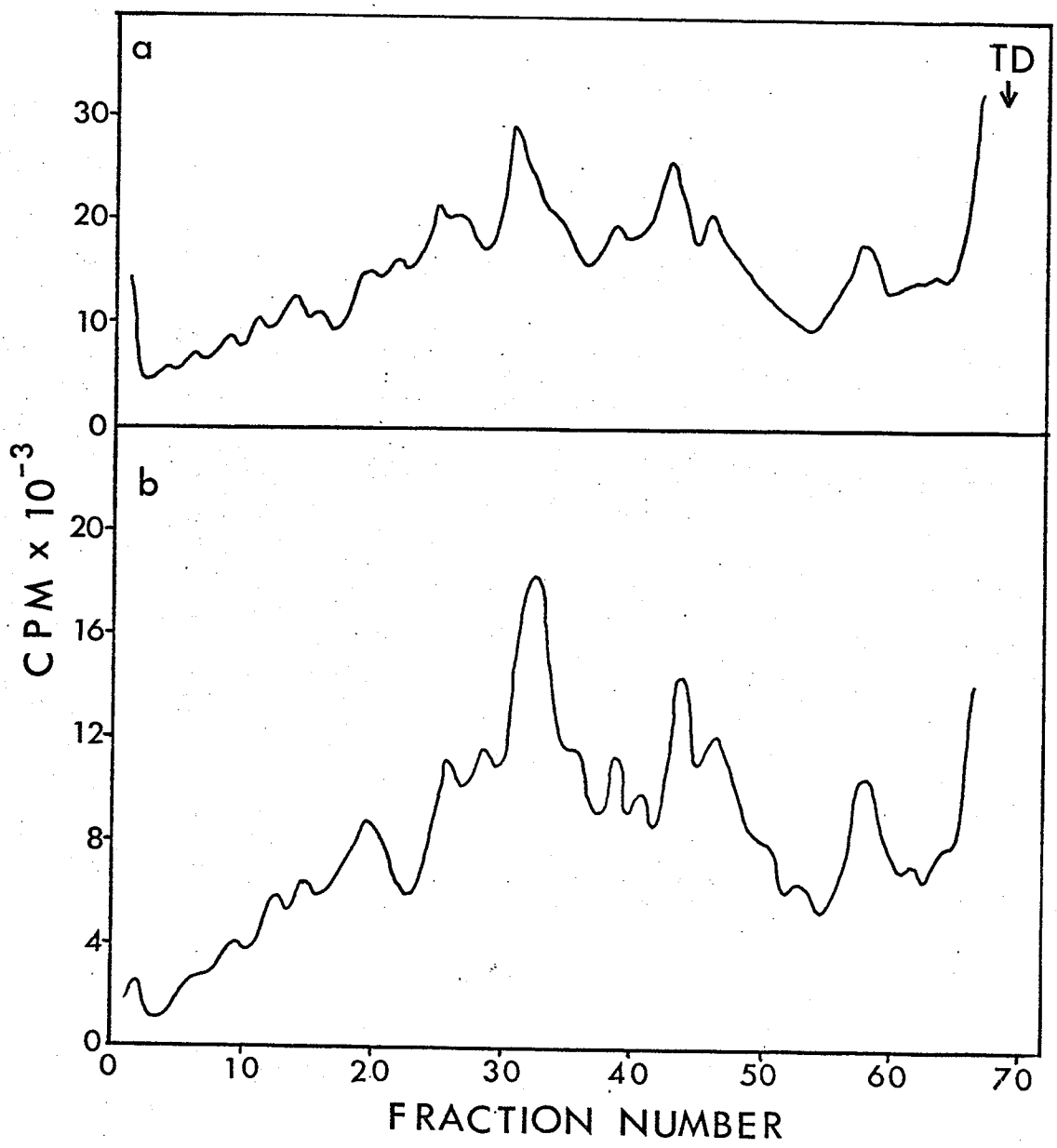

(a).
(b).
20
15
10
5
15
10
5
0
0
5
10
15
20
25
30
35
40
45
^{3}H-CPM × 10^{-2}
FRACTION NUMBER

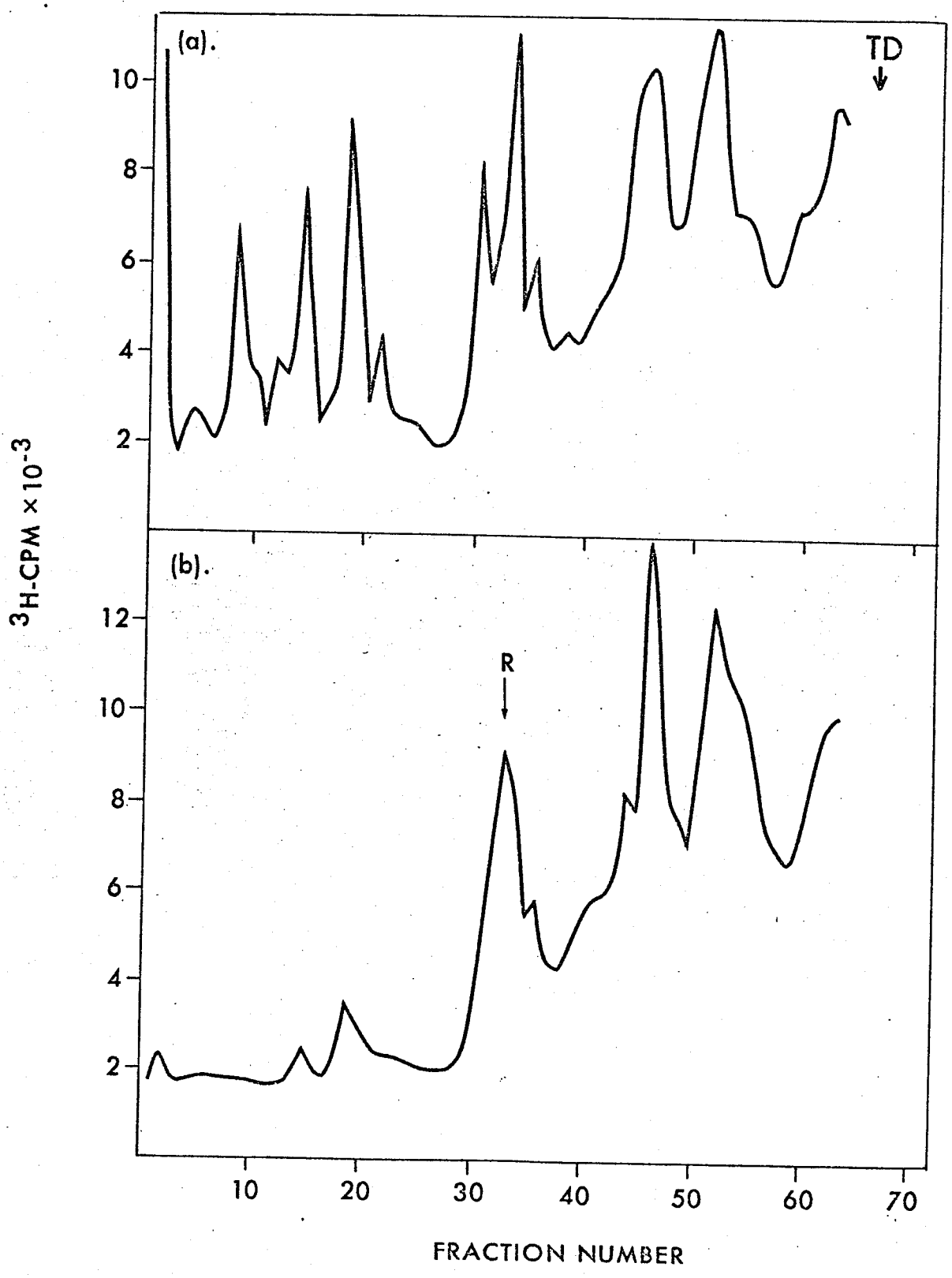
Figure 26: SDS-PAGE analysis on 10% gels of biosynthetically incorporated ^{3}H-amino acids into RBL cell components. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, were washed through FCS and with PBS/BSA. A 10 µl aliquot of a 1.0 ml 0.5% NP-40/PBS solubilized extract was then removed and added to 200 µl of Tris sample buffer containing 2% SDS and heated in a boiling water bath for 90 seconds. (a) Profile from ^{3}H-leucine labelled RBL cells and (b) Profile from ^{3}H-tyrosine labelled RBL cells.


a
TD
30
20
10
0
b
20
16
12
8
4
0
CPM x 10⁻³
0
10
20
30
40
50
60
70
FRACTION NUMBER

demonstrates the profile of NP-40 extracts from RBL cells grown in the presence of ^{3}H-leucine and Fig. 26b demonstrates RBL cell extracts from cells grown in the presence of ^{3}H-tyrosine. The numerous peaks noted in the molecular weight range of 25,000 to 55,000 daltons (fractions 25-50) corresponded quite well to those when surface iodinated cell extracts were used (Fig. 13a). However, it is quite apparent that more molecular species with molecular weights of 25,000 daltons and below, and fewer molecules with molecular weights greater than 75,000 daltons were present in these tritiated cell extracts. These results suggest, in part, that molecules with a molecular weight below 25,000 daltons were not derived from the plasma membrane of the cells and most likely represented proteins of intracellular origin. With both ^{3}H-amino acids, the major peaks appeared in corresponding fractions of the gels.

It was further hoped that the non-specific trapping or binding of non-receptor associated material might be reduced if the direct precipitation procedure or insolubilization of receptor complexes by protein A carrying bacilli, as described under Materials and Methods, were performed. The corresponding SDS-PAGE profiles of isolated tritiated tyrosine labelled cellular material on 10% gels are shown in Fig. 27. In contrast to the 5.6% gels using the Fairbanks system, rather distinct peaks were obtained. Fig. 27a shows an SDS-PAGE profile on 10% gels of ^{3}H-tyrosine labelled proteins isolated by direct precipitation. Considerable resolution of high and low molecular weight components, in addition to material migrating in the region of R receptor (fractions 30-32) with an apparent molecular weight of 45,000 daltons) can be seen. Fig. 27b shows the SDS-PAGE profile on 10% gels of insolubilized immune complexes, which were eluted from protein A carrying *S. aureus* bacilli. Although the amount of high molecular weight material was less prominent than in receptor pre-

Figure 27: SDS-PAGE analysis on 10% gels of ^{3}H-tyrosine labelled RBL cell components isolated by direct precipitation or insolubilization of receptor complexes with protein A-*S. aureus* bacilli. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, were solubilized with 1.0 ml of 0.5% NP-40/PBS and equal aliquots subjected to the following isolation procedure. Direct precipitation was achieved using IgE and ε-specific sheep anti-rat IgE. The precipitate was washed with 0.1% NP-40/PBS and solubilized in Tris sample buffer containing 2% SDS. Insolubilization of receptor complexes consisting of IgE and excess ε-specific sheep anti-rat IgE was achieved by adding the mixture to protein A carrying *S. aureus* bacilli. The bacilli were washed with 0.1% NP-40/PBS and eluted with Tris sample buffer containing 2% SDS. Both samples were heated in a boiling water bath for 90 seconds. (a) Profile of radiolabelled material from direct precipitation and (b) Profile of radiolabelled material eluted from *S. aureus* bacilli.


(a).
TD
(b).
R
^{3}H-CPM x 10^{-3}
FRACTION NUMBER

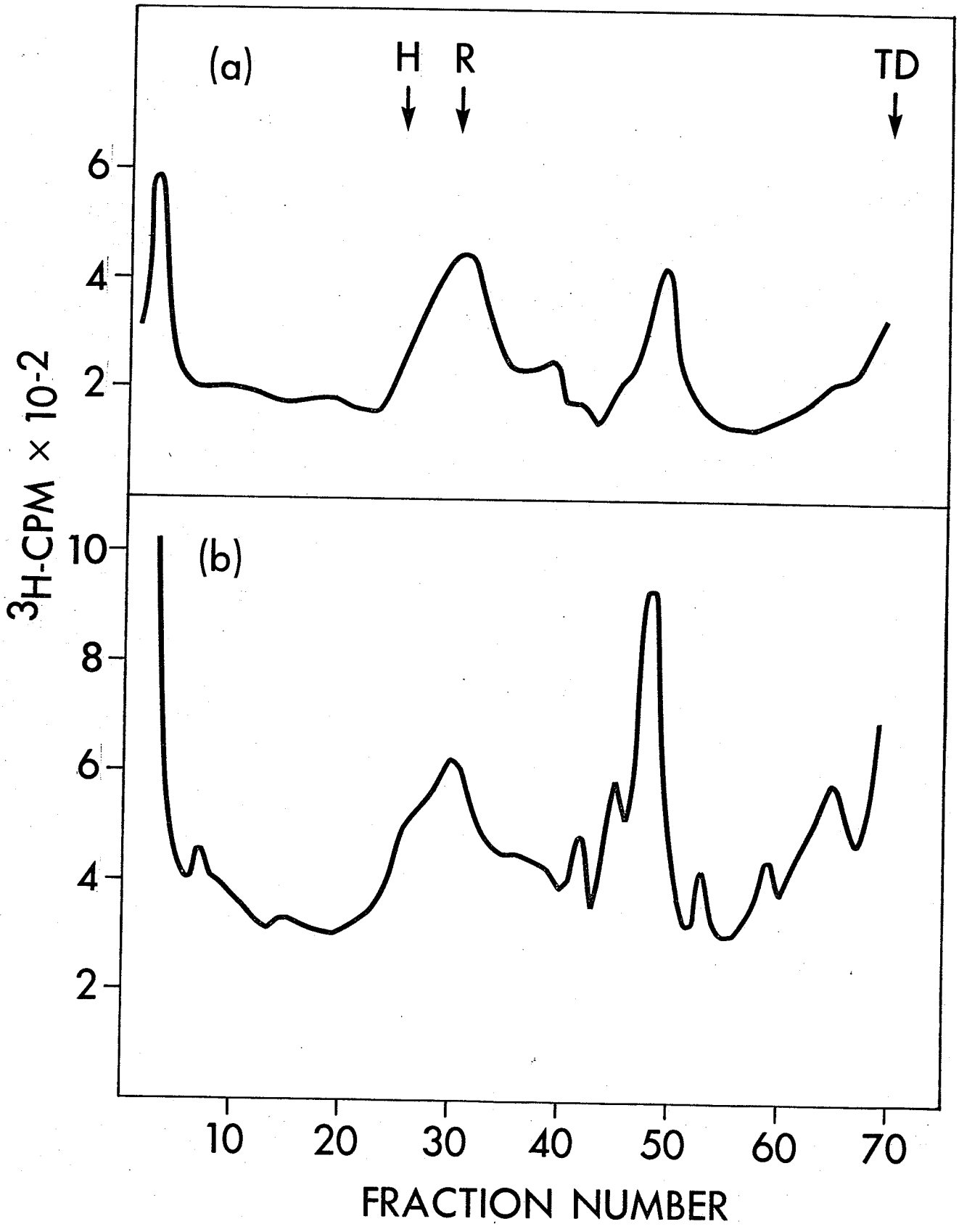
parations, isolated by the direct precipitation procedure (Fig. 27a), there remained a substantial amount of low molecular weight material. With the reduced amount of material appearing in the upper molecular weight regions (Fig. 27b), it was possible to suggest that precipitation (Fig. 27a) may actually lead to the entrapment of high molecular weight molecules. This was also seen when sandwich precipitates of ^{3}H-leucine were analyzed on 5.6% gels (Fig. 24b), where a substantial amount of material appeared at the top of the gel.

It is, however, interesting to note that molecules in the lower molecular weight region persisted irregardless of the method of receptor isolation used. Molecules near fraction 19 (apparent molecular weight of 71,000 daltons), 32 (molecular weight of 45,000 daltons), 47 (molecular weight of 26,000 daltons) and 52 (molecular weight of 23,000 daltons) were isolated by both procedures, as detected on 10% gels (Fig. 27a,b), clearly giving better resolution of material in this region than previously seen in profiles of 5.6% gels (Fig. 24b,c). Since many of these peaks continue to be more prominent than the one peak which could represent R (fraction 32), it is once again difficult to imagine that all of these molecules were receptor-derived or receptor-associated.

In order to eliminate as much as possible the presence of non-specific tritiated labelled material in receptor preparations, the receptor was isolated in the form of IgE-receptor complexes using DNP-IgE and anti-DNP-Sepharose. In this case, elution was achieved with 2,4-dinitrophenolate, which should elute primarily IgE-receptor complexes even if other molecules are bound non-specifically. For this purpose, 5×10^{7} tritiated RBL cells cultured in the presence of tritiated leucine or tyrosine were mixed wth 400 µg of DNP-IgE and incubated for 90 minutes at 37°C. Following washes through FCS and with PBS/BSA, the tritiated cells were solu-

bilized with 0.5% NP-40 in Tris and mixed with 0.3 ml of anti-DNP-Sepharose for 90 minutes at 4°C. The slurry was then transferred to a 1.0 ml Pasteur pipette column, washed with 15-20 ml of 0.1% NP-40/Tris and the DNP-IgE receptor complexes eluted with 0.5 ml of 2,4-dinitrophenolate containing 0.1% NP-40. The DNP eluate was dialyzed against sample buffer with one change and the extract concentrated by surrounding the dialysis bag with dry Sephadex G-200. The sample was then adjusted to 2% (v/v) SDS and run on 10% Laemmli gels. Fig. 28 shows the results obtained for ^{3}H-tyrosine and ^{3}H-leucine labelled RBL cells. In the receptor area of the gel, mainly one tritiated labelled molecule, corresponding in mobility to R, can be seen. The shoulders on the ascending side suggest that some H receptor may also have been present in these receptor preparations. The fact that primarily R was isolated does agree with the results of Conrad and Froese (1978b), and those obtained in Chapter II (Fig. 10) obtained using surface iodinated RBL cells. Once again, a rather dominant peak seen in the vicinity of fractions 45-50 (Fig. 28a,b), raising once more the possibility that molecules in this region of the gel represent receptor-associated material unless, of course, even dinitrophenolate had eluted some non-specifically bound molecules. The results shown in Fig. 28 suggest that many of the high molecular weight tritiated labelled molecules isolated by means of the precipitation schemes or as receptor complexes must have bound non-specifically to either the precipitates or to the affinity matrix used. The continued presence of tritiated material at the top of the gel remains unexplained. Insolubilization of precipitates by SDS-containing sample buffer or elution of the receptor with dinitrophenolate in NP-40 may have also freed similar non-specifically bound molecules, especially in the lower molecular weight ranges of the gel when analyzed on 10% gels.

Figure 28: SDS-PAGE analysis on 10% gels of ^{3}H-amino acid labelled RBL cell components isolated as receptor complexes using the anti-DNP-Sepharose procedure. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, were mixed with 400 µg of DNP-IgE for 90 minutes at 37°C. The cells were washed through FCS and with PBS/BSA to remove free DNP-IgE and solubilized in 1.0 ml of 0.5% NP-40/PBS. Solubilized extracts were mixed with 0.3 ml of anti-DNP-Sepharose for 60 minutes at 4°C, washed with 15-20 ml of 0.1% NP-40/PBS and eluted with 0.1 M 2,4-dinitrophenolate in 0.1% NP-40/PBS. The eluates were dialyzed against Tris sample buffer overnight, adjusted to 2% (v/v) with SDS and heated in boiling water bath for 90 seconds. (a) Profile from ^{3}H-tyrosine labelled cells and (b) Profile from ^{3}H-leucine labelled cells.

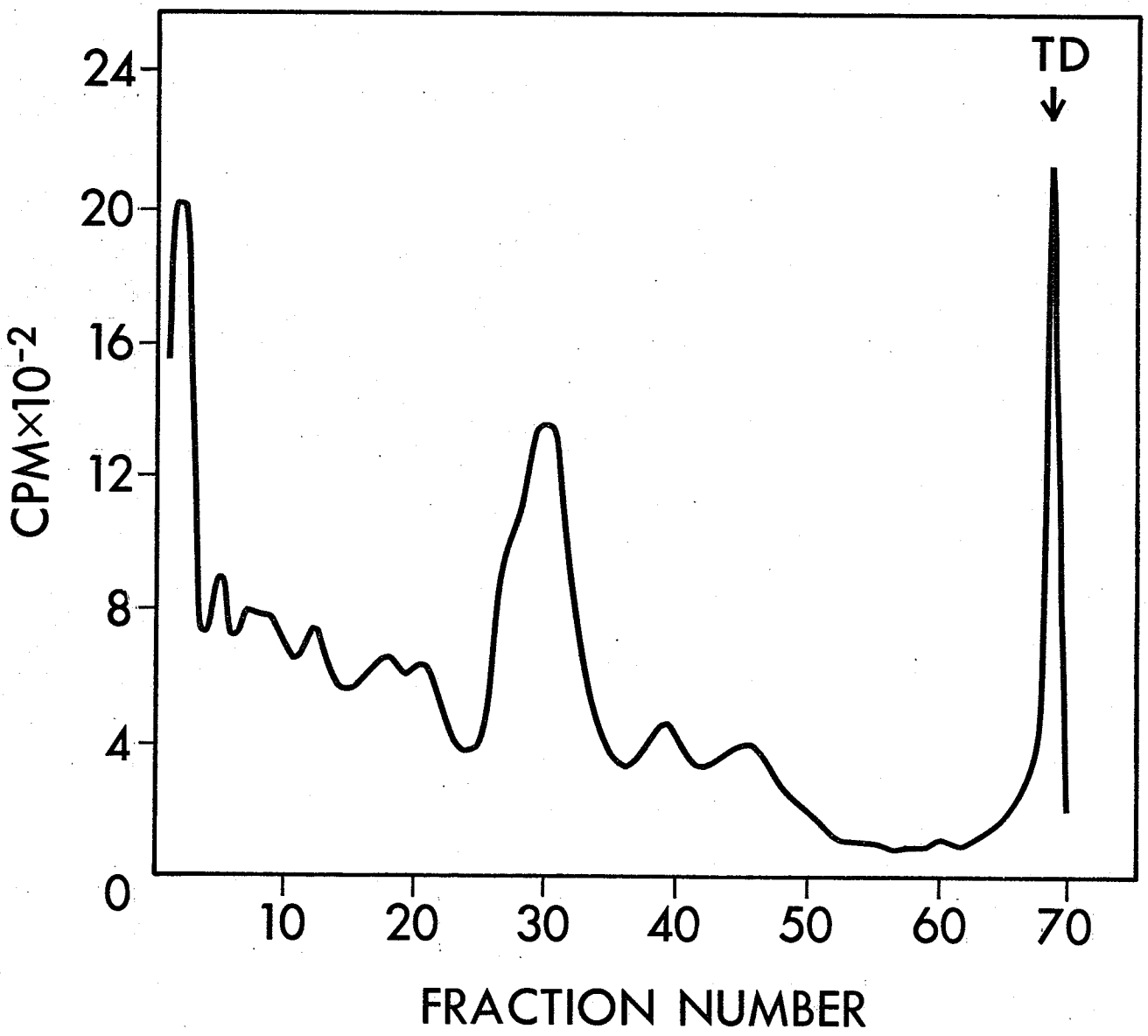

(a)
H
R
TD
6
4
2
(b)
10
8
6
4
2
^{3}H-CPM × 10-2
10
20
30
40
50
60
70
FRACTION NUMBER

The analysis of receptor by receptor-complexes did not always present clearly defined profiles. The presence of R receptor was always consistent as well as material migrating near or at the top of the gel. However, the relative amount of material migrating in the lower molecular weight regions of the gel were inconsistent. This was found to be especially true for the peaks migrating between fractions 40 and 50. To demonstrate this inconsistency, Fig. 29 shows the results obtained when 1.0×10^7 ^{3}H-leucine labelled RBL cells from a 48-hour culture were mixed with 300 µg of DNP-IgE for 90 minutes at 37°C, washed through FCS and with PBS/BSA and solubilized with 0.5% NP-40/PBS. The extract was then mixed with 0.3 ml of anti-DNP-Sepharose for 60 minutes at 4°C, transferred to a 1 ml Pasteur pipette column and washed with 15-20 ml of 0.1% NP-40/BBS and eluted with dinitrophenolate. The dialyzed eluate, when analyzed on 10% gels by SDS-PAGE, shows the characteristic R receptor; however, the peaks in fractions 40-50 are much reduced compared to those seen in the corresponding regions of the gel profile in Fig. 28.

The persistence of material migrating in this region of the gel suggested that at least some form of association must exist with the receptor for IgE on RBL cells. Further evidence for its continued association with the receptor will be shown in the IgE-Sepharose profiles to be presented later.

The experimental data presented so far have demonstrated that receptor material isolated from RBL cells labelled with tritiated amino acids contained, in addition to the receptor for IgE, many different molecules believed to be non-specifically associated with the isolation scheme. Although the IgE-Sepharose profiles of tritiated amino acid labelled receptor material, as seen on 5.6% gels, were quite inconclusive as to the molecules specifically associated with the receptor, it was decided to

Figure 29: SDS-PAGE analysis on 10% gels of ^{3}H-leucine labelled RBL cell components isolated as receptor complexes using anti-DNP-Sepharose. RBL cells (1×10^{7}), biosynthetically labelled for 48 hours, were incubated with 300 µg of DNP-IgE for 60 minutes at 37°C, washed through FCS and with PBS/BSA, solubilized with 1.0 ml of 0.5% NP-40/PBS and the extract mixed with 0.3 ml of anti-DNP-Sepharose for 60 minutes at 4°C. The mixture was transferred to a Pasteur pipette column and washed with 15-20 ml of BBS/BSA containing 0.1% NP-40. Elution was achieved with 0.1 M 2,4-dinitrophenolate in 0.1% NP-40/PBS. The eluate was dialyzed against Tris sample buffer overnight at 4°C and adjusted to 2% SDS (v/v) and heated in a boiling water bath for 90 seconds.


TD
24
20
16
12
8
4
0
CPM×10-2
10
20
30
40
50
60
70
FRACTION NUMBER

persue the isolation of receptor material by means of IgE-Sepharose. This method was chosen because it had been shown that only this procedure isolated both H and R receptors and because it was hoped to get better resolution on 10% gels. Table VII shows the results of insolubilizing tritiated amino acid precursors using IgE-Sepharose and CNBr-activated and ethanolamine quenched Sepharose as a control. It was apparent that substantial amounts of radiolabelled material were being bound by the controls, however, there was a definite increase in the binding of radiolabelled material to IgE-Sepharose.

Analysis by SDS-PAGE of receptors isolated by IgE-Sepharose from cells which had been cultured in the presence of ^{3}H-leucine or ^{3}H-tyrosine, or which had been surface iodinated, is shown in Fig. 30. Fig. 30a depicts the results obtained with surface iodinated cells. Both H and R receptors, as well as the molecule in fraction 19, can be clearly identified. Much more complex patterns were observed as shown in the profiles of Fig. 30b or 30c, where ^{3}H-tyrosine or ^{3}H-leucine labelled cells were used, respectively. Peaks corresponding to H and R can be seen in these profiles, however, in addition, several other biosynthetically labelled molecules appear as well. Compared to previously obtained patterns, these radiolabelled peaks are much better resolved. In addition, the background counts are much more reduced even when control gel profiles are plotted, as shown in Fig. 31. As previously noted, a substantial number of counts was insolubilized by CNBr-activated and quenched Sepharose Cl-4B. However, upon elution and analysis by SDS-PAGE on 10% gels, no prominent peaks were demonstrated.

Peaks migrating in fractions 17-19 and 46-48 (Fig. 30b,c) could be identified in the profiles of eluates from ^{3}H-amino acid labelled cell extracts, iso-

TABLE VII

INCORPORATION OF ^{3}H-AMINO ACID PRECURSORS INTO THE RECEPTORS FOR IgE OF RBL CELLS[(a)]

	CPM BOUND	
AFFINITY GEL	LEUCINE	TYROSINE
IgE-Sepharose	34,240	66,960
Activated Sepharose	24,280	35,600

(a) RBL cells (2×10^{7}) with the respective ^{3}H-precursor incorporated were solubilized and 6×10^{6} cell equivalents were mixed with either 0.3 ml of IgE-Sepharose or 0.3 ml of CNBr-activated and ethanolamine quenched Sepharose. The counts represent the bound material following five washes with 2 - 3 ml of 0.1% NP-40/PBS.

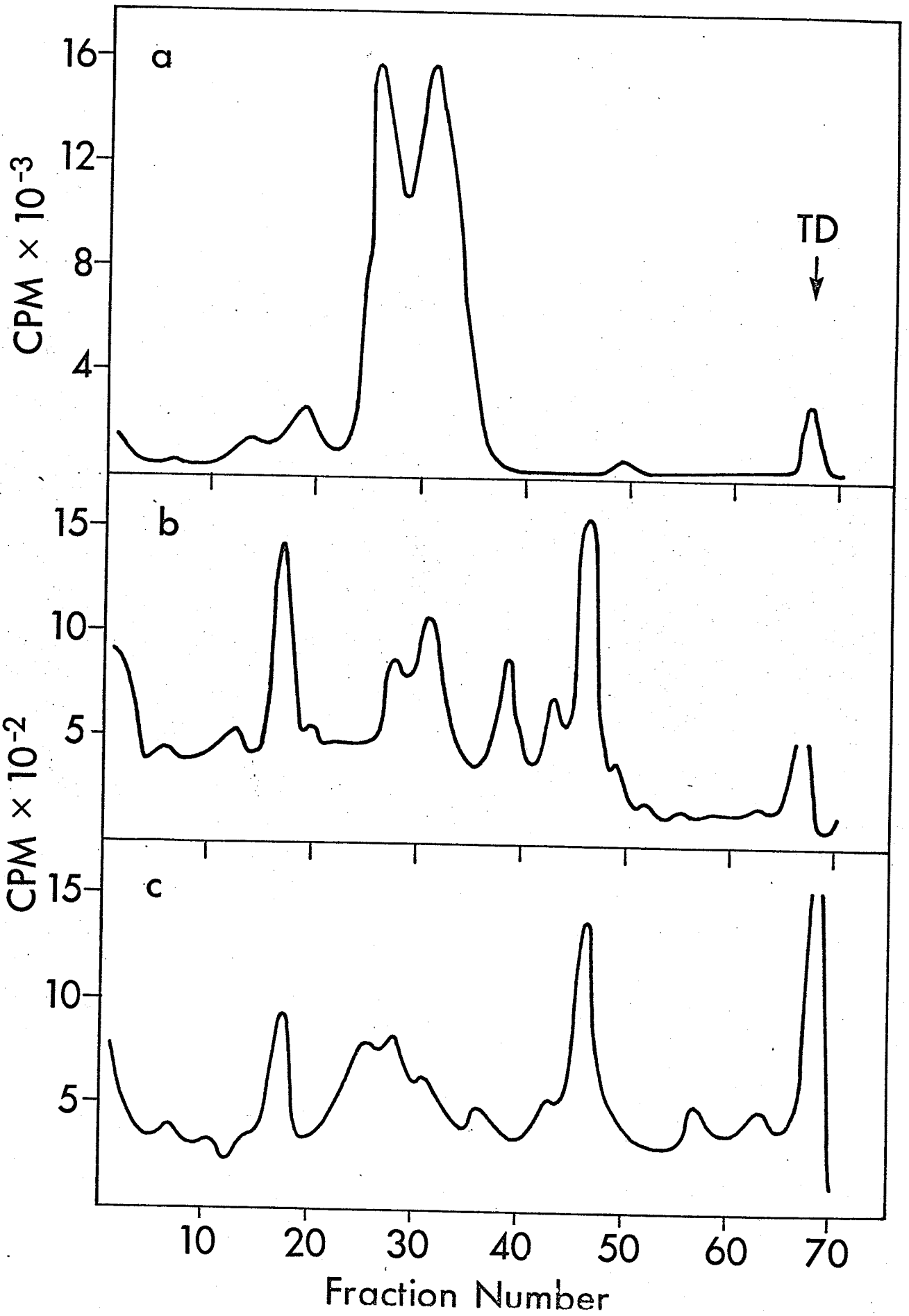
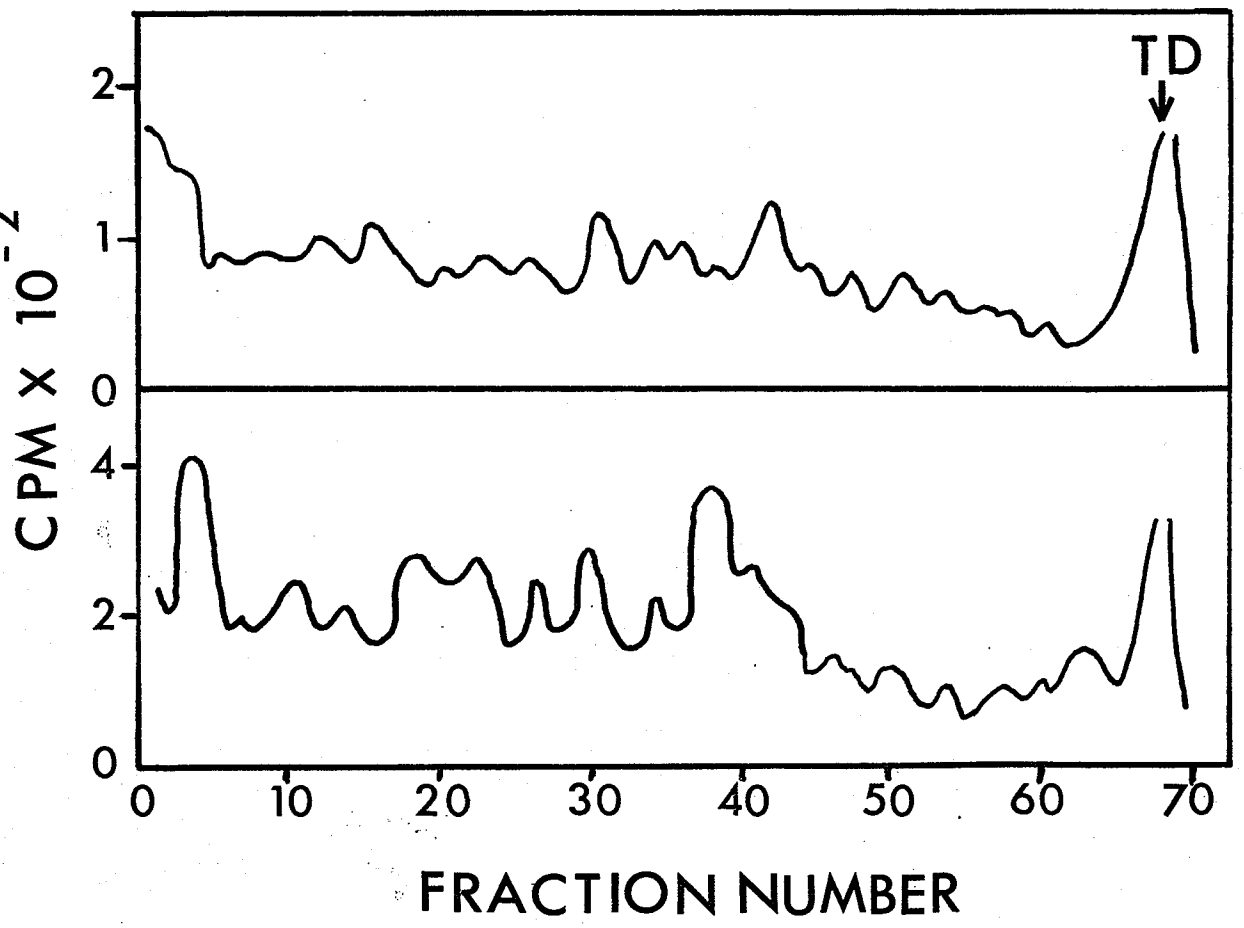

a
b
c
TD
CPM × 10-3
CPM × 10-2
16
12
8
4
15
10
5
15
10
5
10
20
30
40
50
60
70
Fraction Number

Figure 30: SDS-PAGE analysis on 10% gels of ^{3}H-amino acid labelled RBL cell components isolated by IgE-Sepharose. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, or surface iodinated RBL cells (2×10^{7}) were solubilized in 1.0 ml of 0.5% NP-40/PBS and the extracts mixed with 0.3 ml of IgE-Sepharose for 60 minutes at 4°C. The gels were washed with 0.1% NP-40/PBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds. (a) Profile from surface iodinated RBL cell preparation; (b) Profile from ^{3}H-tyrosine labelled RBL cells and (c) Profile from ^{3}H-leucine labelled RBL cells.

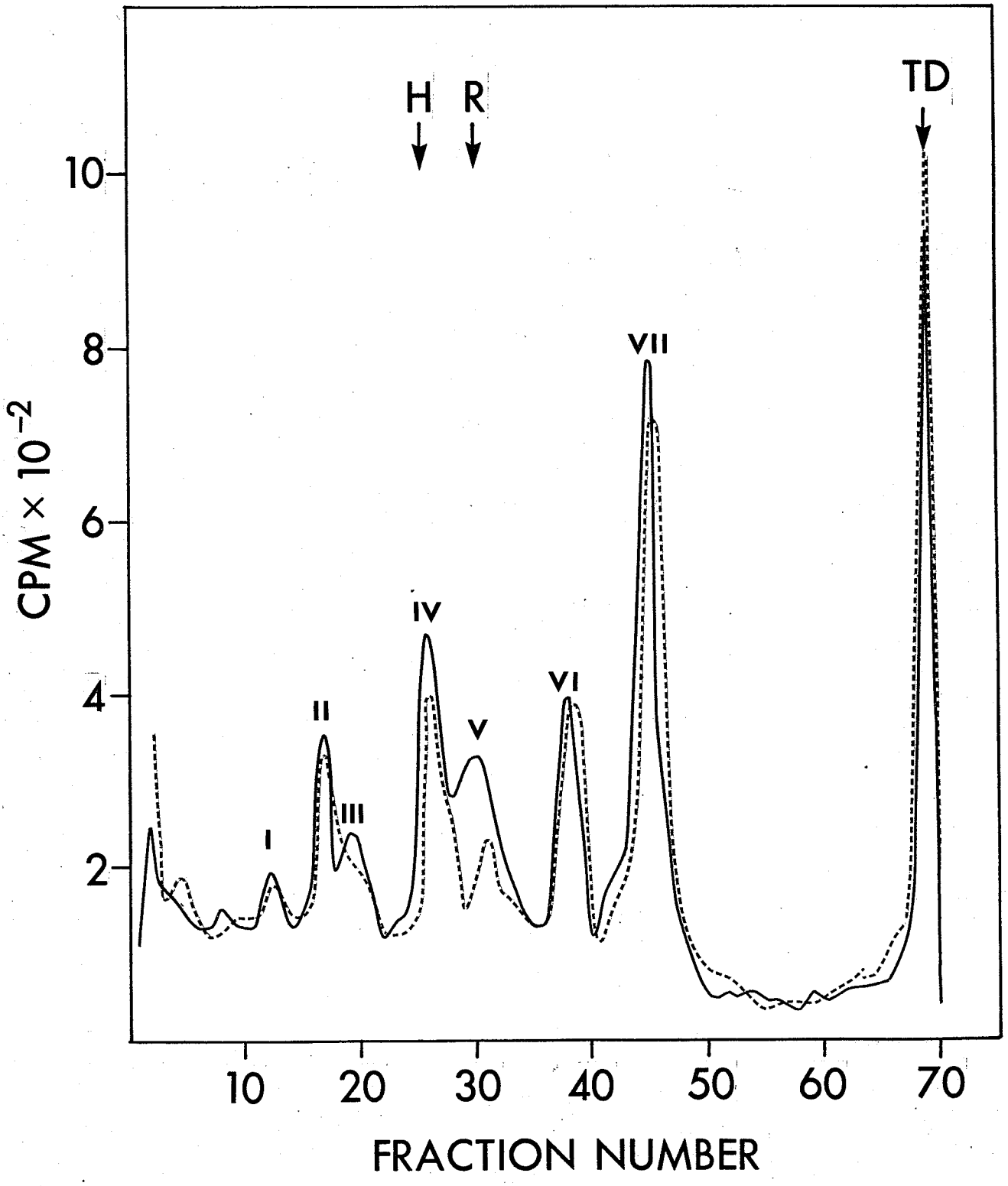
Figure 31: SDS-PAGE analysis on 10% gels of ^{3}H-amino acid labelled RBL components isolated by CNBr-activated and ethanolamine quenched Sepharose Cl-4B. RBL cells (1×10^{7}), biosynthetically labelled for 48 hours, were solubilized in 1.0 ml of 0.5% NP-40/PBS and the extracts mixed with 0.3 ml of CNBr-activated and ethanolamine quenched Sepharose Cl-4B for 60 minutes at 4°C. The gels were washed with NP-40/PBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds. (a) Profile from ^{3}H-tyrosine labelled RBL cells and (b) profile from ^{3}H-leucine labelled RBL cells.


TD
2
1
0
4
2
0
CPM X 10^{-2}
0
10
20
30
40
50
60
70
FRACTION NUMBER

lated from IgE-Sepharose, are still quite prominent, suggesting that molecules making up these two peaks may be somehow related to the receptor for IgE on RBL cells. The peaks present in Fig. 30b and 30c indicated that these molecules contained both leucine and tyrosine; however, the amount of each amino acid incorporated into the various molecules must have varied, as the relative sizes of the peaks shown in the two gel patterns are quite different.

In order to establish that molecules migrating with mobilities corresponding to H and R were indeed specific for IgE and to ascertain if any of the other peaks exhibited specificity for IgE, the following experiments were carried out. RBL cells (2×10^7) were suspended in medium at a concentration of 3×10^6 cells/ml and incubated with 50 µCi of ^{3}H-leucine for 48 hours. Two aliquots of 1×10^7 cells were taken; one was incubated for one hour with 300 µg of IgE, purified by isoelectric focussing, while the other was incubated with BBS/BSA only. The cells were then washed through FCS and once more with BBS/BSA, solubilized and the extract from each aliquot of cells was allowed to react with 0.3 ml of packed IgE-Sepharose for 60 minutes at 4°C. Following five washes with BBS/BSA and one with Tris sample buffer, bound material was eluted with Tris sample buffer containing 2% SDS. When the eluates were analyzed by SDS-PAGE, the results shown in Fig. 32 were obtained. It is evident that peaks corresponding in mobility to H and R were inhibited in the presence of IgE, with the more significant inhibition in the peak corresponding to R. More effective inhibition of R was also seen when similar experiments were carried out with surface iodinated cells (Chapter II). In addition, some inhibition of the molecule migrating in fraction 19 can be seen. Slight differences in counts are also seen in other peaks, particularly the tall peak near fraction 46; however, the effect is too

Figure 32: SDS-PAGE analysis on 10% gels of ^{3}H-leucine labelled RBL cell components isolated in the presence or absence of free IgE by IgE-Sepharose. RBL cells (1×10^{7}), biosynthetically labelled with ^{3}H-leucine for 48 hours, were incubated in the absence (———) or presence (-----) of 300 µg of IgE for 90 minutes at 37°C. The cells were then washed through FCS and with BBS/BSA, solubilized in 1.0 ml of 0.5% NP-40/BBS and the extracts mixed with 0.3 ml of IgE-Sepharose for 60 minutes at 4°C. The gels were washed with 0.1% NP-40/BBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds.

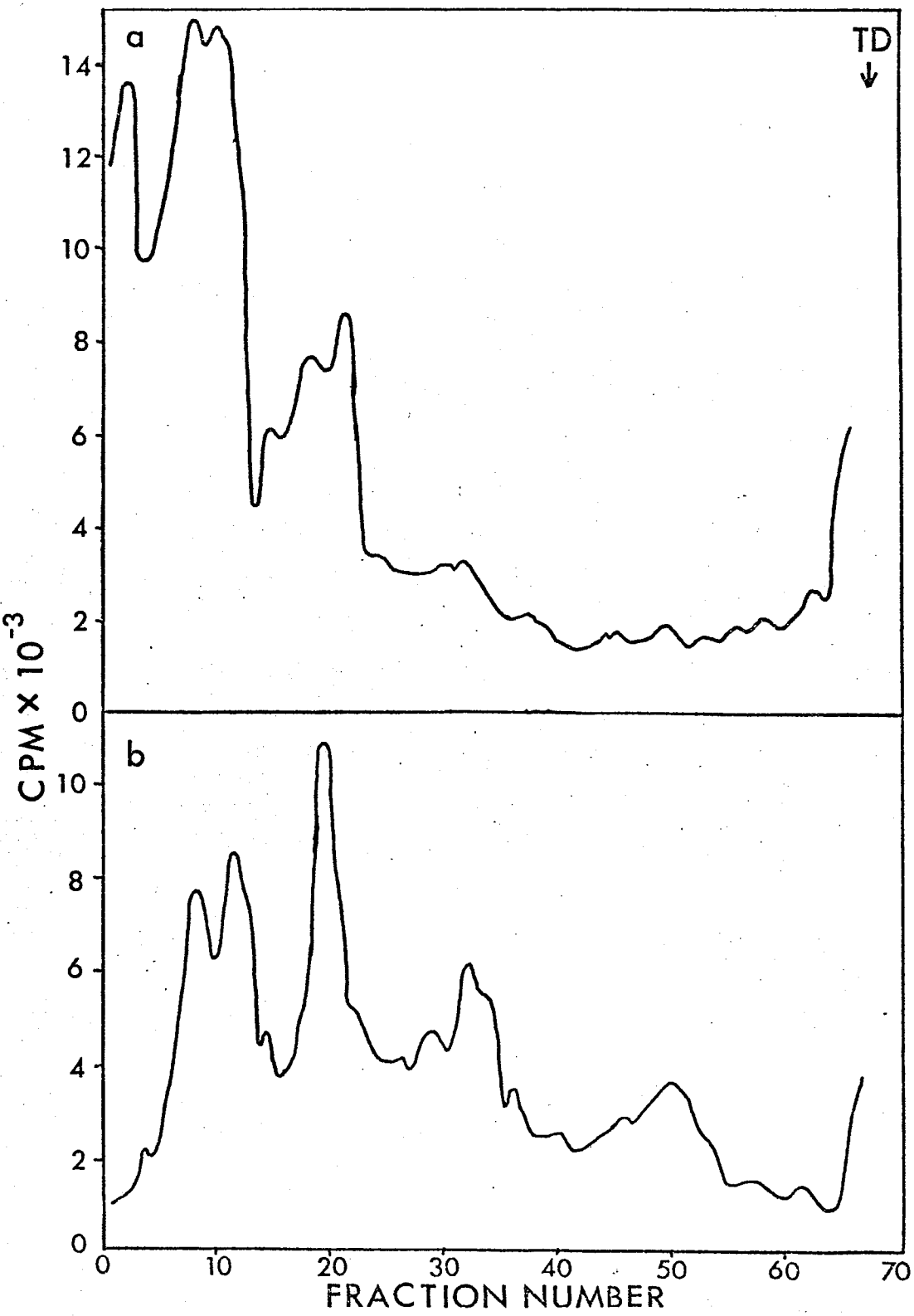

H
R
TD
I
II
III
IV
V
VI
VII
10
8
6
4
2
CPM × 10⁻²
10
20
30
40
50
60
70
FRACTION NUMBER

small to be definitely attributed to inhibition.

As mentioned earlier, most of the preliminary investigations regarding the incorporation of tritiated precursors involved the use of tritiated leucine and tyrosine. These investigations were carried out primarily to reduce the non-specific binding of non-receptor-associated molecules and to achieve, through the use of the Laemmli gel system, a better resolution of various molecules present in the receptor preparations. Although early experiments using the 5.6% Fairbanks gel system had clearly shown incorporation of tritiated sugars into receptor molecules, and relatively little into other molecules, receptor preparations labelled with these precursors were also analyzed on the Laemmli system with 10% gels. This was considered particularly important, since Conrad and Froese (1976), using the 5% gels for SDS-PAGE described by Shapiro *et al.* (1967), found that H and R receptors could not be separated.

(b) Incorporation Studies with ^{3}H-Sugars: To obtain some information on the RBL cell molecules labelled by the ^{3}H-sugars, whole NP-40 extracts of cells were analyzed by SDS-PAGE on 10% gels. Fig. 33 shows the SDS-PAGE profiles of NP-40 solubilized cell extracts from RBL cells grown in the presence of ^{3}H-galactose (Fig. 33a) or in the presence of ^{3}H-fucose (Fig. 33b). In contrast to the amino acid profiles seen in Fig. 26a and 26b, the two tritiated sugars were mainly incorporated into molecules with molecular weights above 55,000 daltons. Furthermore, it is interesting to note that molecules in the higher molecular weight region were also bound by lentil-lectin (Fig. 13), establishing that glycoprotein molecules are mainly associated with the higher molecular weight molecules. Although it is less evident for galactose than for fucose, there appear to be several biosynthetically labelled glycoproteins migrating in the molecular weight range which also contained the receptors H and R.

Figure 33: SDS-PAGE analysis on 10% gels of biosynthetically incorporated ^{3}H-sugar into RBL cell components. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, were washed through FCS and with PBS/BSA. A 10 µl aliquot of a 1.0 ml 0.5% NP-40/PBS solubilized extract was then removed and added to 200 µl of Tris sample buffer containing 2% SDS and heated in a boiling water bath for 90 seconds. (a) Profile from ^{3}H-galactose labelled RBL cells and (b) Profile from ^{3}H-fucose labelled RBL cells.


a
TD
b
CPM X 10^-3
FRACTION NUMBER
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70

To obtain more information about the incorporation of ^{3}H-sugars into both H and R receptors, isolation was carried out by IgE-Sepharose. The results seen in Table VIII suggested that the IgE-Sepharose isolation procedure for receptors labelled with ^{3}H-fucose or ^{3}H-galactose was quite specific. The difference between experimental and control data was much more pronounced than when receptors were isolated from cells labelled with ^{3}H-amino acids. When the bound cellular material was eluted and analyzed by SDS-PAGE, the results shown in Fig. 34 were obtained. As can be seen, only IgE-Sepharose yielded peaks in the receptor area of the gels. Both molecules equivalent to H and R and the minor component near fraction 19 were seen. The control gel patterns clearly demonstrated the specificity for all three molecules bound to and eluted from IgE-Sepharose.

Results from a similar experiment are shown in Fig. 35. In this case, receptors isolated from ^{125}I-labelled cells were shown for comparison. Clearly, the mobility of receptors isolated from ^{125}I- and ^{3}H-sugar-labelled cells was identical.

The percentage of total tritiated sugar counts bound to IgE-Sepharose (approximately 0.4 - 0.8%) was somewhat less than the percentage of total tritiated amino acid counts bound to IgE-Sepharose (approximately 1.0 - 1.4%). However, the sugars were incorporated into fewer molecules bound by IgE-Sepharose. Thus, only two major peaks were seen in Figs. 34 and 35, which correspond in mobility to R and H receptors, whereas control gels consisting of CNBr-activated and ethanolamine quenched Sepharose Cl-4B showed no such specificity. Relatively more of the sugar precursors appeared to be incorporated into a molecule corresponding to H than that corresponding to R. Interestingly, both fucose and galactose were incorporated into the molecule with apparent molecular weight of $\sim$71,000 daltons (fractions 17-19). Similarly, this molecule had earlier been

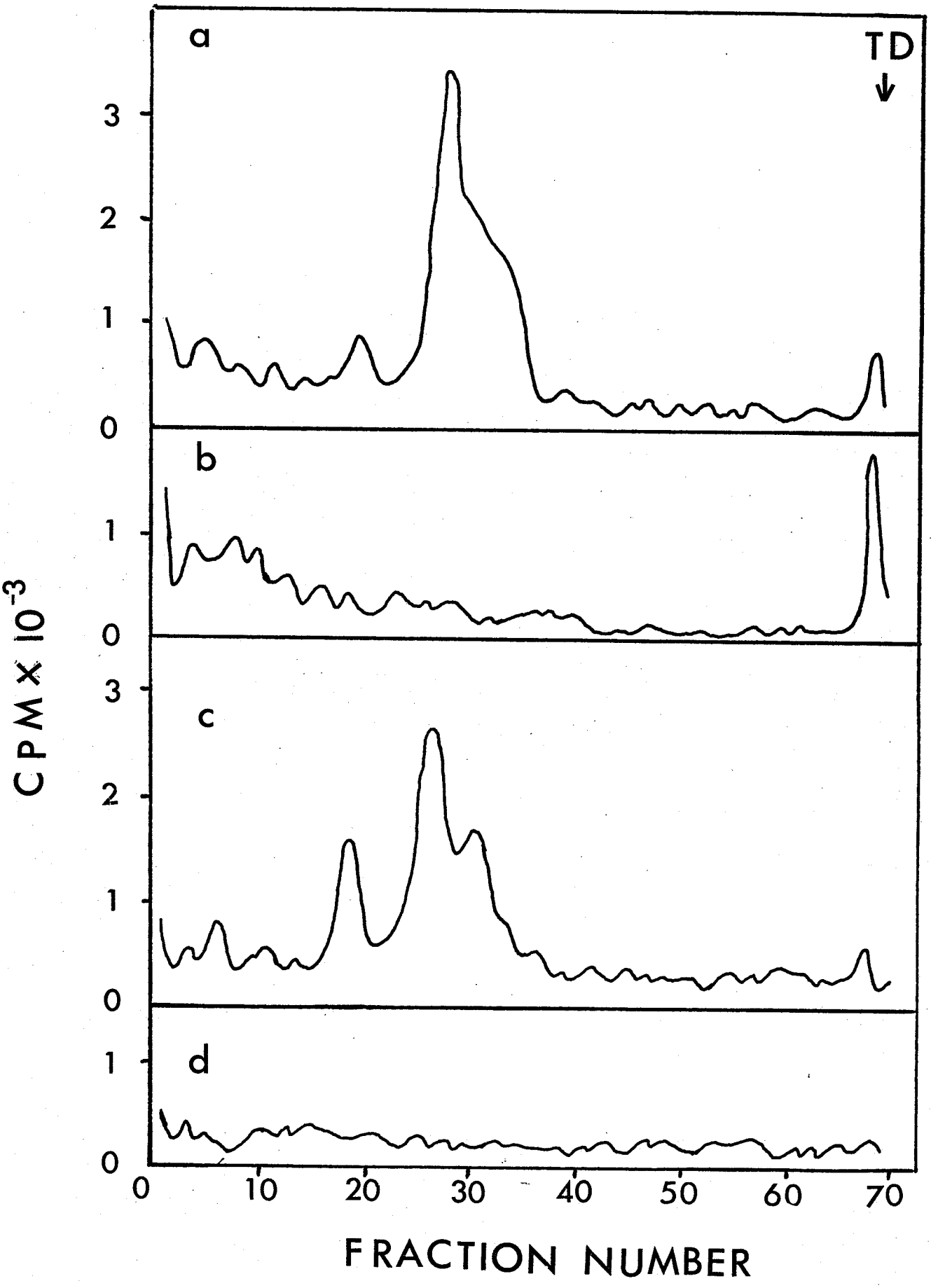
TABLE VIII

INCORPORATION OF ^{3}H-SUGARS INTO THE RECEPTORS FOR IgE OF RBL CELLS (a)

	CPM BOUND	
AFFINITY GEL	GALACTOSE	FUCOSE
IgE-Sepharose	11,160	9,120
Activated Sepharose	3,360	1,960

(a) RBL cells (2×10^{7}) with the respective ^{3}H-precursor incorporated were solubilized and 6×10^{6} cell equivalents were mixed with either 0.3 ml of IgE-Sepharose or 0.3 ml of CNBr-activated and ethanolamine quenched Sepharose. The counts represent the bound material following five washes with 2 - 3 ml of 0.1% NP-40/PBS.

Figure 34: SDS-PAGE analysis on 10% gels of ^{3}H-sugar labelled RBL cell components isolated by IgE-Sepharose. RBL cells (2×10^{7}), biosynthetically labelled for 24 hours, were solubilized in 1.0 ml of 0.5% NP-40/PBS and aliquots representing 6×10^{6} cell equivalents were mixed with 0.3 ml of IgE-Sepharose or CNBr-activated and ethanolamine quenched Sepharose Cl-4B for 60 minutes at 4°C. The gels were washed with 0.1% NP-40/PBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds. (a) Profile from ^{3}H-galactose labelled RBL cell material eluted from IgE-Sepharose; (b) Profile from ^{3}H-galactose labelled RBL cell material eluted from control Sepharose Cl-4B; (c) Profile from ^{3}H-fucose labelled RBL cell material eluted from IgE-Sepharose and (d) Profile from ^{3}H-fucose labelled RBL cell material eluted from control Sepharose Cl-4B.


a
TD
b
c
d
CPM X 10-3
FRACTION NUMBER
0
1
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70

shown in the amino acids profiles and surface iodinated cell extracts isolated by means of IgE-Sepharose.

In order to test if the peaks seen in Fig. 35b and 35c represented receptors for IgE, attempts were made to inhibit their binding to IgE-Sepharose using free IgE. To achieve this, 1.0×10^7 RBL cells were incubated either with 200 µg of IgE in a total volume of 1.0 ml of BBS/BSA or in the same volume in the absence of IgE. The two batches of cells, with either ^{3}H-galactose or ^{3}H-fucose, were then incubated at 37°C for 90 minutes, then washed through FCS and BBS/BSA. Subsequently, the cell pellets were solubilized with 0.5% NP-40 in BBS and the receptor material isolated by means of 0.2 ml of IgE-Sepharose. Laemmli sample buffer eluates of receptors preparations were then analyzed by SDS-PAGE in 10% gels. Fig. 36 clearly demonstrates that when free IgE is mixed with these biosynthetically labelled cells, both H and R can be shown to be inhibited, thereby establishing that these radiolabelled molecules were specific for IgE. As noted with the other inhibition experiments using surface iodinated cells, the molecule seen in fractions 17-19 was also markedly inhibited. This established that H and R, as well as the somewhat larger molecule, were glycoproteins which could be inhibited and as such were specific receptors for IgE on RBL cells.

Although R appeared to be less evident in the eluates obtained from IgE-Sepharose, the use of the DNP-IgE/anti-DNP Sepharose procedure was carried out to isolate R. To achieve this, 3×10^7 RBL biosynthetically labelled RBL cells with either ^{3}H-galactose or ^{3}H-fucose were mixed with 300 µg of DNP-IgE for 90 minutes at 37°C, washed through FCS and solubilized using 0.5% NP-40 in PBS. The solubilized extract was then mixed with anti-DNP Sepharose, washed and eluted as described earlier. The dialyzed eluates were then analyzed on 10% gels by SDS-PAGE and compared to a

Figure 35: SDS-PAGE analysis on 10% gels of ^{3}H-sugar labelled RBL cell components isolated by IgE-Sepharose. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, or surface iodinated RBL cells (2×10^{7}) were solubilized in 1.0 ml of 0.5% NP-40/PBS and the extracts mixed with 0.3 ml of IgE-Sepharose for 60 minutes at 4°C. The gels were washed with 0.1% NP-40/PBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds. (a) Profile from surface iodinated RBL cell preparation; (b) Profile from ^{3}H-galactose labelled RBL cells and (c) Profile from ^{3}H-fucose labelled RBL cells.

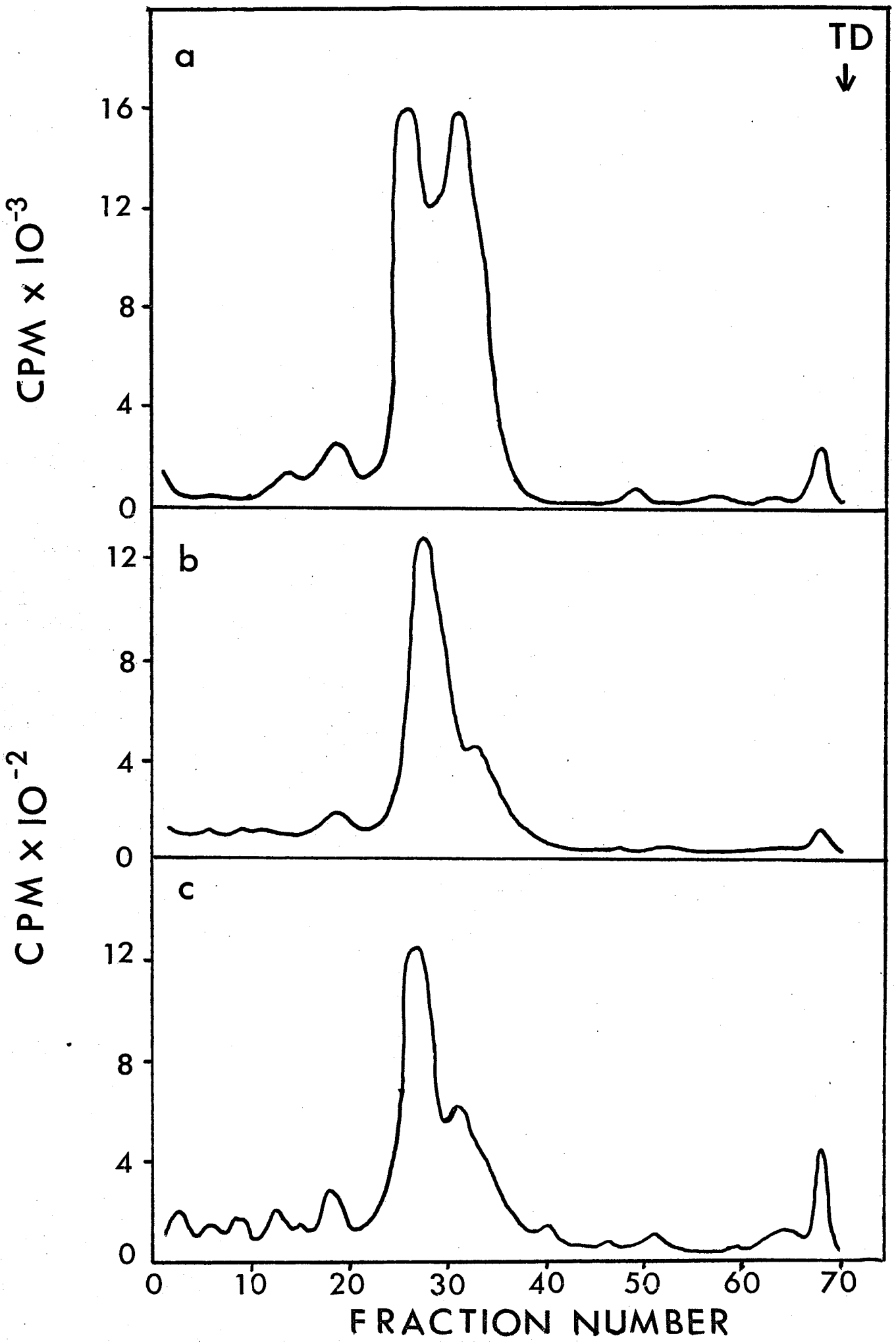
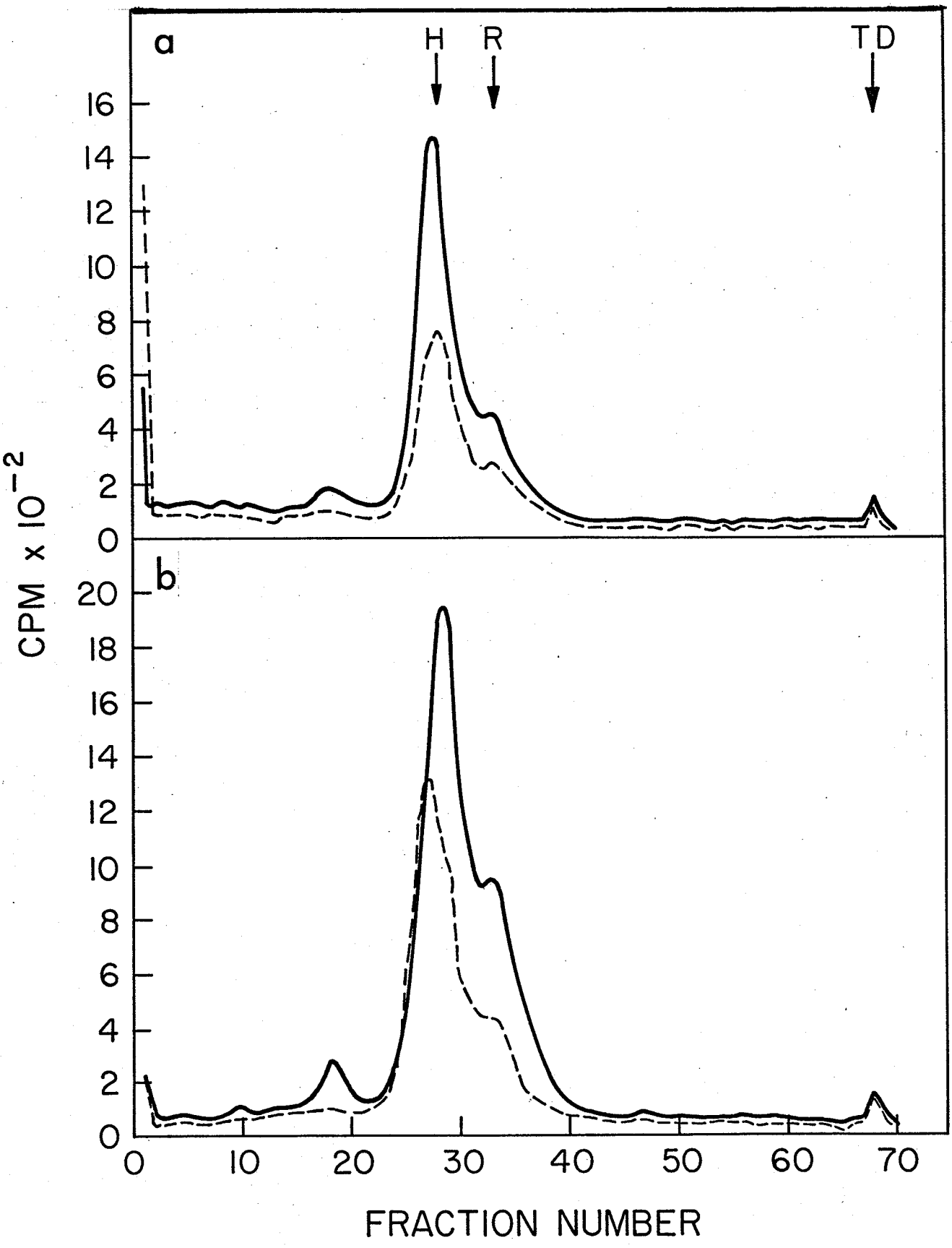
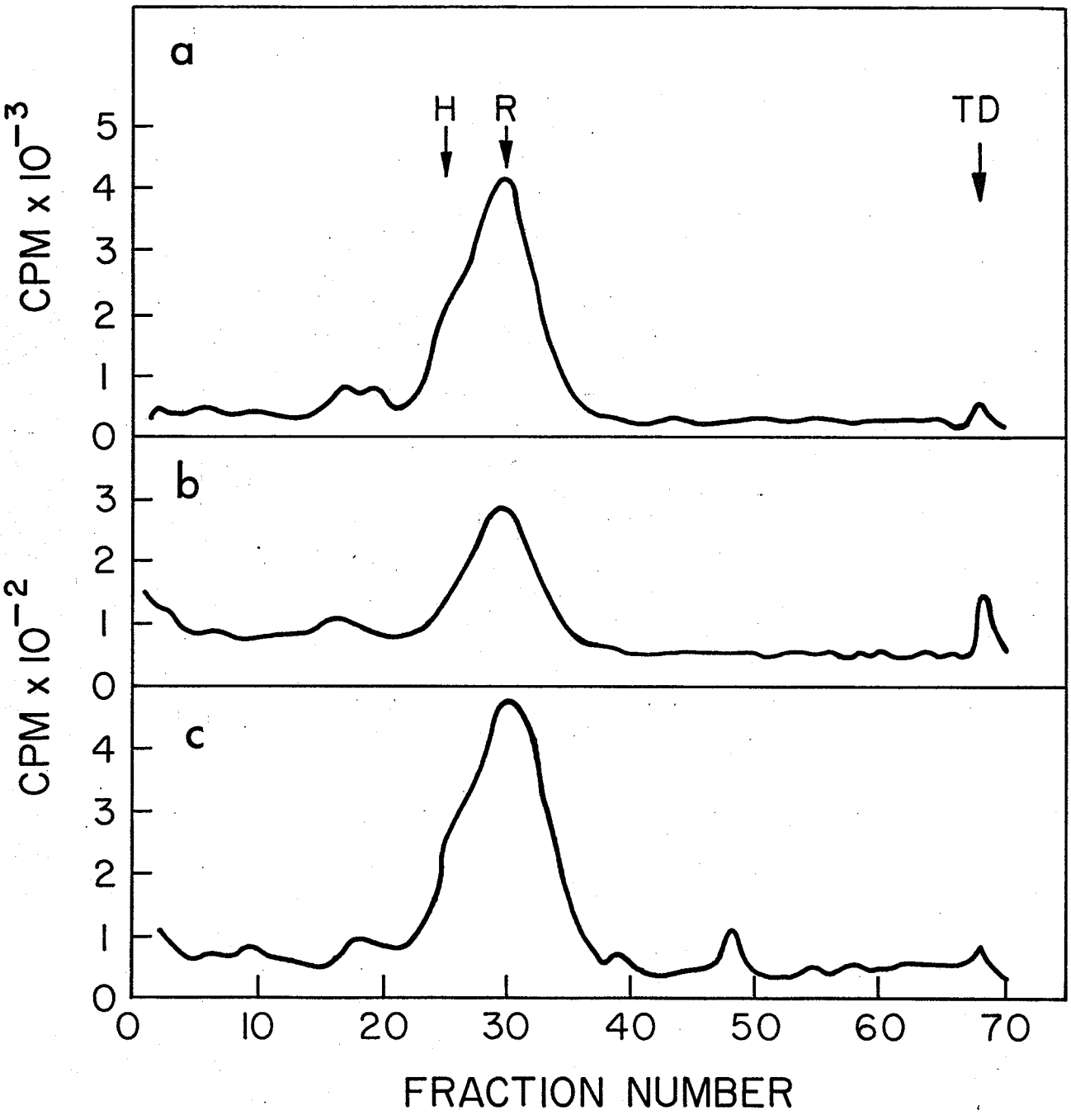

a
TD
16
12
8
4
0
CPM x IO-3
b
12
8
4
0
c
CPM x IO-2
12
8
4
0
0
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 36: SDS-PAGE analysis on 10% gels of ^{3}H-sugar labelled RBL cell components isolated in the presence or absence of free IgE by IgE-Sepharose. RBL cells (1×10^{7}), biosynthetically labelled for 24 hours, were incubated in the absence (———) or presence (-----) of 200 µg of free IgE for 90 minutes at 37°C. The cells were washed through FCS and with BBS/BSA, solutilized in 1.0 ml of 0.5% NP-40 in BBS/BSA and the extracts added to 2.0 ml of IgE-Sepharose for 60 minutes at 4°C. The gels were washed with 0.1% NP-40/BBS and eluted with 200 µl of Tris sample buffer containing 2% SDS by heating in a boiling water bath for 90 seconds. (a) Profiles from ^{3}H-galactose labelled RBL cells and (b) Profiles from ^{3}H-fucose labelled RBL cells.


a
H
R
TD
b
CPM x 10⁻²
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60
70

surface iodinated receptor isolation using the same procedures as shown in Fig. 37. In Fig. 37b and 37c, both galactose and fucose were shown to be incorporated into R, when compared to R receptor isolated from surface iodinated RBL cells shown in Fig. 37a. Once again, there was some evidence of H as an ascending peak on the R receptor isolated by this IgE receptor complex procedure. The appearance of H as a shoulder in these DNP-IgE receptor complexes is not completely understood, although there was often evidence of its appearance in both surface iodinated preparations and biosynthetically labelled RBL cell extracts.

Figure 37: SDS-PAGE analysis on 10% gels of ^{3}H-sugar labelled RBL cell components isolated as receptor complexes using the anti-DNP-Sepharose procedure. RBL cells (3×10^{7}), biosynthetically labelled for 48 hours, or surface iodinated RBL cells (2×10^{7}), were incubated with 300 µg of DNP-IgE for 90 minutes at 37°C, washed through FCS and with PBS/BSA, solubilized with 0.1 ml of 0.5% NP-40/PBS and the extracts mixed with 0.3 ml of anti-DNP-Sepharose for 60 minutes at 4°C. The mixture was transferred to a Pasteur pipette and washed with 15-20 ml of 0.1% NP-40/PBS. Elution was achieved with 0.1 M 2,4-dinitrophenolate in 0.1% NP-40/PBS. The eluate was dialyzed against Tris sample buffer overnight at 4°C and adjusted to 2% SDS (v/v), heated in a boiling water bath for 90 seconds. (a) Profile from surface iodinated RBL cell preparation; (b) Profile from ^{3}H-galactose labelled RBL cells and (c) Profile from ^{3}H-fucose labelled RBL cells.


a
H
R
TD
CPM x 10⁻³
5
4
3
2
1
0
b
3
2
1
0
CPM x 10⁻²
c
4
3
2
1
0
0
10
20
30
40
50
60
70
FRACTION NUMBER

DISCUSSION

In Chapter II of this thesis, it was shown in confirmation to the results of Conrad and Froese (1978a) that, by means of IgE-Sepharose, two receptors named H and R and having apparent molecular weights of 45,000 and 55,000 daltons, respectively, could be isolated from surface iodinated RBL cells. In addition, a third molecule specific for IgE and having an apparent molecular weight of 71,000 daltons was for the first time consistently observed in receptor preparations. Almost identical results were obtained when RBL cells were biosynthetically labelled with either ^{3}H-fucose or ^{3}H-galactose. Counts were incorporated into molecules corresponding to H and R receptors, as well as into the 71,000 dalton molecule. However, fewer ^{3}H-sugar moieties appeared to be incorporated into R than into H. In this respect, Fig. 35 is particularly instructive. The surface iodinated receptor preparation yielded H and R peaks of about equal magnitude, while preparations from cells labelled with the ^{3}H-sugars exhibited significantly lower R peaks. Interestingly, the relative peak heights of H and the 71,000 dalton molecule were about the same, irrespective of the labelling procedure. However, in one case, Fig. 34c, a much more pronounced 71,000 dalton molecule was seen in the case of tritiated fucose-labelled cells.

As expected on the basis of the results shown in Chapter II and obtained by Conrad and Froese (1978b), isolation of receptors by means of DNP-IgE and anti-DNP-Sepharose from ^{3}H-sugar labelled cells (Fig. 37), yielded mainly R receptor. However, evidence for trace amounts of H was observed (Fig. 37a,c). Evidence for trace amounts of H receptor has been reported in preparations of DNP-IgE receptor complexes (Conrad and Froese, 1978b) and in material precipitated by RAR from such complexes (Conrad *et al.*, 1979).

The specificity of the binding of all three receptor molecules to IgE-Sepharose was demonstrated through inhibition experiments with uncoupled IgE (Fig. 36), which was added to intact cells prior to solubilization. The data, particularly those in Fig. 36, do suggest that R is more selectively inhibited than H. A similar observation was made by Conrad and Froese (1978a) with surface iodinated cells. These results suggested that R had a higher affinity for IgE than H. Also, the fact that the 71,000 dalton molecule was almost completely inhibited from binding to IgE-Sepharose suggested that it may also have had a higher affinity for IgE than H. However, this latter conclusion was a very tentative one, in view of the fact that the peak in question was very small and the differences observed may be subject to considerable error margins.

The incorporation of ^{3}H-sugars along with the fact that all three receptors could be radioiodinated demonstrated further that they were glycoproteins and that both had fucose and galactose in their carbohydrate moieties. This was contrasted by the results obtained in Chapter III, Fig. 22, which showed no binding of H to the lectins Pea and Gorse. This suggested that the fucose moieties in H were present in a configuration, which did not interact with the lectins.

Labelling of RBL cells with ^{3}H-amino acids yielded much more complex results. Differences with results obtained with ^{3}H-sugar labelled cells were already apparent when specific binding to immunoprecipitates or IgE-Sepharose was compared with the appropriate control experiments (Tables VI, VII and VIII). In the case of ^{3}H-amino acid labelled cells, the difference between controls and experimentals was always less pronounced. This suggested that more non-specific binding of non-glycosylated proteins than of glycosylated proteins had taken place.

Analysis of receptor preparations by SDS-PAGE on 5.6% gels using

the Fairbanks (1971) system were rather disappointing. Broad peaks were seen in the receptor area of the gels; however, even more counts were observed in other areas of the gels, particularly in the low molecular weight regions. Resolution of these cellular proteins was poor, making it difficult to arrive at a definite conclusion about the nature of this material. It was, however, tentatively concluded that significant non-specific binding had taken place.

In order to obtain better resolution, the Laemmli (1970) SDS-PAGE system using 10% gels was adopted. This system makes use of a discontinuous Tris buffer system. As can be seen in Figs. 27-33, a much better resolution of the peaks was obtained. Material isolated by means of immune precipitates (Fig. 27a) still showed a rather complex pattern with pronounced peaks in the high and low molecular weight regions of the gel. When protein A carrying bacilli were used to bind immune complexes, peaks in the high molecular weight region were significantly reduced (Fig. 27b). A peak with a mobility of R, as well as pronounced peaks in fractions 46 and 52, were seen also.

SDS-PAGE analysis on Laemmli gels of IgE-Sepharose purified receptor preparations also yielded well-defined peaks (Figs. 30 and 32) using either ^{3}H-leucine or ^{3}H-tyrosine. A summary of the major peaks seen (based on Fig. 32) is shown in Table IX. Among the peaks are molecules which, in terms of their molecular weight, have been characterized as receptors. These are peaks III, IV(H) and V(R). That the molecules were indeed specific for IgE was demonstrated by inhibition experiments with uncoupled IgE, which was added to intact cells (Fig. 32). Inhibition of peaks III, IV and V was clearly seen. Some inhibition of peak VII may also have taken place, but this effect was too small to allow a definite conclusion. The inhibition seen in this Figure (32) was not very dramatic. Much higher

TABLE IX

R_f VALUES WITH CORRESPONDING APPARENT MOLECULAR WEIGHTS FOR THE PEAKS IDENTIFIED WITH SDS-PAGE PROFILES FROM BIOSYNTHETICALLY AMINO ACID LABELLED PRECURSORS IN RBL CELL EXTRACTS

PEAK	R_f	MOLECULAR WEIGHT
I	.17	90,000
II	.24	75,000
III	.27	70,000
IV	.37	55,000
V	.43	47,000
VI	.55	36,000
VII	.65	26,000

concentrations of IgE would have had to be used to achieve complete inhibition of the binding. Thus, Conrad and Froese (1978a), when inhibiting the binding of receptors to IgE-Sepharose by the addition of free IgE to solubilized cell extracts, had to add about 4,000 µg of IgE to obtain a 90% inhibition. They used only 3×10^6 RBL cell equivalents and 0.1 ml of IgE-Sepharose. In the present study, in order to obtain substantial amounts of tritiated IgE-Sepharose bound materials, 1×10^7 cells and 0.3 ml of IgE-Sepharose were used.

In spite of the fact that only three of the peaks in Fig. 32 (Table IX) could be inhibited from binding to IgE-Sepharose, it is hard to believe that all the other molecules were non-specifically bound to IgE-Sepharose. In order to reduce the presence of non-specifically bound molecules in receptor preparations, DNP-IgE and anti-DNP-Sepharose were used for receptor isolation. Less non-specifically bound material should have been eluted with dinitrophenolate. This was indeed the case (Fig. 29) and mainly R was found by SDS-PAGE analysis; however, in other experiments, a molecule corresponding to peak VII (Table IX, Fig. 32) and having an apparent molecular weight of 26,000 daltons was observed (Fig. 28). This molecule was rather prominent in most other receptor preparations of ^{3}H-amino acid labelled RBL cells when analyzed on 10% gels by SDS-PAGE (Fig. 31a,b and Fig. 27b). These various observations provided compelling circumstantial evidence that the 26,000 dalton molecule may, in fact, be associated with R receptor (since it is isolated by IgE-Sepharose and by DNP-IgE and anti-DNP-Sepharose). It is most likely not exposed to the ectomembrane of RBL cells, as it does not become iodinated by the lactoperoxidase catalyzed reaction and because it does not seem to be a glycoprotein.

The fact that the peak corresponding to the 26,000 dalton molecule

varied in size in different receptor preparations isolated by means of haptenated IgE does suggest that its putative association with R may be weak. Thus, it may sometimes be washed off anti-DNP-Sepharose, depending on how extensively affinity gels are washed before elution is initiated. Similar observations were made by others (H. Metzger, personal communication).

At present, it is not clear why peak VII was not more significantly inhibited in Fig. 32. It is is associated with R, peaks V and VII should have been inhibited to the same extent. Thus, it is obvious that more research is required before the nature of the 26,000 dalton molecule is clearly established.

As for the other molecules seen in receptor preparations from ^{3}H-amino acid labelled cells (i.e. peaks I, II, VI or Fig. 32), they are most likely either cytoplasmic or ectomembrane derived. An association of these molecules with any of the receptors for IgE can so far not be postulated.

GENERAL DISCUSSION

During the past few years, as more information on the receptors for IgE on both RMC and RBL cells became available, a picture has begun to emerge which points to an increasing complexity of these receptors. Early studies by Conrad and Froese (1976) had suggested that receptor preparations isolated from both RMC and RBL cells by a sandwich precipitation technique involving IgE and anti-IgE and a second antibody, contained a single cell surface receptor for IgE with an apparent molecular weight of 62,000 daltons, as determined by SDS-PAGE on 5% gels. These results were confirmed by Kulczycki *et al.* (1976), who also demonstrated that on 10-12% gels, the apparent molecular weight was 45,000 - 50,000 daltons. This value must, therefore, be considered to be closer to the true molecular weight of the receptor. The difference in apparent molecular weights on gels of different porosity can be attributed to the glycoprotein nature of the molecules (Weber and Osborn, 1975). Somewhat later, Conrad and Froese (1978a) were able to show that when IgE-Sepharose was used for receptor isolation, two molecules having molecular weights of 45,000 and 55,000 daltons were obtained in the case of RBL cells. These have been named R and H receptors, respectively, and they have now also been observed in the case of RMC (Froese, 1980). In both of these studies, only one molecule of 45,000 daltons (R) could be detected by SDS-PAGE analysis on 10% gels if IgE and anti-IgE were used for receptor isolation.

The present investigation confirmed these observations by showing that indeed two major receptors could be isolated from surface-iodinated RBL cells using IgE-Sepharose (Chapter II) and that only one receptor molecule was obtained if IgE and anti-IgE were used. It also extends these studies by demonstrating that these molecules are not only seen in the case

of surface-iodinated RBL cells, but also when RBL cells were biosynthetically labelled with tritiated sugars (^{3}H-fucose and ^{3}H-galactose) (Figs. 23 and 34) or with tritiated amino acids (^{3}H-leucine and ^{3}H-tyrosine) (Figs. 24 and 30). Kulczycki *et al.* (1976) and Kulczycki and Parker (1979) had also shown the incorporation of precursor sugars and amino acids into the receptors for IgE. However, they had only detected a single molecule when they used IgE-Sepharose for receptor isolation (Kulczycki and Parker, 1979).

Single receptor molecules were also isolated by other groups of investigators. Thus, Kanellopoulos *et al.* (1979), using haptenated IgE and antibodies to the hapten, isolated a surface labelled molecule from RBL cells with an apparent molecular weight of 53,000 daltons, and Isersky *et al.* (1978) purified a molecule of 58,000 daltons from the same cells using IgE-anti-IgE or anti-receptor antibodies. The experiments carried out by Conrad and Froese (1978b) and the data presented in this study also detected only one kind of receptor molecule by these methods of receptor isolation. The molecular weight, however, was significantly lower (∿45,000 daltons). It now appears that these differences in molecular weight can be attributed not to the differences in methodology, but differences in the RBL cell lines maintained in the respective laboratories (Froese *et al.*, to be published).

In addition to H and R receptors of RBL cells, the present study has clearly established the presence of a third molecule with specificity for IgE. This molecule, which was usually detected in fraction 17-19 on 10% gels, was calculated to have an apparent molecular weight of 70,500 (±2,000) daltons, which has been referred to as the 71,000 dalton molecule. It was regularly detected in receptor preparations from surface-iodinated RBL cells. Significantly, its binding to IgE-Sepharose could be inhibited either by adding free IgE to NP-40 extracts of RBL cells or by saturating the receptors on intact RBL cells (Table III). When cells labelled with

^{3}H-leucine were used, the incubation of intact cells with IgE and the subsequent binding of solubilized extracts to IgE-Sepharose resulted in a decrease of the binding of the 71,000 dalton molecule (Fig. 32). Molecules corresponding to H and R receptor were, of course, also inhibited from binding. These results definitely point to the 71,000 dalton molecule as being a receptor for IgE. The fact that blocking exposed intact receptor sites on RBL cells with IgE, followed by washing, eventually prevented the binding of this molecule to IgE-Sepharose, clearly shows that IgE binds to this molecule not only in NP-40 extracts, but also on intact RBL cells. In some of its properties, this 71,000 dalton molecule is similar to R, since it can be isolated by IgE-Sepharose as well as by means of IgE and anti-IgE. Some evidence for this molecule in receptor preparations had already been presented by Conrad and Froese (1978a) and Conrad *et al.* (1979). However, because of its somewhat irregular appearance in receptor preparations, it apparently was not identified as a receptor for IgE.

At present, it is not clear which of the receptors for IgE on RBL cells or RMC is analogous to the receptor for IgE on human basophils. The latter have been described by Hempstead *et al.* (1979). They were isolated by means of IgE-Sepharose and exhibited an apparent molecular weight of 58,000-68,000 daltons.

The studies described in this thesis have also helped to delineate the chemical nature of the receptors for IgE on RBL cells. Thus, further evidence was obtained which clearly showed that all receptor molecules (H, R and the 71,000 dalton molecule) were, in fact, glycoproteins. Thus, ^{3}H-fucose and ^{3}H-galactose could be incorporated into all three molecules (Chapter IV). Also, the protein nature of these molecules was demonstrated by the fact that both ^{3}H-tyrosine and ^{3}H-leucine could be incorporated into molecules migrating with mobilities corresponding to H, R and 71,000 daltons

(Figs. 24 and 30). These molecules could also be prevented from binding to IgE-Sepharose when IgE was added to intact cells (Fig. 32). Others had also shown the incorporation of amino acids and sugar precursors into receptors (Kulczycki *et al.*, 1976; Kulczycki and Parker, 1979), however, these studies differed from those presented here in that only a single receptor molecule was identified. However, on the basis of a recent collaborative study (Froese *et al.*, to be published), it is reasonable to assume that the RBL cells used by Kulczycki *et al.* (1976) and Kulczycki and Parker (1979) carried H- and R-like molecules and that, therefore, the earlier studies (Kulczycki *et al.*, 1976), which used IgE and anti-IgE for receptor isolation, dealt mainly with R, while more recent studies (Kulczycki and Parker, 1979) which made use of IgE-Sepharose for receptor isolation, could have dealt with either H or R or both. These authors never detected a molecule which was similar to the 71,000 dalton molecule described in this thesis.

Identification of the receptors for IgE as proteins was also aided by the fact that they can be iodinated. Moreover, when solubilized, the receptor isolated by means of IgE and anti-IgE can be degraded by trypsin (Conrad and Froese, 1976; Kulczycki *et al.*, 1976) or by pepsin and pronase (Kulczycki *et al.*, 1976). The protein nature of the receptors for IgE was also evident from the observation that they were prone to denaturation. Thus, of a receptor preparation isolated by means of IgE-Sepharose and KSCN elution, only about 15-20% could be reprecipitated by IgE and anti-IgE (Conrad and Froese, 1978a). However, as pointed out by the authors, this value was most likely low since IgE and anti-IgE would only have isolated R receptor. Indeed, when rebinding to IgE-Sepharose was determined in this thesis (Table IV and Fig. 18), more rebinding (30-50%) was observed. Similar observations were made by Kulczycki and Parker (1979), who found that acid elution from IgE-Sepharose yielded a receptor preparation of

which only 33% rebound to IgE-Sepharose. Elution with guanidine was limited to only 21% rebinding, whereas urea or acid-detergent free eluates showed little or no specific rebinding to IgE-Sepharose. This denaturation could be reduced to give a 50% yield by rapidly flowing columns and quick neutralization of eluates (Kulczycki *et al.*, 1979).

The glycoprotein nature of both H and R was also clearly exhibited by binding of various lectins as seen in Chapter III. Binding of receptor to these lectins has suggested the presence of mannose, N-acetylglucosamine and galactose in both H and R receptors, although these specificities were not established more firmly. Only in one case was specificity demonstrated by inhibition of binding to lentil-lectin by α-methyl mannoside. Incorporation studies have confirmed the presence of N-acetylglucosamine (Kulczycki *et al.*, 1976) and galactose (Chapter IV). Lectin binding studies with pea-lectin-Sepharose and gorse-lectin-Sepharose (fucose binding lectins) have suggested that H may not be associated with fucose. However, this was not borne out by incorporation studies (Chapter IV), which clearly showed the presence of this sugar in both H and R recpetors. Thus, fucosyl residues in the H receptor may not be readily accessible to the two lectins. The lectin binding properties of the 71,000 dalton molecule could not be clearly established, primarily because of the small peaks representing this molecule.

The first studies to establish that the receptors for IgE were glycosylated were performed by Bach and co-workers (Bach *et al.*, 1971; Bach and Brashler, 1973). Using intact RMC, they treated the cells with neuraminidase and observed decreased histamine release relative to controls. However, the results cannot be considered as clear-cut evidence that the receptors were glycoproteins with sialic acid in the carbohydrate moiety. The effect could have been indirect, produced by an alteration of the net charges in the cells, or by changes in the mediator release process at the point

which does not involve the receptor.

The lectin binding studies of this thesis have helped to confirm that the two receptors for IgE, H and R, can be separated and are thus most likely not associated with one another in NP-40 extracts. Similar conclusions can be derived from studies with anti-receptor antibodies. One specifically purified antibody preparation reacted only with R (Conrad *et al.*, 1978), while another one reacted primarily with H (Conrad *et al.*, 1979). Therefore, it is unlikely that only R was specific for IgE coupled to Sepharose and H was being isolated because it was associated by non-covalent bonds with R. It is still not fully understood why only R was isolated when IgE and anti-IgE or DNP-IgE and anti-DNP-Sepharose were used. Conrad and Froese (1978a) had suggested that H forms complexes with IgE but is released when the latter reacts with an antibody.

One of the original aims of the biosynthetic incorporation studies carried out during the course of this investigation was to establish the possible association of non-receptor molecules with the receptors for IgE. This search was prompted by the observations of Conrad *et al.* (1976) that IgE-receptor complexes had a molecular weight of $3.5 - 5.0 \times 10^5$ daltons, while that of the receptor as determined by SDS-PAGE analysis on 5% gels was only about 60,000 daltons (Conrad and Froese, 1976). These authors thus speculated that in NP-40 extracts, the receptor may, in fact, be isolated as a "receptor complex" consisting of several identical monomers, which were dissociated in SDS or, alternatively, the receptor may have been associated with an unlabelled molecule(s) which were dissociated by SDS and thus not detected by SDS-PAGE. The likelihood of this large difference in molecular weight being due to solely noniodinated molecules was considerably reduced by the observations that in the presence of NP-40, the molecular weight of the receptor, as measured by density gradient centrifugation or

gel filtration, was of the order of 130,000 - 150,000 daltons (Newman *et al.*, 1967; Conrad and Froese, 1978a). These authors attributed the higher molecular weight of the receptor in NP-40 extracts to be the results of binding of this detergent in the form of large micelles. However, when Newman *et al.* (1977) calculated a hypothetical molecular weight for a detergent-free receptor, they obtained a value of 77,000 daltons. Similarly, Conrad and Froese (1978a) determined a molecular weight of about 65,000 daltons in the presence of SDS, as measured by gel filtration. Thus, the possibility of a small molecule being associated with the receptor was still left open.

Isolation of receptor for IgE by means of IgE-Sepharose from RBL cells labelled with ^{3}H-amino acids yielded two prominent peaks (Fig. 30) in fractions and in fractions 46-48, which had not been observed in receptor preparations from iodinated cells or from cells labelled with ^{3}H-sugars. Of these two molecules, the one appearing in fraction 46-48 was considered to be the more likely candidate for association with the receptor. It has a low molecular weight of 25,500 (±1,500) daltons, which will be referred to as the 26,000 dalton molecule, and would thus yield a complex with R in the order of 71,000 daltons. Moreover, inhibition experiments with IgE (Fig. 32) have suggested, even though only marginally, some inhibition of the binding of this molecule to IgE-Sepharose. Unfortunately, the inhibition data were not clear-cut. Actually, if the 26,000 dalton molecule was associated with R in a 1:1 stoichiometric ratio, equal inhibition of both molecules should have been seen.

Interestingly, the 26,000 dalton molecule was sometimes observed as a major peak when DNP-IgE and anti-DNP-Sepharose were used for receptor isolation (Fig. 28) from ^{3}H-leucine labelled cells. On other occasions, only small amounts of this molecule were detected (Fig. 29). This variation does suggest that possible association of R with the 26,000 dalton molecule may be weak. Thus, factors like the degree of washing anti-DNP affin-

ity columns may determine the amount of the 26,000 dalton molecule isolated. Interestingly, other investigators have also detected a similar molecule in their receptor preparations. Isersky *et al.* (1978) observed a small amount of a molecule of 30,000 daltons in their receptor preparations obtained from iodinated RBL cells. A 30,000 dalton molecule was also demonstrated by Kulczycki and Parker (1979) in their receptor preparations. These authors considered the molecule to be a contaminant, as it was not found in highly purified receptor preparations. The loss of this molecule upon repeated chromatography may once again be indicative of a relatively low affinity for either H or R receptors. The results of Kulczycki and Parker (1979) do not allow a definite conclusion as to whether the major receptor molecule which they describe can be compared to H or R of the present study, since IgE-Sepharose isolates both of these molecules.

The most direct evidence of an association between a receptor for IgE (most likely R) and a smaller molecular weight molecule comes from a recent report presented by Metzger *et al.* (1980). Using a cross-linking agent, these authors could show the association of the receptor and a 30,000 dalton molecular at a molecular ratio of 1:1. Cross-linking of the two molecules could be achieved either on intact cells or in NP-40 extracts. In view of the posssible association of a 30,000 dalton molecule with the receptor (most likely R), it is tempting to speculate that the 71,000 dalton molecule seen in this thesis may actually represent a more stable adduct of these two molecules.

It should also be mentioned that Conrad and Froese (1976) and Froese (1980) observed a low molecular weight molecule in receptor preparations from RMC. On 10% gels, this molecule migrated in fractions 48-50 and it, therefore, seemed to have a molecular weight smaller than the 26,000 dalton molecule found in experiments described in this thesis. On the basis of the data available to date (Froese, 1980), this molecule does not seem to

be the same as the 26,000 dalton molecule described here. Thus, the molecule of RMC origin was bound by immune complexes free of IgE and which did not appear to bind either H or R. Also, the binding of this molecule to IgE-Sepharose could not be inhibited by free IgE, while that of H and R was completely inhibited (Froese, 1980). This molecule was thus considered to be some hetero-Fc receptor reacting with the Fc portion of IgG.

As pointed out earlier, in addition to H, R, the 71,000 and 26,000 dalton molecules, several other ^{3}H-amino acid labelled molecules were isolated in receptor preparations by means of IgE-Sepharose. Many "non-receptor" molecules were also observed by Kulczycki and Parker (1979) in their eluates from IgE-Sepharose of ^{3}H-leucine labelled material. The molecules observed by these authors and in the present study were most likely not of ectomembrane origin, since they were not labelled by ^{125}I nor by tritiated sugars. These molecules may well represent non-specifically bound material of either cytoplasmic or endomembrane origin. Alternatively, some of them may possibly represent cryptic receptors, i.e. receptors destined for incorporation into the plasma membrane but not yet incorporated into it. It should, however, be pointed out that Rossi *et al.* (1977) could not find any evidence for the presence of such cryptic receptors. One possibility for obtaining some information about these molecules might have been to use IgG-Sepharose as a control. However, an ongoing study in this laboratory has shown that rat IgG coupled to Sepharose also binds both H and R receptors, but less effectively than IgE-Sepharose (Kepron and Froese, 1980).

The knowledge about Fcγ receptors on RBL and RMC is still rather limited. Thus, monomeric IgG2a does not bind to RBL cells, while immune complexes involving this immunoglobulin do (Halper and Metzger, 1976). Ishizaka *et al.* (1979) suggested that on human basophils, receptors for IgE and IgG are different molecules. However, data by Kepron and Froese (1980) indicate that on RBL cells the receptor for IgE cross-reacts with IgG.

By contrast, Conrad and Froese (1978a) could not inhibit IgE receptor binding to IgE-Sepharose using rat IgG. Moreover, immune precipitates consisting of heterologous Ig (most likely IgG) did not show any receptor binding (see Figs. 3 and 5) (Conrad and Froese, 1976).

In view of these somewhat contradictory results, IgG-Sepharose was not considered to be an appropriate control for the experiments described in this thesis.

At present, relatively little is known about the function of the various receptor molecules. Only R has so far been implicated in a biological function. Thus, an antiserum which reacts primarily with R (Conrad *et al.*, 1978) was capable of releasing histamine in a non-cytotoxic fashion from RMC (Ishizaka *et al.*, 1977). More recently, the same antibodies were also shown to stimulate phospholipid methylation and Ca^{++} influx in a manner similar to the one induced by anti-IgE (Ishizaka *et al.*, 1980).

Another antiserum (Isersky *et al.*, 1978) was shown to trigger a subline of RBL cells which do release histamine and which had previously been considered to be a mouse mastocytoma (MCT-1). However, the effects of this antiserum on normal rat mast cells were not tested. This antiserum was produced to purified receptors isolated from RBL cells by means of IgE and anti-IgE and should thus have reacted primarily with R receptor; however, a recent study (Froese *et al.*, to be published) suggests that this antiserum reacts better with H than R. Therefore, it is not clear which of the receptor molecules was involved in the release of ^{3}H-serotonin with which the cells had previously been incubated.

A third antiserum which had been directed to receptors isolated from RBL cells by means of IgE-Sepharose and which reacted primarily with H could not trigger histamine release from RMC (Conrad *et al.*, 1979), in spite of the fact that it could react with determinants of H exposed on the RBL cell surface. One possible reason for this failure to trigger normal

RMC may be sought in the fact that RMC appear to carry only relatively few H molecules on their surfaces (Froese, 1980).

One of the most interesting questions to be answered in the future will be whether or not the various receptors for immunoglobulins (Fc receptors) on the diverse cells of the immune system are related. One common feature is that they all appear to be glycoproteins. However, this is not surprising, since many of the surface molecules on cells belong to this category.

It is interesting to note that a subpopulation of B lymphocytes (Wil-2WT) are the source of IgE receptors which, after isolation by means of IgE-Sepharose, demonstrate molecules of 86,000, 47,000 and 23,000 daltons as determined by SDS-PAGE (Meinke *et al.*, 1978). An anti-receptor antiserum was shown to react with the same three molecules. The predominant molecule was the one with an apparent molecular weight of 47,000 daltons which, of course, is very similar to R receptor of RBL cells and RMC. Nevertheless, this finding does not mean that the two molecules are identical. Thus, the affinity of the receptors on (Wil-2WT) cells was shown to be $10^7 M^{-1}$ (Hellstrom *et al.*, 1978), while that of the receptors for IgE on RMC and basophils is in the range of 10^9 - 10^{10} M^{-1} (Ishizaka *et al.*, 1973; Kulczycki and Metzger, 1974; Conrad *et al.*, 1975). In addition, the receptors for IgE from cultured lymphoblastoid cells seem to be more susceptible to enzymatic degradation, since they could only be isolated in the presence of protease inhibitors (Meinke *et al.*, 1978). The presence of protease inhibitors was not needed for the isolation of IgE receptors on RBL or RMC.

In general, it seems that the apparent molecular weights of many Fcγ receptors and those of IgE receptors fall into the range of 40,000 - 65,000 daltons. Thus, Rask *et al.* (1975) isolated molecules of 65,000, 18,000 and 15,000 daltons from murine spleen cells. The two smaller molecular weight molecules were considered to be degradation products of the larger. A

molecule of about 70,000 daltons was also isolated by Wernet and Kunkel (1975). Cooper and Sambray (1976) found a receptor molecule for IgG of about 45,000 daltons to be associated with murine leukemia cells (L1210), and Loube *et al.* (1980) found a 57,000 dalton molecule on macrophage-like cells ($P388D_1$). They also saw two minor membrane components of 28,000 and 24,000 daltons.

A molecule of about 60,000 daltons was observed on human mononuclear cells by Cunningham-Rundles *et al.* (1978). However, a dimer of this molecule (120,000 daltons) was also found.

Larger Fcγ receptor molecules were also found by Frade and Kourlisky (1977), who isolated a molecule of 110,000 daltons from a T cell lymphoma (L-5178-Y). Upon reduction, this yielded molecules with molecular weights of 56,000, 36,000, 25,000, 18,000 and 15,000 daltons. Bourgois *et al.* (1977) also found a molecule of 110,000 - 130,000 daltons on murine B cells, which broke down into smaller units when isolation was carried out in the absence of protease inhibitors. Human B cells also appear to have fairly large receptors of 100,000 daltons (Molenaar *et al.*, 1977).

At present, it appears that receptors for IgE on RBL cells and RMC are not very sensitive to endogenous proteases. However, it remains to be established what the relationship of the 71,000 dalton molecule to H and R receptors is. The addition of protease inhibitors to PBS/NP-40 used for cell solubilization does not appear to affect the relative amounts of H, R and the 71,000 dalton molecules (unpublished observations).

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