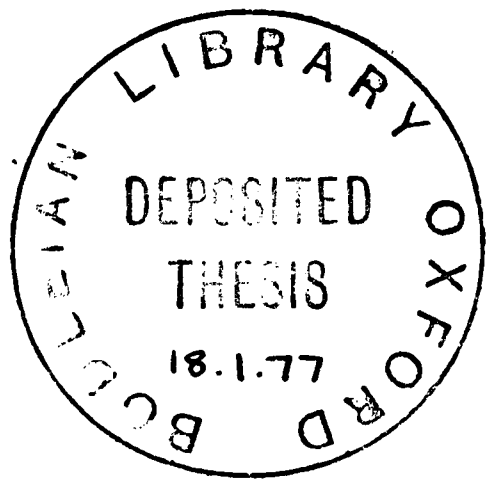
THE PURIFICATION AND CHARACTERISATION OF THE THY-1 DIFFERENTIATION ANTIGEN FROM RAT BRAIN

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ABSTRACT

Many of the specialised functions of the tissues of higher organisms will involve cell-cell interactions mediated by tissue-specific molecules at the cell surface. Very little is known about these interactions but a possible approach is to use antisera to recognise tissue-specific molecules (differentiation antigens) of the cell surface and then to follow the antigenicity in purification studies. The biochemical characterisation of cell surface differentiation antigens should help in understanding the molecular basis of cell-cell interactions.

It was decided to study the Thy-1 antigen because (i) it has a differential tissue distribution being present mainly in thymus and brain of rats and mice, (ii) it is clearly defined and (iii) it is present at the cell surface in amounts suitable to attempt purification from rat thymus or brain. This thesis is concerned mainly with the purification and characterisation of the Thy-1 antigen from rat brain.

As a first step in the purification, the solubilisation of the Thy-1 antigenic determinants was attempted. Effective solubilisation from a crude membrane fraction was obtained with deoxycholate following extraction with the non-ionic detergent lubrol-PX. Studies in deoxycholate by gel filtration and sucrose density gradient centrifugation in H_2O and ^2H_2O showed that the Thy-1 antigenic determinants were associated with a single homogenous component with a $S_{20,w}$ of 2.2S, partial specific volume of 0.72 ml/g and Stokes radius of 3.0 nm. The value for the partial specific volume indicated that the Thy-1 antigenic activity was not associated with large amounts of low density material i.e. lipids and it is typical of a protein or glycoprotein. From these parameters a molecular weight of 27,000 was calculated for Thy-1 plus any bound deoxycholate. The hydrodynamic parameters obtained were very similar to those obtained for thymocyte Thy-1 solubilised in deoxycholate (Letarte-Muirhead et al, 1974).

Further purification involved affinity chromatography on a lentil lectin-Sepharose 4B and gel filtration on Sephadex G-200 in deoxycholate. More than 80% of the solubilised brain Thy-1 was bound by the lentil lectin and could be eluted with methyl α-D-glucopyranoside in deoxycholate and after gel filtration the Thy-1 antigenic activity was present in a fraction containing only one component, a glycoprotein with an apparent molecular weight of 24,000 after electrophoresis on 12.5% polyacrylamide gels in sodium dodecyl sulphate. This glycoprotein was shown to carry the Thy-1 antigenic determinants by a number of criteria including (i) the glycoprotein fraction contained the Thy-1 antigenic determinants at a high specific activity which was near that predicted for the pure antigen, (ii) the antigenic activity electrophoresed on polyacrylamide gels in sodium dodecyl sulphate in an identical manner to the glycoprotein band, and (iii) antisera raised against the purified glycoprotein recognised antigens with the same characteristic tissue distribution as Thy-1.

Thy-1 purified from rat thymocytes (Letarte-Muirhead et al, 1975) was also a glycoprotein of about 25,000 molecular weight but two forms were distinguished and purified; Thy-1_{L+} which bound to lentil lectin and Thy-1_{L-} which did not bind to lentil lectin. The thymocyte and brain forms of Thy-1 could not be distinguished antigenically although small differences in electrophoretic behaviour on polyacrylamide gels in sodium dodecyl sulphate were noted.

In order to facilitate further analysis of brain and thymocyte Thy-1, the deoxycholate was removed by precipitating the glycoproteins with ethanol. The glycoproteins were recovered in a water soluble form without loss of antigenic activity. Equilibrium ultracentrifugation studies showed that Thy-1 was present in an aggregated form in the absence of deoxycholate with weight average molecular weights in the range 230,000-370,000. Addition of deoxycholate caused dissociation into

the monomeric form with a molecular weight of 29,000 (including bound deoxycholate).

Chemical analysis of brain Thy-1 and thymocyte Thy-1_{L+} and Thy-1_{L-} showed that their amino acid compositions were virtually indistinguishable but differences were observed in the carbohydrate compositions. These differences were substantial between brain and thymocyte Thy-1 but much smaller between thymocyte Thy-1_{L+} and Thy-1_{L-}. The differences in carbohydrate composition between brain and thymocyte Thy-1 were very unusual and could result from the polypeptide chain of Thy-1 carrying different carbohydrate structures in thymus and brain. All three forms contained about 30% by weight of carbohydrate.

The nature of the antigenicity of Thy-1 was investigated by exposing the purified preparations to treatments which should destroy antigenic determinants dependent on the protein conformation but not affect carbohydrate determinants, viz. heating, reduction and alkylation, or proteolytic digestion. Each of these treatments destroyed all the antigenic determinants defined on the Thy-1 molecule and thus they are almost certainly present on the protein part of the molecule.

With regard to the function of Thy-1 it is likely that the molecule has a role in the tissues where it is abundant, i.e. thymus and brain. Although the chemical analysis does not provide a simple explanation of the function it does set some limitations. Thus, if the specific function of Thy-1 is mediated by the polypeptide chain then presumably the function is the same in thymus and brain and the differences in carbohydrate are simply due to the different glycosyl transferases present in the two tissues. Alternatively the carbohydrate could mediate the specific functions of Thy-1 and in this case the polypeptide chain would serve as a backbone to display carbohydrate structures and to anchor the molecule into the membrane. In view of the molecular characteristics of Thy-1 and in particular the presence of large amounts

of carbohydrate which is presumably present at the cell surface, the latter alternative is more attractive. Thus the carbohydrate structures of Thy-1 could act as ligands for cell surface glycosyl transferases or lectins which are currently being implicated in cell-cell interactions. In this case the differences in carbohydrate composition between brain and thymocyte Thy-1 could reflect differences in the specificity of recognition in brain and thymus.

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*Letarte-Muirhead, M., Barclay, A.N. & Williams, A.F. (1975), Purification of the Thy-1 Molecule, a Major Cell-Surface Glycoprotein of Rat Thymocytes, Biochem. J. 151, 685-697.

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*Copies of these publications are included at the end of this thesis.

ABBREVIATIONS

B-lymphocytes	Lymphocytes derived from the bursa or its equivalent in mammals
BBSS	Balanced buffered salt solution
BSA	Bovine serum albumin
SDS	Sodium dodecyl sulphate
S.E.M.	Standard error of the mean
T-lymphocytes	Lymphocytes derived from the thymus
$Thy\text{-}1_{L+}$	Thymocyte Thy-1 which binds to lentil lectin
$Thy\text{-}1_{L-}$	Thymocyte Thy-1 which does not bind to lentil lectin (Letarte-Muirhead et al, 1975)

Other abbreviations used are standard see Biochem. J. (1975) 145, 9-20.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

A characteristic of higher organisms is the organisation of tissues with specialised functions. Many of these functions will involve cell-cell interactions mediated by membrane molecules which are probably specific to the tissue. The determination of the molecular and biochemical properties of these membrane molecules is essential in understanding interactions involving the cell surface. Furthermore, if these molecules are purified, strong specific antisera can be raised and together with the purified molecule, used as reagents to investigate aspects of their functions.

Conventional biochemical analysis of membrane molecules is difficult because of the large number of different components present in the membrane and the difficulty in identifying the cell surface molecules which may be of interest. In this regard a serological approach in which antisera are used to recognise cell surface molecules has certain advantages; with this approach it is possible to (i) study only molecules which are at the cell surface by using intact cells in assays for the antigens, (ii) define cell surface molecules which are tissue specific i.e. differentiation antigens and (iii) follow the antigenicity of the differentiation antigen in conventional purification studies on the antigen.

When studies for this thesis were commenced, no membrane molecule had been purified by the above procedures. Thus it was important to select a differentiation antigen which was suitable to develop the necessary techniques to purify it. In the laboratory of A.F. Williams

it was decided to study the Thy-1 antigen in the rat because (a) it was substantially tissue specific being present mainly in thymus and brain, (b) it was present at the cell surface, (c) preliminary evidence suggested that it was present in large amounts in these tissues and (d) the antigen is clearly defined and specific antisera are easily produced. Thus the Thy-1 antigen appeared to be a suitable molecule to develop the techniques to purify and characterise membrane differentiation antigens and it may have interesting functions in thymus and brain. Thus attempts were made to purify the Thy-1 antigen from both rat thymus and brain and this thesis is concerned mainly with the purification and characterisation of the molecule from brain. The Thy-1 antigen, purified from thymocytes in parallel studies, was also analysed and the results compared to those obtained for brain Thy-1. The results are also discussed with respect to the function of Thy-1 in brain and lymphoid cells.

This introductory chapter describes some aspects of lymphoid and brain function which involve interactions at the cell surface together with methods to analyse membrane molecules which may mediate these interactions. The identification of differentiation antigens is described with examples from both lymphoid and brain tissues and the properties of the Thy-1 antigen are discussed in more detail.

1.2 The role of the cell surface in the lymphoid system and brain

1.2.1.1 The lymphoid system

In higher vertebrates the lymphoid system consists of a number of discrete organs e.g. thymus, spleen, lymph nodes, Peyer's patches, tonsils and appendix, which contain mainly lymphocytes in a network of reticular cells (reviewed in Owen, 1973). Small lymphocytes are a key cell type in this system since they are responsible for the initial recognition of antigen (Gowans & McGregor, 1965). The interaction

between antigen and small lymphocytes causes these cells to differentiate and divide to give the cells that mediate the immune response (see below). In many cases other cells which lack specific antigen recognition, for example macrophages, may be involved in the immune response in conjunction with the lymphocytes. A characteristic of the lymphoid system is that the lymphocytes do not remain within a single organ but recirculate rapidly via the bloodstream to be reabsorbed into various lymphoid organs (reviewed in Gowans, 1970; Ford, 1975).

1.2.1.2 B-lymphocytes and T-lymphocytes

Two distinct types of immune response have been distinguished; the humoral response leading to the production of circulating antibodies directed against the stimulating antigen and the cell mediated response where effector cells interact directly with the foreign antigen e.g. in delayed hypersensitivity reactions. It is generally accepted that these responses are mediated by two classes of lymphocytes; B-lymphocytes (derived from the bursa or its functional equivalent in mammals; Cooper & Lawton, 1972) and T-lymphocytes (derived from the thymus). Fig. 1.1 shows diagramatically the development of the two classes of lymphocytes from precursors in the bone marrow. On antigenic stimulation the B-lymphocyte differentiates and divides to give antibody producing plasma cells and T-lymphocytes differentiate and divide to give effector cells for cell-mediated immune reactions (reviewed in Greaves et al, 1973; Ford, 1973; Cantor & Weissman, 1976).

The B- and T-lymphocytes make up the majority if not all of the class of small lymphocytes and are morphologically indistinguishable. However B-lymphocytes are characterised by the presence of large amounts of cell surface immunoglobulin which T-lymphocytes lack, and in the mouse, the Thy-1 antigen (formerly called theta, (Θ)) is a valuable marker for T-lymphocytes and their precursor cells (Raff,1971). The distribution and characterisation of the Thy-1 antigen is described

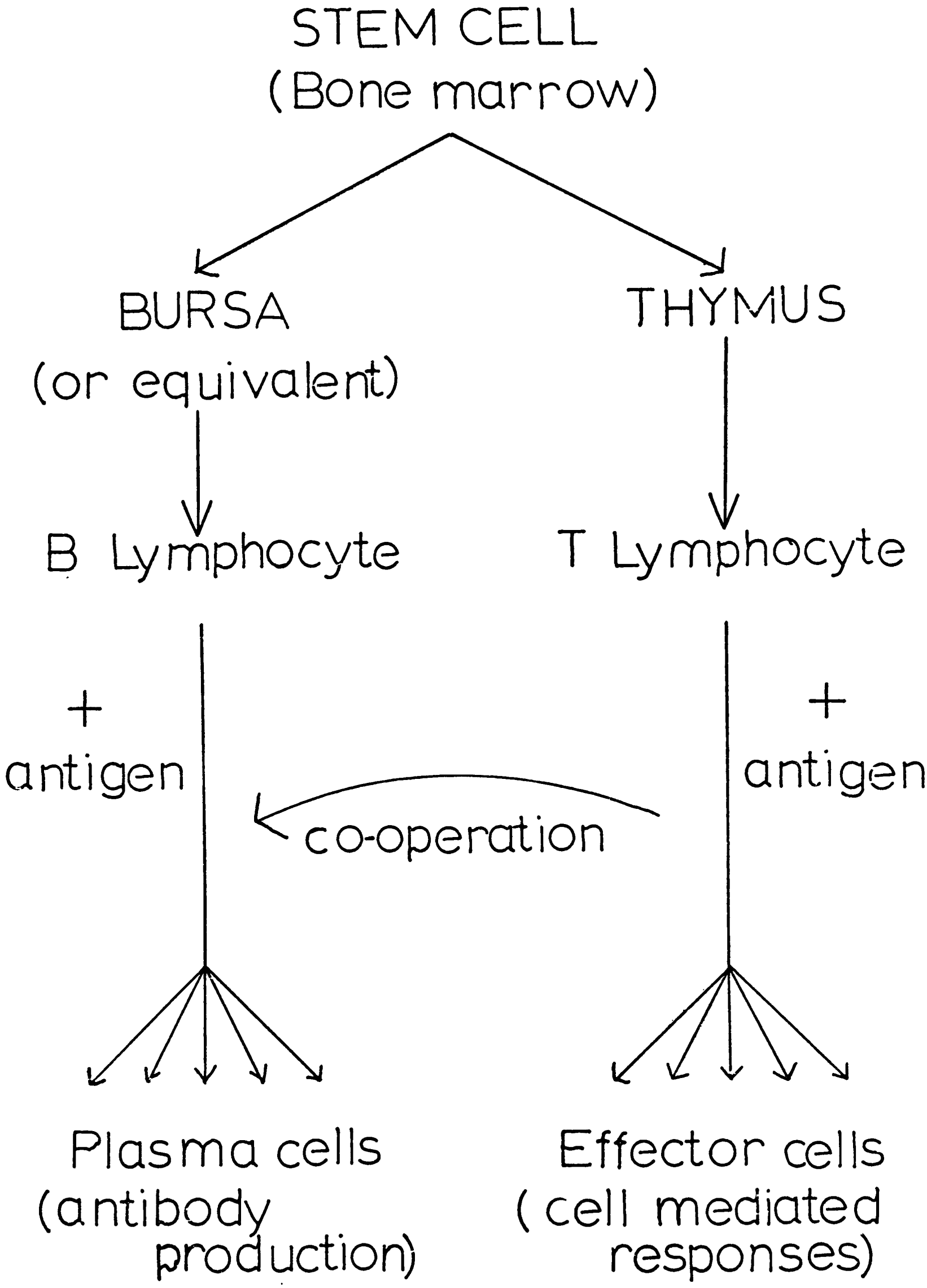


Fig. 1.1 Scheme of the pathways of lymphocyte differentiation

below (Ch. 1.4.6).

1.2.1.3 The reaction of lymphocytes to antigen

Although macrophages have been shown to bind antigen, only lymphocytes have the ability to bind antigen specifically (Nossal & Ada, 1971). The antigen receptor on B-lymphocytes is generally accepted to be immunoglobulin present at the cell surface (reviewed in Warner, 1974) but the nature of the antigen receptor of T-lymphocytes has been the subject of much controversy and is not conventional immunoglobulin (Paul, 1973; Hunt & Williams, 1974; Binz & Wigzell, 1975; Hämmerling et al, 1976). Both B- and T-lymphocytes can, upon suitable antigenic stimulation be induced to divide and differentiate, but as shown in Fig. 1.1, there is a requirement for the co-operation of T-lymphocytes in many B-lymphocyte responses to antigen (Miller & Mitchell, 1969; Davies, 1969). The nature of this T-lymphocyte B-lymphocyte co-operation is unclear with suggestions that there is direct contact between the B- and T-lymphocytes, or an indirect contact via another cell type or via soluble factors (Miller & Mitchell, 1968; Mitchison, 1971; Taussig,1974).

The B- and T-lymphocyte classes can be further subdivided in a number of ways; thus types of B-lymphocytes express a variety of different classes and subclasses of immunoglobulin with different functional capabilities (Warner, 1974; Vitetta & Uhr, 1975) e.g. in the class of immunoglobulin on memory cells (Mason, 1976; Abney et al, 1976). Subclasses of T-lymphocytes can be distinguished by their surface markers and other properties and there is evidence for interactions of these subclasses which may result in synergistic or antagonistic effects (Gershon, 1974; Canton & Weissman, 1976). Despite these complications the central and specific role that B- and T-lymphocytes play in the antibody and cell mediated responses to antigen is reasonably clear. The strength and nature of this response is likely to be modified specifically by recirculating antibodies (Fitch, 1975) and non-specifically

by other factors e.g. lymphokines (Morley et al, 1973) and other cell types e.g. macrophages (reviewed in Nossal & Ada, 1971).

1.2.1.4 Migration of lymphocytes

Both the B and T classes of lymphocytes recirculate rapidly between the bloodstream and the lymphoid system (see above) and presumably this maximises the opportunities for lymphocytes to recognise foreign antigens. In the recirculation both B- and T-lymphocytes enter the lymph nodes by the same post capillary venules but then they migrate to specific regions of the lymph nodes and spleen - the so-called B- and T-areas (Parrott & de Sousa, 1971; Ford, 1975). Stimulation with particulate antigen at a lymph node causes a rapid recruitment of lymphocytes, specific for the antigen, at the node (Rowley et al, 1972). This is followed by differentiation of the T- and B-lymphocytes into large activated blast cells which can then be released and migrate to other sites, e.g. IgA bearing blast cells present in the thoracic duct have been shown to migrate mainly to the small intestine with smaller numbers going to the spleen and bone marrow (Williams & Gowans, 1975; Pierce & Gowans, 1975; Ford, 1975).

1.2.1.5 The role of the cell surface in the lymphoid system

The above outline of the lymphoid system indicates many areas where specific reactions are likely to be mediated by the cell surface. For instance in the development of the B and T classes of lymphocytes, in their recirculation and migration to specific areas of the lymphoid organs, the interactions of B- and T-lymphocytes with antigen, the co-operation between B- and T-lymphocytes in antibody responses, the interactions of lymphocytes with other cell types, and the migration of the activated B- and T-lymphocytes. Of all these interactions only that between the immunoglobulin receptor of B-lymphocytes and antigen is well established (Warner, 1974) and even this cell bound immunoglobulin

has not been purified. There is also much controversy about the role of the different classes of immunoglobulin present on populations of B-lymphocytes (see Vitetta & Uhr, 1975; Mason, 1976).

1.2.2.1 The Cell types of the brain

The complex network of cells and their multiple intercellular connections which characterise this organ also hinders the definition and study of the constituent cell types. However two distinct classes of brain cells can be made; the neurons and the neuroglia (reviewed in Johnston & Roots, 1972).

The neurons are characterized by their direct involvement in the initiation and conduction of nerve impulses (see below). The cells consist of a cell body and a number of processes, the dendrites and axons. These cells are not homogenous and the arrangement of the processes can be quite characteristic for a given type of neuron e.g. the Purkinje cells have an elaborate system of dendrites which arise in one plane and are directed towards the outer surface of the cerebellum and are quite different from the granule cells, which usually have only four processes and are also present in the cerebellum. The neuroglia have not been the subject of such extensive study as neurons whose role in the transmission of electrical signals has attracted interest. However the neuroglia are very numerous and may outnumber neurons by 10:1 in some regions of the human brain. Two classes of neuroglia have been described, the macroglia of ectodermal origin and the microglia of mesodermal origin. The macroglia can be divided into astroglia and oligodendroglia and further categories can be distinguished morphologically. The function of the neuroglia is not known although the oligodendroglia have been shown to be involved in myelination of the central nervous system (Peters, 1960); the role of neuroglia relative to neurons is discussed below.

1.2.2.2 Neuron-neuron inter-relationships

The processes of neurons make contact with other neurons at a specialised contact site, the synapse (reviewed in Pappas & Purpura, 1972). This is the site where electrical signals are transmitted between neurons via a chemical transmitter and synapses are also characterised by common morphological features e.g. a narrow cleft - the synaptic cleft - between the neurons prevents direct electrical contact, and is surrounded by the presynaptic and post-synaptic densities of the two neurons in contact (see Fig. 1.2). The method of transfer of the electrical signal involves the release of the chemical neurotransmitter from synaptic vesicles in the presynaptic area, into the cleft. Here they react with specific receptors on the post synaptic membrane and may lead to the reinitiation or inhibition of electrical impulses in this neuron; (the mechanisms involved are discussed in Hall, 1972; Katz, 1966).

Despite this common strucutral feature, considerable differences can be detected in the synapses, in their morphology as shown by electron microscopy, and biochemically from studies on their neurotransmitters. Thus synapses can be categorised further on account of the nature of all its elements (viz. size and shape of the presynaptic and post synaptic densities, the synaptic cleft, and the synaptic vesicles (see Palay & Chan-Palay, 1976)), and by the neurotransmitter present at the presynaptic site. More than 15 putative neurotransmitters have been proposed, some causing excitatory effects at the post synaptic membrane, e.g. acetylcholine, dopamine, and other inhibitory effects e.g. gamma-aminobutyric acid, glycine (Agranoff, 1975; Snyder & Bennett, 1976; Hughes et al, 1975). However each neuron is presumed to contain only one type of neurotransmitter (Dale, 1935). The complex and characteristic organisation of the various types of neurons necessitates highly specific mechanisms to ensure that only the "correct" connections are made during the development of the brain (see below).

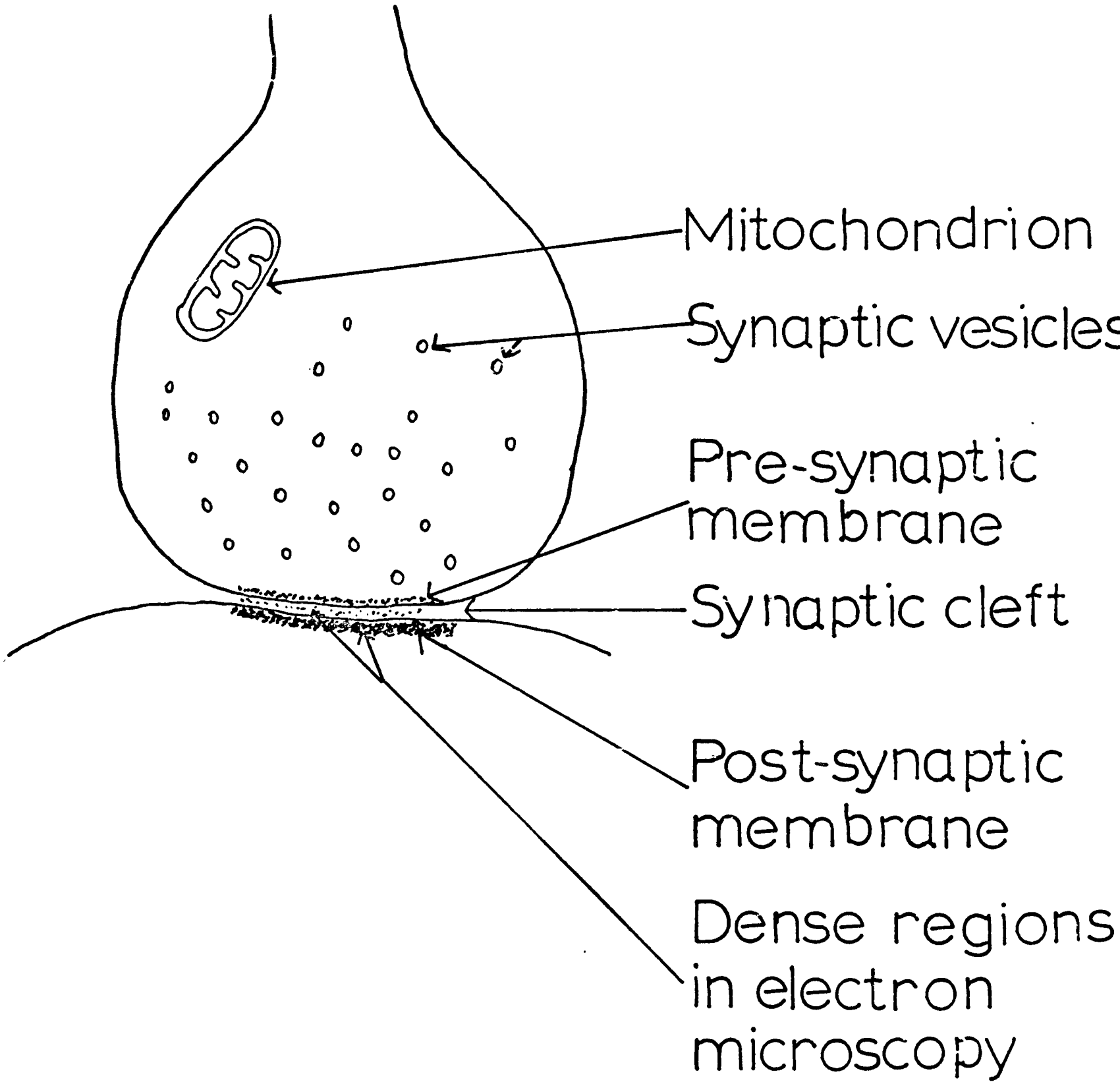


Fig. 1.2 The synapse - a diagramatic representation

1.2.2.3 Neuron-neuroglia inter-relationships

The positioning of the neuroglia both between and around the neurons implied that these cells might have some supportive role in the function of the neurons e.g. in regulating the metabolites for the neurons (reviewed in Watson, 1976; Somjen, 1975). However the neuroglia have been shown to have electrophysiological properties of their own and it has been suggested that these cells may modulate the normal neuronal excitation patterns (Galambos, 1971; Schrier & Thompson, 1974; Somjen, 1975). However the role of the neuronal-neuroglial interaction is not well established although the properties of the neuroglia suggest that they may play an important role in brain function.

1.2.2.4 The development of the brain

The development of the brain must provide the correct inter-neuronal contacts and arrangement of the various types of neurons and neuroglia so far distinguished and outlined above. The highly integrated structure of nervous tissue prevents the application of many techniques but studies by light microscopy, electron microscopy and autoradiography have shown that the basic features of brain development are comparable to the genesis of other organs and involve aspects of cellular proliferation, migration, differentiation and death (Angevine, 1970; Prestige, 1970). The early stage of development is dominated by cell division and then migration from the ventricular zone. In general, as these cells differentiate, more cells migrate through these differentiating cells and so on to give a band of cells with the older ones at the inside. This migration pattern is believed to depend on the interactions with adjacent cells. These may by epithelial cells, other neurons or neuroglia (reviewed in Sidman & Rakic, 1973; Sidman, 1974).

Evidence for cell surface interactions has been obtained from studies on behavioural mutants of mice which have distinctive malformations in the structural organisation of the cerebellum or cerebrum

(reviewed in Sidman, 1972), and also from the reaggregation of dissociated neuronal tissues in a number of different systems (Roth et al, 1971; Garber & Moscona, 1972; De Long & Sidman, 1970; Gottlieb & Glaser, 1975). Programmed cell death is a recognised cellular event in organ genesis (Glücksmann, 1951) and could be important in regulating the cell numbers, in brain development in addition to genetic controls. It could also effect the patterns of cell aggregation and connectivity (reviewed in Prestige 1970; 1974).

Thus the development of the brain is characterised by complex cell interactions as the cells differentiate and migrate to reach their final positions and make their specific interconnections that characterise the mature brain.

1.2.2.5 The role of the cell surface in the brain

Cell-cell contact is likely to be very important in the developing brain and involve several cell types. This development could be modified by soluble factors analogous to nerve growth factor in the peripheral nervous system (reviewed in Levi-Montalcini, 1966), but the variety of organisational changes occurring within small regions suggests that the specificity of most of the interactions lies in cell-cell interactions. In the mature brain cell-cell contact must maintain the integrity and function of the synapses with the likelihood that the glial-neuronal interaction may also be of importance in brain function.

1.2.3 Conclusion

The above outline indicates that the lymphoid and nervous systems require many specific cell surface interactions both in the development and organisation of their respective cell types and in effecting their functions. The molecular basis of the majority of these interactions is not understood and the complexity of cellular organisation in the brain makes these reactions more difficult to study than in the lymphoid system.

1.3 Plasma membranes - structure, function and analysis

1.3.1 Introduction

There are several different types of membranes in the cells of higher organisms which mediate specific functions for the cell e.g. endoplasmic reticulum, lysozomal, mitochondrial, nuclear and plasma membranes. However it is the plasma membrane which mediates the cell interactions about which this thesis is concerned and this section describes the structure of these membranes, how they medicte their functions and methods to characterise their components.

1.3.2 The structure of plasma membranes

Plasma membranes consist of three major constituents, protein, lipid and carbohydrate. A typical composition is 50:45:5 percentage by weight of protein:lipid:carbohydrate as found for rat thymocyte plasma membranes (Misra et al, 1975). The main exception is myelin, (which is also an atypical membrane in other ways), which contains an exceptionally high lipid content, and has a protein:lipid:carbohydrate ratio of 18:79:3 (Guidotti, 1972). The carbohydrate present in membranes is invariably covalently bound to either proteins or lipids to give glycoproteins and glycolipids respectively. Staining techniques and chemical modification studies have shown that the carbohydrate is present at the outside of the plasma membrane, viz. on the outer surface of the cell (reviewed in Steck, 1974; Hughes, 1976).

The generally accepted model for the arrangement of these components is the fluid mosaic model of Singer & Nicolson (1972). In this model the lipids are arranged in a bilayer with the hydrophilic groups at the outside and the hydrophobic sidechains at the inside of the bilayer; this lipid bilayer accounts for the general impermeability of membranes to ions and water soluble molecules. Two classes of protein

(or glycoprotein) are distinguished; peripheral proteins which can be released from the membrane by relatively mild conditions such as high ionic strength or changes in pH; and integral proteins which require the use of organic solvents or detergents to release them from the membrane. Thus the peripheral proteins are only loosely bound by predominantly ionic interactions whereas the integral proteins are anchored into the lipid bilayer by means of a hydrophobic region in the protein i.e. these are amphipathic molecules and some can traverse the whole of the lipid bilayer, and be exposed to the aqueous environment at each side of the membrane. There is now considerable evidence for this type of molecule (Steck, 1974; Tomita & Marchesi, 1975).

As the name, "fluid mosaic model" suggests, an important aspect of this model is the fluidity of the membrane which allows the lateral movement of its components. The amphipathic structure of integral proteins, which precludes rotation across the bilayer on thermodynamic grounds, facilitates lateral movement as shown for histocompatibility antigens in cell fusion experiments (Frye & Edidin, 1970) and for other molecules by the phenomena of antigenic modulation and capping (Boyse et al, 1967; Taylor et al, 1971). Studies with fluorescent labelled lipids indicated that the lipids can diffuse rapidly within each layer of the lipid bilayer but not between layers (Kornberg & McConnell, 1971a; 1971b).

1.3.3 Function of plasma membranes

The plasma membrane is vital both in maintaining the integrity and metabolism of the cell, and in the cell interactions of particular interest to this study. The latter could be mediated by a number of possible mechanisms:

a) direct uptake onto the cell (for soluble effectors)

b) binding of effector or cell leading to altered function of receptor - as shown for many hormone interactions (reviewed in Cuatrecasas, 1974)

c) rearrangement of surface components leading to pinocytosis (Taylor et al, 1971) or transmission of the effect via alteration of cytoplasmic molecules linked to membrane components (reviewed in Nicolson, 1976)

d) modification of the membrane component so as to alter its function e.g. reaction with other cells as proposed for glycosyl transferase modification of cell surface glycoproteins (Roth et al, 1971 and Ch. 7).

Glycoproteins, proteins and glycolipids are all exposed at the cell surface and are possible candidates for specific cell interactions. The range of specificities required for this type of interaction (see Ch. 1.2) suggests that the proteins or glycoproteins are more likely to mediate these reactions than the glycolipids which have a much more limited range of possible structures. The evidence concerning the molecular interactions which mediate cell interactions is discussed further in Ch. 7.

1.3.4 Biochemical analysis of membrane components

It is clear from the examples given in Ch. 1.2 that specific interactions at the cell surface are vital for the development and function of tissues and that these interactions are likely to be complex. In general there is no simple specific assay for the individual components which mediate these effects but there are a number of possible approaches to characterise these molecules biochemically e.g.

(i) purify and characterise many membrane components and test them or antisera to them in functional tests e.g. cell aggregation assays.

(ii) select a particular class of components which might be involved e.g. glycoproteins and study them as in (i).

(iii) use the target cell as an affinity ligand to purify solubilised components of other cell surfaces which bind to this cell.

(iv) use antigenicity as a marker to select likely molecules, such as tissue specific cell surface molecules, and to follow the antigenicity in the purification of the molecules (see below).

A large number of functions are mediated by the cell surface and methods (i) and (ii) do not provide any means of selecting for those molecules which are involved in the specific cell-cell interactions. However these approaches have yielded the characterisation of some membrane components e.g. the major glycoprotein and "band 3" of human erythrocytes but the nature of their functions is unknown (Marchesi et al, 1972; Yu & Steck, 1975a). Method (iii) is theoretically possible if one could obtain fixed target cells without loss of binding activity and non-specific binding could be minimised. However only those reactions where the binding of the cell interaction was stable, would be detected i.e. all reactions involving enzymatic modification would not be followed. Method (iv) would seem to be the most suitable approach and as outlined in Ch.1.1, this approach via the identification and purification of differentiation antigens is that used in this thesis. The types of molecules that are likely to be detected by this method are discussed below (Ch. 1.4.1).

1.3.5 Preparation and analysis of plasma membranes

A number of different methods have been used to prepare plasma membranes from a variety of tissues. Firstly the cell membrane is disrupted e.g. by freezing and thawing, hypotonic lysis, homogenisation, non-ionic detergents, nitrogen cavitation or by mechanical disruption (reviewed in Hughes, 1976). The membrane is separated from the nuclei and soluble components by differential centrifugation. The plasma membranes can then be separated from other membranes, such as endoplasmic reticulum, by density gradient centrifugation e.g. for rat lymphocyte

membranes (Misra et al, 1975) and brain synoptosomal membranes (Whittaker, 1964). The purity of the membrane is generally estimated by the level of various marker enzymes for the different types of membrane and by electron microscopy. However the resultant membranes are only of limited purity, and this could be important if a component is found in low yield i.e. it could be either a major component of a contaminant or a minor component of the plasma membrane. Erythrocytes from some species are exceptional in that they lack all membranes other than the plasma membrane which can then be obtained with a high degree of purity.

The technique of polyacrylamide gel electrophoresis in sodium dodecyl sulphate has been widely used to characterise membrane proteins and glycoproteins in terms of their electrophoretic mobilities (Fairbanks et al, 1971), e.g. it has been used for comparing membrane fractions for contaminants (e.g. for brain membranes, Banker et al, 1974) and for comparing membrane components between species (Hamaguchi & Cleve, 1972; Schmidt-Ullrich et al, 1974) or within a species (Neville & Glossmann, 1971); it can also give some indication of proteolysis which can be caused by endogenous enzymes in the membrane (Glossmann & Neville, 1971). This technique can be used on a preparative scale but it has the disadvantage that denaturing conditions are used. However it is a valuable analytical technique.

There are two ways in which one can select for molecules at the cell surface (a) by following antigens present on whole cells or (b) by labelling studies on intact (viable) cells. The approach via antigenicity is that used in this thesis. This is achieved simply by using intact cells as the target cells in the binding assays for quantitating antigens (see Ch. 1.4). If the antigen is present inside the cell as well as at the surface, this will be revealed by comparing the amount of antigen present on intact cells and on cells after lysis.

The alternative approach is to selectively label the components of the cell surface using intact cells and a reagent which will not

enter the cell itself. The most commonly used method is to iodinate the proteins with ^{125}I, using the enzyme lactoperoxidase to provide the oxidation conditions (Phillips & Morrison, 1971; Marchalonis et al, 1971). However the labelling is not proportional to the number of molecules present as it will depend on the number of tyrosine residues available for iodination (Phillips & Morrison, 1973). Furthermore the presence of a significant number of dead cells could lead to appreciable labelling of internal membranes and cytoplasmic components (Marchalonis et al, 1971; Schmidt-Ullrich et al, 1974). Other labelling methods can also be used e.g. galactose oxidase to incorporate ^{3}H into galactose residues at the cell surface (Gahmberg & Hakomori, 1973; reviewed in Carraway, 1975).

A widely used technique is that of immunoprecipitation of radioactively labelled surface molecules. The surface membrane is labelled as above or by biosynthetic labelling, solubilised in detergent, reacted with the specific antisera of interest and the immunocomplexes precipitated are analysed by electrophoresis on polyacrylamide gels (Marchalonis et al, 1971; Nathenson & Cullen, 1974). The information obtainable is limited to the characteristics of a band on a gel. No quantitative data can be obtained because of the nature of the labelling procedure and because the technique is dependent on the solubilisation of the antigen for which there is no assay. Thus it is not surprising that variable results are obtained and are well illustrated with those obtained for Thy-1 (discussed in Ch. 7). However this method has been of value where good specific reagents and suitable controls are available. For example Vitetta et al (1971) were able to show that the surface IgM of murine lymphocytes was in a monomeric form rather than the pentameric form found in serum. Recently this technique has been used to separate small amounts of internally labelled histocompatibility antigens from which some amino acid sequence data has been obtained (Silver & Hood, 1975; Vitetta et al, 1976; Ewenstein et al, 1976).

1.3.6 Purification of membrane components

Solubilisation. The components of the plasma membrane most likely to be involved in the mediation of cell interactions are proteins and glycoproteins (see Ch. 1.3.3). The first problem in attempting to purify and characterise these components is to obtain them in a solubilised form, separated from the lipids, and suitable for further purification. Many methods have been used to solubilise membranes (reviewed in Hughes, 1976) and include: organic solvents (e.g. butanol) chaotropic reagents (e.g. potassium thiocyanate, lithium di-iodo salicylate), limited proteolysis or autolysis, (e.g. with papain), extremes of ionic strength, ionic detergents (e.g. sodium dodecyl sulphate), non-ionic detergents (e.g. Triton X-100, NP-40) or weakly ionic detergents (e.g. deoxycholate). In studies where antigenicity is being used to follow the membrane protein it is important to use non-denaturing conditions of solubilisation. For this the most suitable reagents for obtaining the intact molecule are the non-ionic or weakly ionic detergents. These mild conditions would also permit the use of affinity chromatography techniques.

Criteria for solubilisation. The first indication that a molecule may be solubilised is if it is not sedimented following high speed centrifugation. The molecular parameters can then be determined by gel filtration and sucrose density gradient centrifugation. These techniques should indicate whether multiple forms of the molecule exist and the approximate size of the solubilised molecule (or complex). However when membrane proteins are solubilised by detergents they tend to bind considerable amounts of detergent (up to 1.2 x their own weight for non-ionic detergents, Clarke, 1975; Helenius & Simons, 1975). As non-ionic detergents have a low density ($\bar{v}$ = 0.9 - 1.1 ml/g; Helenius & Simons, 1975) the sedimentation parameters of molecules solubilised in these detergents can

be affected and it is important to estimate the partial specific volume of the complex e.g. by sedimentation on sucrose density gradients in H_2O and ^2H_2O (Meunier et al, 1972 and discussed in Ch. 3). Membrane components solubilised by bile salts (weakly ionic detergents) are not affected in the same way because they have higher densities than the non-ionic detergents e.g. deoxycholate has a $\bar{v}$ = 0.76 ml/g (Small, 1968).

Methods of separation. Many conventional biochemical techniques can be carried out in the presence of detergents e.g. gel filtration, ammonium sulphate precipitation, velocity and equilibrium ultracentrifugation, affinity chromatography, electrophoresis, etc. Problems may be encountered with particular detergents as indicated above for non-ionic detergents. Also deoxycholate must be used at pH's greater than 7.6 to prevent precipitation of the acid form and also in conditions of low ionic strength to prevent gelling. However detergents would appear to be the best general approach to the purification of membrane proteins and glycoproteins in conditions where their antigenic activity and hopefully biological activity are not lost.

1.4 Differentiation antigens of the cell surface

1.4.1 Introduction

Antigens are molecules which can be recognised by the reaction of antibodies to antigenic determinants present on these molecules. Those antigens expressed on one or a small number of tissues are termed differentiation antigens to distinguish them from those present on many tissues, as for example the histocompatibility antigens. One can select for differentiation antigens of the cell surface by serological analysis of intact cells (see below). In this case one is most likely to be recognising proteins or glycoproteins and to a lesser extent glycolipids of the plasma membrane (see Ch. 1.3) as proteins and carbohydrates are generally more immunogenic than lipids. As discussed in

Ch. 1.1 these tissue specific cell surface molecules are suitable candidates for mediating the tissue specific cell interactions pertinent to this study.

This section describes the recognition of cell surface differentiation antigens with examples from both the lymphoid and nervous systems and the Thy-1 differentiation antigen is discussed in more detail.

1.4.2 Definition of cell surface differentiation antigens

Two main categories of differentiation antigens can be distinguished: alloantigens recognised by alloantisera raised between different strains in the same species and xenoantigens recognised by xenoantisera raised between different species. The alloantisera only detect those polymorphisms between strains which result in antigenic differences and as such are less complex than xenoantisera, which can detect the many differences that have arisen between the divergence of the species used in the immunisations. The alloantigens have the advantage that they can be defined genetically and by selective breeding, congenic strains which differ only in the polymorphism of interest can be obtained (e.g. for Thy-1, John et al, 1972). Some simplification of xenoantisera is possible if the immunogen contains a limited number of components and if the resulting antisera is absorbed extensively with other tissues (e.g. Morris & Williams, 1975). The antigens recognised can be further defined by the hydrodynamic properties of the solubilised antigen (Morris et al, 1975) or by the characteristics of a precipitin arc obtained by crossed immunoelectrophoresis in the presence of detergents which permits the study of membrane molecules (Jorgensen & Bock, 1974; Laurell, 1965). However the only clear method of defining xenoantigens is to use the purified antigen to raise the xenoantisera, and then use the tissue distribution of the antigens recognised as a confirmation of the specificity.

1.4.3 Recognition of cell surface differentiation antigens

A number of different techniques have been used to recognise cell surface antigens and these include cytotoxicity assays, indirect antibody binding assays and immunoprecipitation techniques in the presence of detergents. The most versatile and quantitative assay for cell surface antigens is the indirect antibody binding assay and its advantages are discussed fully in Morris & Williams (1975). This is the type of assay used throughout this thesis and the basic principles are outlined in Fig. 1.3. Target cells bearing the antigen of interest ("X" in Fig. 1.3) are incubated with antisera which recognises that antigen. The cells are washed to remove unbound antibodies and reacted with ^{125}I-labelled anti-immunoglobulin antibodies. After washing the target cell-antibody-^{125}I-anti-(immunoglobulin) complex, the amount of ^{125}I-anti-(immunoglobulin) bound is quantitated in a gamma counter. This will be proportional to the amount of the first antisera bound specifically to the target cell. The amount of antigen in other tissues or extracts can be measured by incubating the specific (first) antisera with the tissue, removing the tissue-antibody complex by centrifugation and quantitating the residual antibodies as before (see Fig. 1.3). If the target cells are fixed with glutaraldehyde, antigens can be assayed in the presence of detergents. Thus one can follow membrane antigens after solubilisation with detergents without the necessity of removing the detergent.

The indirect antibody binding assay can also be used to estimate the number of antigenic sites per cell or the amount of specific antibody in an antiserum by altering the conditions of the assay as outlined in Table 1.1. The distribution of a cell surface antigen within a population of cells can be followed by autoradiography (see Fig. 1.3). Alternatively one can use a fluorescent labelled anti-(immunoglobulin) reagent in place of the radioactive labelled one (Fig. 1.3) and follow the distribution with a fluorescence

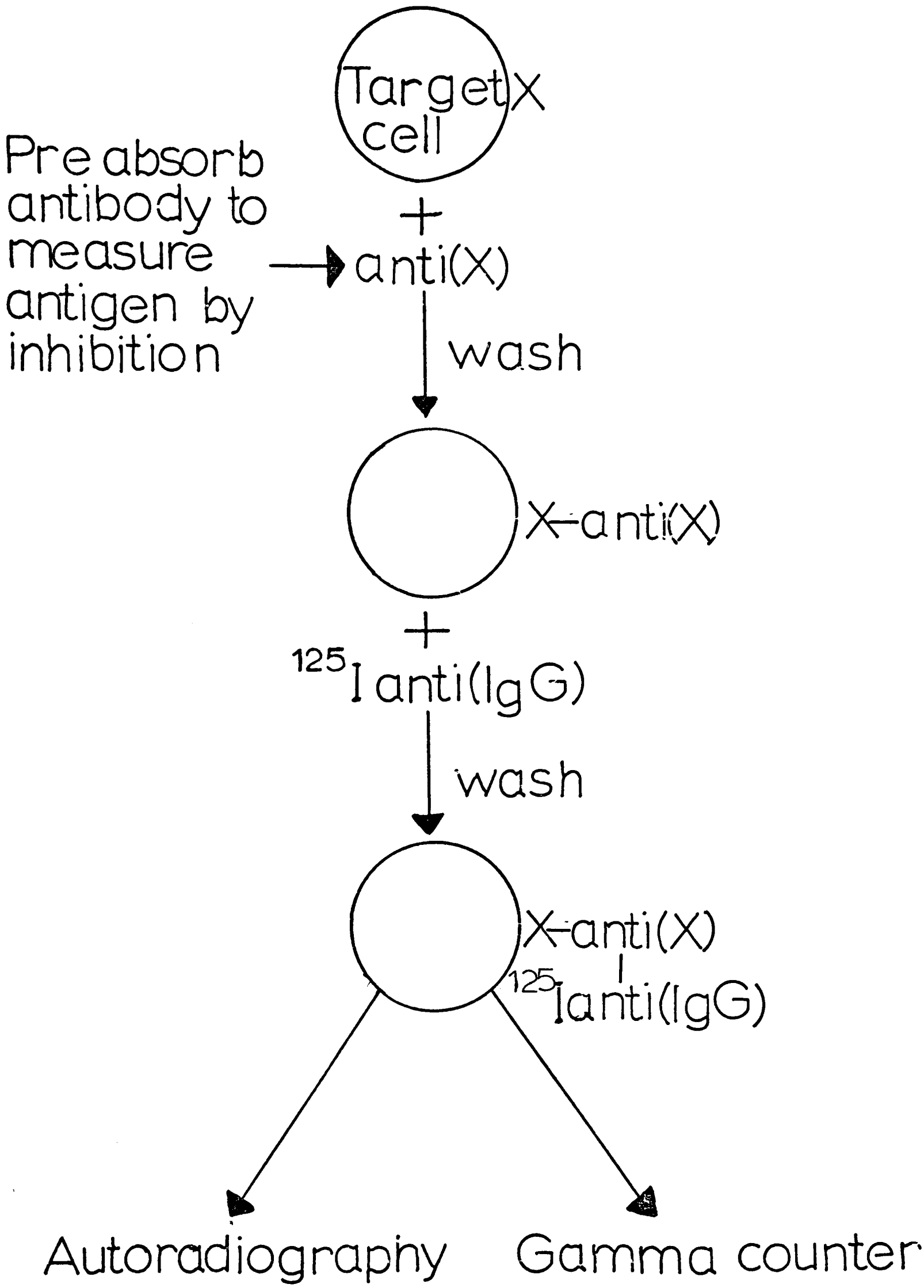


Fig. 1.3 The indirect binding assay for cell surface antigens

Table 1.1 The use of indirect antibody binding assays in studies on cell surface antigens

Aim of assay	Conditions of assay		
	Target cells	Test serum (first incubation)	^{125}I-anti(Ig) (second incubation)
1) To measure antibody concentration and analyse the specificities of a serum	in excess	limiting	saturating
2) To measure number of antigenic sites per cell	limiting	saturating	saturating
3) To provide a sensitive quantitative assay for purification studies	in excess	limiting	trace levels but not limiting

Table from Morris & Williams (1975).

activated cell sorter (Bonner et al, 1972).

Thus the indirect binding assay provides the most versatile and quantitative assay for studying cell surface antigens with the further advantage that the antigens can readily be followed in the presence of detergents.

1.4.4 Differentiation antigens of the lymphocyte cell surface

Several alloantigens have been identified which are specific to the surface of cells of the lymphoid system of mice and rats as shown in Table 1.2. A number of xenoantigens have also been described in terms of the method of immunisation and the tissue distribution of the antigen or antigens recognised following absorption of the antisera (reviewed in Williams, 1977). Of these xenoantigens only the xenoantigenic determinants of Thy-1 and the membrane bound immunoglobulin of B-lymphocytes are clearly defined. However both these examples are exceptional as the Thy-1 molecule was already defined by its alloantigenic determinants and because specific anti-(immunoglobulin) xenoantisera can be easily prepared with immunoglobulin purified from serum or secretions. The membrane immunoglobulin is also the only example of a lymphocyte differentiation antigen whose function is known (see Ch.1.2.1.3). However differentiation antigens have been valuable as markers for cell populations that express them (see Table 1.2) and of these Thy-1 and membrane immunoglobulin have been particularly useful as markers for T- and B-lymphocytes respectively in the mouse (see Ch. 1.2.1.2 and reviewed in Raff, 1971; Greaves et al, 1973).

1.4.5 Differentiation antigens of the brain

Unlike the lymphoid system, the cells which make up the brain are so interconnected as to make the separation of intact viable cells from mature brain impossible, and hence complicate the study of their cell surface antigens. However two types of approach are possible.

Table 1.2 Differentiation alloantigens of the lymphocyte cell surface

Mouse Antigens	Tissue distribution
Thy-1	Thymocytes, T-lymphocytes, brain fibroblasts and epidermal cells
Ly-1, Ly-2,Ly-3,Ly-5	Thymocytes and T-lymphocytes
Ly-4	Possibly on B-lymphocytes only
T1a	Thymocytes and leukemia cells
G IX	Lymphoid cells of normal mice but this is a product of leukemia virus
Ia	B-lymphocytes, macrophages, sperm, epidermal cells and possibly some on T-lymphocytes
Pca-1	Plasma cells liver, kidney, brain
Rat Antigens	
Thy-1	Thymocytes, some bone marrow cells, fibroblasts and brain
T-cell alloantigen	Peripheral T-lymphocytes
Ly-1	Thymocytes & peripheral lymphocytes

Data are from Williams (1977).

Firstly one can study those differentiation antigens which are present on nervous tissue and some other tissue which permits a clear definition as shown in Table 1.3. Thy-1 falls into this class and is discussed in detail below (Ch. 1.4.6). Histocompatibility antigens are not differentiation antigens but have been included as they could have a differential distribution within nervous tissue. All these antigens are absent at birth and rise rapidly during the post-natal development of the brain (see below, Ch. 1.4.6). However this approach is obviously limited as all antigens completely specific to the nervous system would go undetected.

The second approach is to raise and analyse antisera with cell lines derived from nervous tissue or the cell populations that can be obtained from neonatal rodent brain (Barkley et al, 1973) or fractions obtained following mechanical disruption of brain tissue e.g. synaptosomes, synaptic vesicles (Whittaker et al, 1964). Many cell lines have now been obtained and characterised into neuronal-like or glial-like cells according to their biochemical, morphological and electrophysiological properties (Schubert et al, 1974; Pfeiffer & Wechsler, 1972; Stallcup & Cohn, 1976). A number of brain differentiation antigens have been analysed by these methods and are illustrated in Table 1.4. Those antigens which have been clearly shown to be on the intact cells of the cell lines must be differentiation antigens of the cell surface. There is no evidence as to the number of antigens being recognised in each case except in the case of antigens D_1 D_2, D_3, C_1 (see Table 1.4) which are defined in terms of a precipitin arc, and no quantitative data is available concerning the expression of these antigens nor about their chemical nature. Indeed from the distribution analyses and methods of immunisation used it is possible that the antigens described by 3 groups (see Table 1.4) are identical (Martin, 1974; Akeson & Herschman, 1974a, 1974b; Schachner & Wortham, 1975).

To date cell surface antigens of the brain have not been of the same value as markers for cell types as in the lymphoid system (see above)

Table 1.3 Differentiation antigens of mouse brain defined on other tissues

Antigen	Tissue distribution: Neonatal Brain[a]	Tissue distribution: Adult Brain	Tissue distribution: Other tissues	References
Thy-1	-	+	thymocytes, T-lymphocytes epidermal cells, fibroblasts	1
H-2	±	+	most tissues	2
Sk-1	-	+	epidermal cells	3
Pc-1	-	+	plasma cells, liver, spleen	4
N2-B	-	+	thymocytes	5
H-Y	±	+	most tissues in males	6
Ea-2	-	+	most tissues	2

a) the data for the development of these antigens is from Schachner & Hammerling (1974).

References

1 See Ch. 1.4.6.2

2 Klein (1975)

3 Scheid et al (1972)

4 Takahashi et al (1970)

5 Shirai & Mellors (1971)

6 Gasser & Silvers (1972)

Nomenclature of antigen	Tissue Distribution				Method of raising antisera	References
	Brain		Cell lines			
	Neonatal	Adult	Glial	Neuronal		
NS-1	-	+	+	-	mouse anti-(mouse glioma G-26)	1
NS-2	-	+	+	-	rabbit anti-(mouse glioblastoma)	2
NS-3	-	+	+	-	rabbit anti-(mouse C1300 neuroblastoma)	3
NS-4	+	+	±	-	rabbit anti-(mouse-4day-cerebellum)	4
Nervous System antigen	±	+	-	-	rabbit anti-(mouse brain)	5
MBA-1		+		+	rabbit anti-(mouse C1300 neuroblastoma)	6
MBA-2		+	-	+	natural mouse antibodies	7
Akeson & Herschman cntigen		+	-	+	rabbit anti-(mouse neuroblastoma)	8, 9
Common antigen	+	+	+	+	mouse anti-(rat glioma)	10
D_1	±	+	-*	+*	rabbit anti-(rat synaptosomal membrane)	11, 12
D_2	±	+	-*	+*	"	11, 12
D_3	±	+	-*	+*	"	11, 12
C_1	±	+	-*	+*	Rabbit anti-(rat synaptic vesicles)	13

+ & - indicate the presence or absence of a particular antigen in the respective tissues where tested.

* determined on short term cultivated rat astroglial cells and rat synaptosomes (see ref. 12)

References

1 Schachner (1974)
2 Schachner & Carnow (1975)
3 Schachner & Wortham (1975)
4 Schachner et al (1975)
5 Schachner et al (1976)
6 Martin (1974)
7 Martin & Martin (1975)
8 Akeson & Herschman (1974a)
9 Akeson & Herschman (1974b)
10 Fields et al (1975)
11 Jorgensen & Bock (1974)
12 Bock et al (1975)
13 Bock & Jorgensen (1975)

Table 1.4 The distribution of differentiation antigens of rodent brain

and nothing is known about their function. However if these antigens could be defined clearly and quantitatively, purified, and then strong specific antisera raised against them, then good reagents for studying the various cell populations should become available. At present the only specific antisera available are those defined on other tissues (Table 1.3) or those raised against molecules which have been purified because of their exceptional properties e.g. myelin basic protein because of the low density of myelin and its simple polypeptide composition (Eng et al, 1968; Morrell et al, 1972); S-100 because it is soluble in saturated ammonium sulphate and 14-3-2 and glial fibrillarly acidic protein because these are highly acidic molecules (Moore, 1975).

1.4.6 The Thy-1 antigen

1.4.6.1 Definition of the Thy-1 antigen

The Thy-1 antigen (formerly called theta (Θ)) was one of the first differentiation antigens to be found on lymphoid cells. It was identified in terms of two alloantigens, Θ-AKR and Θ-C3H by the analysis of antisera raised against mouse thymocytes in mice of a different strain. The alloantigens were subsequently renamed Thy-1.1 and Thy-1.2 respectively (Snell & Cherry, 1972) and their expression is controlled by the Thy-1 locus on chromosome 9 of the mouse (Itakura et al, 1972; Blankenhorn & Douglas, 1972). The Thy-1 antigen is also expressed on mouse brain (see below) and Douglas (1972) showed that rat brain and thymocytes expressed Thy-1.1 antigenic activity as defined with the mouse antisera, but as yet no rat strain has been found to express Thy-1.2 antigenic activity.

The molecule which carries the Thy-1 alloantigenic determinant is called the Thy-1 molecule. Two other Thy-1 antigenic activities have been recognised on the rat Thy-1 molecule and are termed the rat-specific and the rat-mouse cross-reacting xenoantigenic determinants (Morris &

Williams, 1975). These determinants are present on all Thy-1 molecules because antibody affinity chromatography against one determinant gave depletion of all the Thy-1 determinants (Morris et al, 1975; Letarte-Muirhead et al, 1975). Similar xenoantigenic determinants have been described on mouse Thy-1 (Clagett et al, 1973; Thiele & Stark, 1974).

1.4.6.2 The distribution of Thy-1 in mouse and rat

The distribution of Thy-1 is summarised in Table 1.5. The quantitation of the antigen in the various tissues is from studies by the inhibition of cytoxicity assays (Reif & Allen, 1964, 1966; Douglas, 1972) and indirect antibody binding assays (Acton et al, 1974; Williams, 1976) and the percentage of cells labelled is from analysis by autoradiography (Morris & Williams, 1975; Williams, 1976); the amount of antigen on epidermal cells and fibroblasts was not quantitated as direct cytotoxity was used to demonstrate the presence of the antigen (Scheid et al, 1972; Stern, 1973). The distribution (Table 1.5) clearly shows that only two tissues, brain and thymus express large amounts of the Thy-1 antigen in both rats and mice. In mice, Thy-1 is present on many peripheral lymphocytes although it is expressed at a lower level than on thymocytes and Thy-1 has been widely used as a marker for T-lymphocytes (Raff, 1971). The distribution of Thy-1 in rat differs from that in mice in two ways: firstly Thy-1 is absent from most peripheral lymphocytes (Acton et al, 1974) and secondly it is present on many rat bone marrow cells (Williams, 1976).

The Thy-1 antigen is present in large amounts in all regions of adult mouse brain and at lower levels in peripheral nervous tissue (Table 1.6); a similar distribution has been found for rat brain (R.T. Acton, personal communication). An attempt to localise the Thy-1 antigen within brain by immunofluorescence gave inconclusive results as fluorescence was not obtained where Thy-1 was known to be present from inhibition assays (Moore et al, 1971).

Table 1.5 The tissue distribution of Thy-1 antigen in mice and rats

	% absorptive capacity*		% cells labelled	
	mice	rats	mice	rats
Thymus	100	90	95	92
Lymph node	14	0.7	55	2
Spleen	7	3.5	30	15
Bone marrow	<1.5	10	1	40
Adult brain	80	70		
Neonatal brain	1.3	4		
Fibroblasts	+	+		
Epidermal cells	+			
Erythrocytes, liver, kidney, lung, heart	<1.0	<1.0		

*expressed on a packed tissue basis relative to that for mouse thymus (100%)

Data from Reif & Allen, 1964, 1966; Douglas, 1972; Scheid et al, 1972; Stern, 1973; Acton et al, 1974; Morris & Williams, 1975; Williams, 1976.

Table 1.6 The distribution of Thy-1 antigen in mouse nervous tissue

Tissue used for absorption	Absorptive capacity* relative to thymus (%)
total brain	107
hemisphere	152
pituitary	89
brain stem	76
cerebellum	57
spinal cord	54
olfactory bulb	41
sciatic nerve	29

*calculated relative to absorption by mouse thymus on a packed tissue basis. The data are for AKR mouse brain and are from Reif & Allen (1966).

The Thy-1 antigen is absent from neonatal brain of rats and mice but rises rapidly to reach adult levels after about 25 days (e.g. for mouse brain see Fig. 1.4) (Reif & Allen, 1966; Douglas, 1972). This developmental pattern is interesting as it correlated with many other developmental events in the brain e.g. the onset of bioelectric activity (Deza & Eidelberg, 1967), the formation of synaptic junctions (Aghajanian & Bloom, 1967), the adoption of behavioural patterns (Tilney, 1933), the appearance of various enzymes (Van den Berg, 1974) and other cell surface antigens (Schachner & Hämmerling, 1974). However it precedes the main period of myelination (Davison & Peters, 1970; Cohen & Guarnieri, 1976). Thy-1 is present on several rat neuronal cell lines but not on glial cell lines (R.J. Morris, personal communication). Schachner (1973a) detected Thy-1 in small amounts on the mouse C1300 neuroblastoma whilst Joseph & Oldstone (1974) failed to detect Thy-1 on this or any other cell line tested. This indication that Thy-1 is associated with neuronal cells rather than glial cells is also suggested from studies on cultures of 15-19 day old fetal mouse brain: after 4-11 days of culture, Thy-1 was expressed on cells with a neuronal like morphology and an indistinct class of cells which included fibroblasts, but was absent from the glial-like cells (Mirsky & Thompson, 1975). Also studies on the preparation of synaptosomes indicated that Thy-1 was associated with the synaptosomal membrane fraction (R.T. Acton, J. Addis, C.F. Carl & W.F. Bridgers, unpublished work), although it may be present on other brain membranes as well. Thus a clear localisation of Thy-1 in brain is lacking but the available evidence suggests an association with neuronal rather than glial cells; this is discussed further in Ch. 7.

1.4.6.3 The Quantitation of Thy-1 at the cell surface

Binding studies with alloantisera have indicated that there are on average, more than 400,000 Thy-1.2 antigenic determinants on mouse

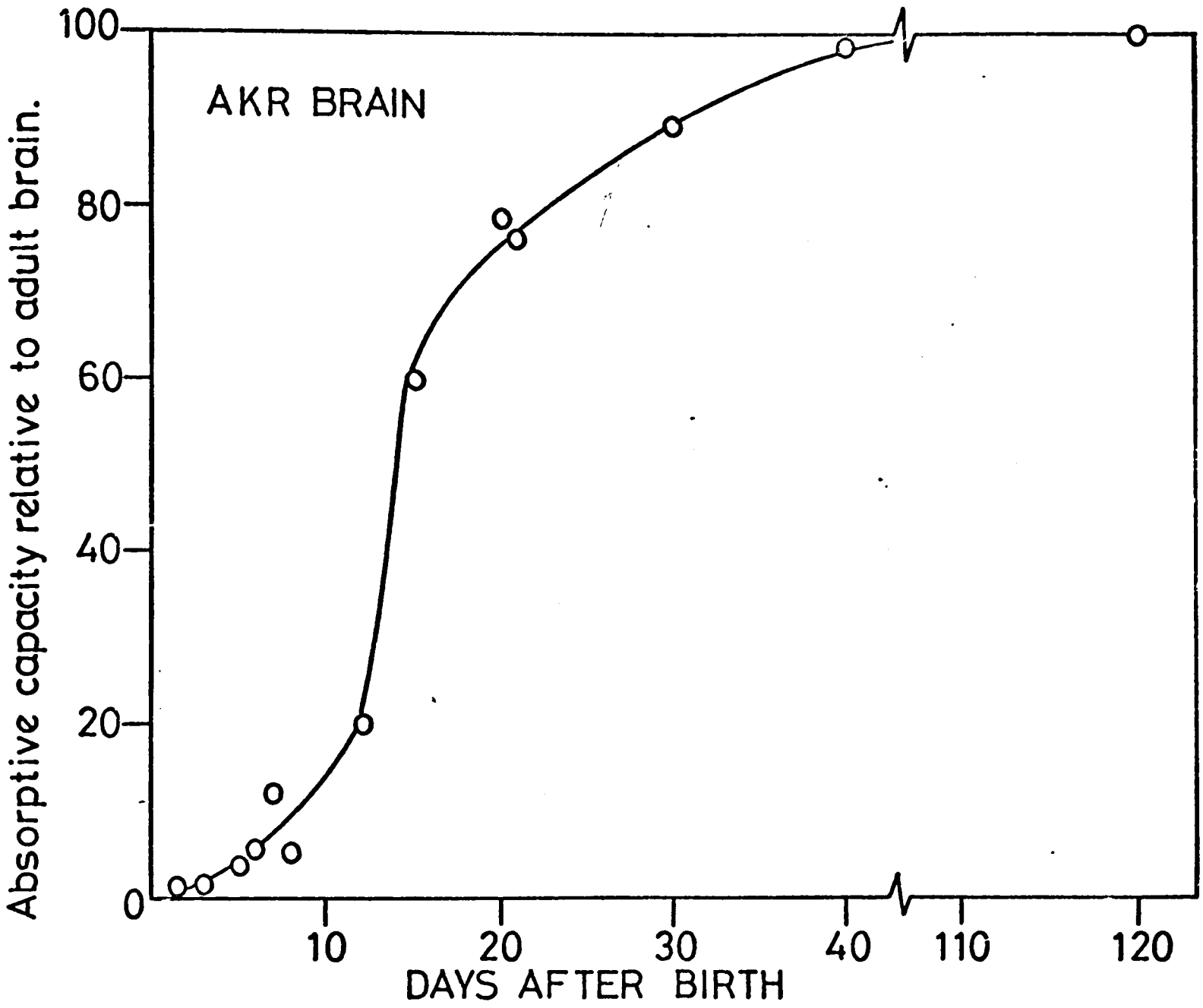


Fig. 1.4 The post natal development of Thy-1 in mouse brain.

The data are for AKR mouse brain and are from Reif & Allen (1966).

thymocytes which express this alloantigen (Hämmerling & Eggers, 1970) and 600,000 Thy-1.1 antigenic sites on rat thymocytes (Acton et al, 1974). The latter figure was confirmed with studies using rabbit antisera recognising the Thy-1 xenoantigenic determinants (Morris & Williams, 1975). All the Thy-1 is likely to be present at the cell surface of thymocytes as lysis of the cells failed to reveal more antigen, and as detergents were necessary for solubilisation of the antigen it is presumably an integral membrane component of the plasma membrane of thymocytes (Letarte-Muirhead et al, 1974). The value of 600,000 antigenic sites per cell, suggests that Thy-1 is a major component of the thymocyte membrane and is comparable in number to the well studied major membrane glycoprotein (500,000 molecules/cell) and "band 3" (940,000 molecules/cell) of human erythrocytes (Steck, 1974). As Thy-1 is present in similar quantities in rat brain as in thymocytes on a packed tissue basis (see Table 1.5) it is presumably also a major component of some brain membranes (see Ch. 3).

1.4.6.4 The nature of the Thy-1 molecule

The majority of studies to determine the chemical nature of Thy-1 have been on the Thy-1 of mouse thymocytes and the results obtained are remarkable for their variety e.g. a glycolipid (Vitetta et al, 1973); a protein of molecular weight of 65,000 (Atwell et al, 1973) and of 40,000 (Sauser et al, 1974); a protein or glycoprotein (Kucich et al, 1975); and a glycoprotein with a molecular weight of 25,000 (Letarte-Muirhead et al, 1975; Trowbridge et al, 1975). Finally it has been suggested that the Thy-1 alloantigenic determinants are carried by specific gangliosides in mouse brain and thymocytes (Esselman & Miller, 1974; Miller & Esselman, 1975). Studies on the solubilisation of rat thymocyte Thy-1 have shown that the Thy-1 antigenic activity was associated with a protein or glycoprotein with a molecular weight of 28,000 including

bound deoxycholate (Letarte-Muirhead et al, 1974). Possible reasons for the variety of results obtained are discussed in detail in Ch. 7 in view of the studies on brain Thy-1 in this thesis and studies carried out in parallel on rat thymocyte Thy-1 (Letarte-Muirhead et al, 1975).

1.5 Conclusion

The study of cell surface differentiation antigens is a method with the necessary resolving power to select for molecules which might mediate the specific cell interactions that are characteristic of both the lymphoid and nervous systems. Very little is known about the molecules which mediate these reactions but the Thy-1 molecule appears to be a suitable candidate for such functions. It has the advantages that it can be defined genetically and is present in large amounts in two rat tissues - the thymus and brain. There are suitable tissues to attempt to purify and characterise the molecule.

This thesis describes the purification of rat brain Thy-1 and the characterisation of the purified antigen together with thymocyte Thy-1 which was purified in parallel studies by M. Letarte-Muirhead and A.F. Williams.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1 Animals

AKR, C3H, CBA and Balb/c inbred strains of mice and Wistar rats were obtained from Oxfordshire Laboratory Animal Colonies, Bicester. Wistar rats were also from M.R.C. Radiobiology Unit, Harwell, Oxon. A/Thy-1.1 and A/Jax mice were from Dr. D.A.L. Davies, Searle Laboratories, High Wycombe. Park grey rabbits were from Oxford University Park Farm, Northmoor, Oxon.

2.1.2 Chemicals

Tris(hydroxymethyl)aminomethane (Tris), Lubrol PX, sodium deoxycholate, 3,5 diaminobenzoic acid, n-acetyl neuraminic acid (synthetic), methyl α-D-glucopyranoside, periodic acid; Sigma Chemical Co., London S.W. 6.

Acrylamide, bisacrylamide, sodium dodecyl sulphate, TEMED (NNN'N'-tetramethylethylene diamine), 4-amino-3-hydroxynapthalene-1-sulphonic acid; BDH Chemicals Ltd., Poole, Dorset.

Freund's complete and incomplete adjuvants, Schiff's (Feulgen) stain; Difco Labs., Surrey.

Coomassie Blue; Michrome, Edward Gurr Ltd., London, S.W. 14.

^{14}C -labelled deoxycholate and carrier free ^{125}I (IMS 30); Radiochemical Centre, Amersham, Bucks.

$^{2}H_2O$; Ryvan Chemical Co., Southampton.

Lentils (lens culinaris) -green variety; Palm's Delicatessen, The Market, Oxford.

p-Toluene sulphonic acid, 3-(2-aminoethyl)indole-HCl; Pierce Chemical Company, Rockford, Illinois.

Glutaraldehyde; Koch Light, Colnbrook.

2.1.3 Proteins

Bovine serum albumin (Cohn Fraction V), avidin, fetuin, myoglobin cytochrome c, bovine IgG, pepsin; Sigma Chemical Co., London S.W. 6.

Papain; Worthington Biochemical Corp., New Jersey.

Pronase P; Kaken Chemical Co., Tokyo.

Chymotrypsin; Boehringer, Mannheim.

Trypsin (treated L-1-chloro-3-tosylamino-4 phenylbutan-2-one) and rabbit IgG were from Dr. L.E. Mole, M.R.C. Immunochemistry Unit, Oxford.

All other proteins were prepared by standard procedures in this laboratory.

2.1.4 Buffers

Balanced buffered salt solution (BBSS) (Shortman, 1968) was used for all experiments with viable cells. Bovine serum albumin (BSA) was added to 0.5% (w/v) except where this would interfere. The BSA had been neutralised as described by Legge & Shortman (1968).

Sodium deoxycholate was in 0.01M Tris-HCl, 0.02% (w/v) NaN_3 pH 8.0 and is referred to as "deoxycholate buffer". The pH was adjusted to 8.0 at 20°C and at 4°C the pH was 8.4. All other buffers are described in the text.

2.1.5 Antisera

The following antisera were used:

Balb/c mouse anti-(Wistar rat brain) serum was raised by 2 subcutaneous injections of 0.5 mg wet weight of Wistar rat brain homogenate in complete Freund's adjuvant at an interval of one week, followed by 4, weekly,

interperitoneal injections of 0.5 mg of homogenate. The mice were bled 7-10 days after the last injection (Acton et al, 1974).

CBA mouse anti-(Wistar rat brain) serum was raised as for the Balb/c mouse anti-(Wistar rat brain) serum (see above) except that 2 mg wet weight of rat brain homogenate was used instead of 0.5 mg.

CBA mouse anti-(AKR mouse thymocyte) serum was raised by the following schedule: day 0, 10^7 viable AKR mouse thymocytes subcutaneously; day 7, 10^7 glutaraldehyde-fixed AKR thymocytes in complete Freund's adjuvant subcutaneously; days 14, 21, 28, 35, 42, 51, 61, 70, 10^7 glutaraldehyde-fixed AKR thymocytes interperitoneally (no adjuvant). Mice were tail bled on days 21, 35, 49, 58, 68 and bled out on day 77.

Rabbit anti-(rat brain Thy-1) serum was raised in two rabbits which had been primed with brain membrane glycoproteins obtained after affinity chromatography with lentil lectin (see Ch. 4) and were further immunised with pure Thy-1 5 months later. The priming consisted of two intramuscular injections of 1 mg of glycoproteins in complete Freund's adjuvant and one subcutaneous injection of 1 mg in incomplete Freund's adjuvant at weekly intervals. After 5 months, three injections of 40 μg of pure Thy-1 were given intravenously at weekly intervals. Animals were bled weekly after this.

Rabbit anti-(thymocyte Thy-1) serum was raised against Thy-1 purified from rat thymocytes by affinity chromatography with lentil lectin (Letarte-Muirhead et al, 1975). Two rabbits were immunised 3 times with 30 μg of Thy-1 at 8 day intervals. The first two immunisations were in complete Freund's adjuvant and were given intramuscularly; the third was in incomplete Freund's adjuvant and given subcutaneously. The animals were bled 8 days after the third injection.

Horse anti-(rabbit IgG) serum: This serum was a gift of the Wellcome Foundation Ltd., London. It had been raised by repeated injections of a horse with alum precipitated rabbit IgG. It contained 6 mg/ml antibody to rabbit IgG.

Rabbit anti-(mouse IgG) serum was raised by 2 weekly intramuscular injections of 0.1 mg of mouse IgG in complete Freund's adjuvant, followed by a third of 0.5 mg intravenously after 2 months. The rabbits were bled weekly two weeks after the last injection and purified rabbit anti-(mouse IgG) antibodies prepared (see below).

2.2 Analytical Techniques

2.2.1 Protein and phospholipid estimation

Protein was estimated by Hartree's (1974) modification of the method of Lowry et al (1951) using bovine serum albumin as a standard. Samples in deoxycholate were estimated either by comparing them with standards in the same concentration of deoxycholate or by removing the deoxycholate by extraction with acetone at 0°C after precipitation with trichloroacetic acid at a final concentration of 20% (w/v) for 1h at 0°C.

Phospholipid was estimated from the phosphorus present in a chloroform-methanol (2:1 v/v) lipid extract (Folch et al, 1957). Phosphorus was determined by the method of Bartlett (1959) modified as follows: samples containing 1-5 μg phosphorus were dried down in vacuo; 1.0 ml 70-72% perchloric acid was added and heated for 1 h at 220°C under reflux. When cool, the sample was made up to 5 ml with water and 0.4 ml of 5% (w/v) ammonium molybdate added, followed by 0.4 ml of reducing reagent, mixing each time. This reducing reagent was 0.25% (w/v) 4-amino-3-hydroxynaphthalene sulphonic acid, 15% (w/v) sodium bisulphite to which 0.5% (w/v) anhydrous sodium sulphite had been added, mixed and the solution filtered. After the addition of this reducing reagent, water was added to a final volume of 10 ml and tubes heated at 100°C for 7 min. On cooling the absorbance at 830 nm was measured.

The phospholipid content of the samples was calculated by multiplying the observed weight of phosphorus by 25, assuming that the phospholipids have an average phosphorus content of 4% and a molecular

weight of 775 (Kopaczyk et al, 1966).

2.2.2 Polyacrylamide gel electrophoresis in sodium dodecyl sulphate

Disc gels were prepared and run as described by Fairbanks et al, (1971). Several different concentrations of acrylamide were used and these were prepared by varying the proportion of the acrylamide plus bisacrylamide mixture used.

Samples were solubilised by heating at 100°C for 3 min in SDS (sodium dodecyl sulphate) at 1% or more so as to give an SDS:protein ratio of more than 7:1, and 4M urea plus either 2mM iodoacetamide or 1% (w/v) dithiothreitol; after heating, the reduced samples were alkylated with 1.5M iodoacetamide at a final concentration of 0.12M. Dilute samples in deoxycholate were concentrated by precipitation with 20% (w/v) trichloroacetic acid at 0°C for 1h and extracted twice with acetone at 0°C to remove deoxycholate and trichloroacetic acid. Some variation in the effectiveness of this precipitation had been observed (Letarte-Muirhead et al, 1975); thus in all cases where purity was being estimated samples were also concentrated by ultrafiltration (see below).

After electrophoresis gels were stained for protein with Coomassie Blue (Weber & Osborn, 1969) and destained electrophoretically in a Canalco destainer. Gels were stained for carbohydrate with the periodic acid-Schiff method as described by Glossmann & Neville (1971) except that the gels were fixed with 25% (v/v) propan-2-ol, 10% (v/v) acetic acid and all procedures were at room temperature.

The relative mobility of the bands was determined by comparison with the mobility of bromophenol blue dye which was run with each sample. The apparent molecular weight was estimated from linear relationship between log (molecular weight) and the relative mobility as determined with marker proteins (Weber & Osborn, 1969). Those used were (molecular weights given in parentheses): heavy chain (52,000) and light chain (23,500)

of rabbit IgG, bovine serum albumin (68,000), hen ovalbumin (43,000), whale myoglobin (17,000). Fetuin and avidin were also run on gels stained for carbohydrate but they were not used in determining molecular weights as they give anomalous values (Glossmann & Neville, 1971).

Densitometric traces of gels were carried out on a Gilford 2410-S gel scanner at 550 nm for gels stained with Coomassie Blue and 560 nm for gels stained with periodic acid-Schiff stain.

In order to assay Thy-1 antigenic activity after electrophoresis on polyacrylamide gels, the following modifications were made: the concentration of SDS in the gel and running buffer was reduced to 0.1% (w/v); the sample was loaded directly in 0.1% (w/v) SDS, 0.01M Tris-HCl, pH 8.0. ^{125}I -myoglobin and ^{125}I -ovalbumin were loaded in trace amounts as internal markers. After electrophoresis, the gels to be assayed were cut into 3mm slices which were crushed in 0.5 ml 0.05% (w/v) SDS in 0.01M Tris-HCl pH 8.0, and left overnight at 4°C. The position of the marker proteins was determined by counting the slices in a Nuclear Enterprises gamma counter. The antigenic activities were determined after the fractions were diluted with 0.25% (w/v) BSA in 0.01M Tris-HCl, pH 8.0 to a final SDS concentration 0.017%.

2.2.3 Amino acid analysis

Samples for amino acid analysis were dried down in vacuo in acid washed pyrex glass tubes. 0.5 ml constant boiling HCl and 0.025 ml 0.1M phenol (to prevent loss of tyrosine; Sanger & Thompson, 1963), were added and the samples frozen. Dissolved gases were removed by evacuation while allowing the samples to thaw. After refreezing, the tubes were sealed under vacuum and hydrolysed at 110°C for 22h. After hydrolysis the HCl was removed in vacuo over NaOH.

Analysis was by the method of Moore & Stein, (1951) on a modified Beckman analyser (see Reid, 1974) or on a Locarte analyser. Cysteine was estimated as cysteic acid following performic acid oxidation (Hirs, 1967).

A correction for the loss of threonine and serine was made by extrapolation from values obtained from 72h and 24h hydrolyses. Samples for the estimation of tryptophan were hydrolysed with 0.3 ml 3M p-toluene sulphonic acid, 1% (w/v) 3-(2-aminoethyl)-indole-HCl at 110°C for 48h and analysed on the short column of the Beckman analyser using the pH 4.25 and pH 5.28 buffer steps as described by Liu & Chang (1971).

Norleucine was added as an internal marker to those samples which were also being analysed for carbohydrate residues so that an accurate estimate of the protein:carbohydrate ratio could be obtained.

2.2.4 Carbohydrate analysis

2.2.4.1 Gas liquid chromatography. Sugars were estimated as O-trimethylsilyl ethers of methyl glycosides by gas liquid chromatography (Clamp, 1974). 100 μg of sample plus about 40 nmoles arabitol (as an internal standard) were dried down in vacuo over P_2O_5. Then 0.5 ml 1M HCl in methanol was added and the tubes were sealed under N_2 and heated at 85°C for 6h, after which the samples were neutralised with Ag_2CO_3. 50 μl acetic anhydride was added, the tubes were sealed and left for 6h at 20°C. The precipitates were washed 3 times with 0.5 ml methanol and the supernatants dried down by rotary evaporation. 50 μl of silylating reagent (Sweeley et al, 1963) was added, the tube sealed and left for 30 min at 20°C. After centrifugation 3 μl of supernatant was analysed on a Hewlett Packard Model 5750 gas liquid chromatograph with one glass column (3m x 2mm) containing 3% SE-30 Ultraphase on Chromosorb W(H/P) 80-100 mesh. The temperature was 120°C for 20 min, rising by 1°C/min to 210°C which was maintained until sialic acid came off.

As a minor peak (possibly lipid) was found in the region of glucosamine in some early analyses, subsequent samples were extracted with hexane immediately after methanolysis to remove any lipids (Esselman et al, 1972).

2.2.4.2 Estimation of amino sugars by ion exchange chromatography. 100μg sample was dried down in vacuo over P_2O_5 and 0.5 ml 3M p-toluene sulphonic acid, 0.2% tryptamine-HCl added. The tubes were sealed under N_2 and heated at 110°C for 72h (Liu & Chang, 1971). 40nmole norleucine was added as an internal standard. 0.75 ml 1N NaOH, and 0.1 ml thioglycol were added and the sample was made up to 3.0 ml with water. Samples were analysed on a Biocal Model BC 100L using a two column technique (Jacobs, 1964). The values obtained for glucosamine and galactosamine were corrected on the basis of a 70% recovery as obtained for ovomucoid run similarly.

2.2.4.3 Estimation of sialic acid. Sialic acid was determined by the fluorometric method of Hess & Roide (1964). Samples containing 0.5-2nmole sialic acid in glass tubes (which had been carefully acid washed and rinsed 10 times with double distilled water) were dried down in vacuo. 100μl 0.005M 3,5-diaminobenzoic acid, 0.125M HCl was added, the tube sealed and heated at 100°C for 16h. An aliquot was diluted in 0.05M HCl and analysed in a Perkin-Elmer MPF-2A fluorescence spectrometer, exciting at 436 nm and following emission at 525 nm. A range of sugar standards were run in each experiment using N-acetyl neuraminic acid as the standard sialic acid. In these conditions hexoses gave about 5% of the fluorescence of sialic acid and hexose standards were run in each experiment so that their contribution to the fluorescence could be calculated from the hexose composition obtained by gas liquid chromatography.

2.3 Preparation of reagents

2.3.1 Lentil lectin

Lentil lectin was prepared from green lentils - lens culinaris- by a modification of the method of Hayman & Crumpton (1972). Lentils were soaked overnight at 4°C in 0.9% (w/v) NaCl and homogenised with a Waring Blender. The larger debris was removed by filtration through muslin and centrifugation for 60,000 g.min. The fraction precipitated

between 30% and 80% saturated ammonium sulphate was dialysed against 0.05M Tris-HCl,0.005M $CaCl_2$, 0.005M $MnCl_2$, pH 7.5, and passed through a Sephadex G-75 column (3 x 25 cm). The adsorbed fraction was eluted with 0.2M methyl α-D-glucopyranoside, 0.05M Tris-HCl, pH 7.5. About 1 g of pure lectin (as judged by polyacrylamide gel electrophoresis in SDS) was obtained in a typical preparation from 2 kg of lentils. The lectin was dialysed against, and stored in 0.025M Tris-HCl, 0.05M NaCl, 0.005M $CaCl_2$, 0.005M $MnCl_2$, 0.02% NaN_3, pH 7.5.

Lentil lectin columns were prepared as described below (Ch.2.3.3).

2.3.2 Purification of Antibodies.

Two purified antibody reagents were used, rabbit anti-(mouse IgG) and horse anti-(rabbit IgG). These were prepared from the immune sera by immunoabsorption as described by Jensenius & Williams (1974), e.g. for horse anti-(rabbit IgG) the serum was passed through an immunoadsorbent column containing rabbit IgG bound to Sepharose 4B beads (see below). After washing with 0.01M Tris-HCl, 0.5M NaCl, 0.02% NaN_3, pH 7.4, the antibodies were eluted with 1M propionic acid, neutralised with solid Tris and dialysed against the buffer for pepsin digestion which was as described by Nisonoff et al (1966). The $F(ab')_2$ fragment was purified by gel filtration on Sephadex G-200 in the pH 7.4 buffer as above. The $F(ab')_2$ from the purified antibodies was used routinely in binding assays (see below) rather than intact IgG because of the tendency of IgG to aggregate after acid elution from the affinity columns. These IgG aggregates can give anomalous binding (Jensenius & Williams, 1974).

2.3.3 Preparation of affinity columns.

Proteins were bound to Sepharose 4B beads by the method of Cuatrecasas (1970) for anti-insulin antibodies. Sepharose 4B beads were activated with CNBr and proteins coupled at about 10-20 mg protein/ml beads at pH 6.8 in 0.2M citrate buffer. The efficiency of coupling was

always greater than 90%. The columns were washed extensively and stored at 4°C.

Columns were prepared with lentil lectin, rabbit anti-(rat brain Thy-1) serum and various IgG fractions for the preparation of anti IgG reagents.

2.3.4 Iodination of Proteins.

^{125}I was coupled to proteins by the Chloramine T method (Byrt & Ada, 1969) as modified by Jensenius & Williams, 1974. 10μl chloramine T (2 mg/ml in 0.3M sodium phosphate buffer, pH 7.3) was added to 10 μl ^{125}I (80-140 mCi/ml). 25μl of protein (1 mg/ml) was added and reacted for 2 min. at room temperature. The reaction was stopped by the addition of 25μl tyrosine (0.4 mg/ml in 0.3M sodium phosphate buffer pH 7.3) and 100μl 10% (w/v) BSA in BBSS added (except for the iodination of myoglobin and ovalbumin where this step was omitted.) The free ^{125}I was removed by gel filtration on a 5 ml Sephadex G-50 (fine) column in BBSS, 0.02% NaN_3 and the "^{125}I-labelled" protein was stored at 4°C. Under these conditions, 1.0-2.0 atoms of ^{125}I were incorporated into each immunoglobulin molecule. For the iodination of myoglobin and ovalbumin where a lower specific activity was sufficient, the ^{125}I was diluted to 5 mCi/ml with 0.048 mM KI, 0.3M sodium phosphate buffer pH 7.3, and iodination was as above.

The ^{125}I was released slowly from the protein and after 4-5 weeks the preparation was either discarded or the free ^{125}I was removed by dialysis.

2.3.5 Glutaraldehyde fixation of cells.

Thymocytes from AKR mice or Wistar rats were teased from thymus glands in BBSS. The cells were filtered on cotton wool, washed, twice with 0.5% BSA/BBSS and once with BBSS, by centrifugation. After resuspension at 2 x 10^8/ml in BBSS at 20°C, an equal volume of 0.25% (w/v)

glutaraldehyde was added. After 5 min a tenth volume of 10% BSA in BBSS was added to stop the reaction, the cells washed, resuspended in 10% BSA in BBSS at 10^8 cells/ml by passing through a gauge 26 needle and stored in aliquots at -70°C. Sheep red blood cells were glutaraldehyde fixed in a similar manner except that they were stored at 5×10^8 cells/ml.

2.4 Radioactive binding assays.

2.4.1 Assay for Thy-1 antigenic activity.

All procedures were at 0-4°C and the Thy-1 antigenic activities were assayed by the inhibition of radioactive binding assays (Williams, 1973; Letarte-Muirhead et al, 1974) as follows: 0.1ml of tissue or extract was incubated for 1h with 0.1 ml of diluted antiserum in 0.5% BSA in BBSS. After centrifugation for 100,000 g. min 2 x 50 μl aliquots were added to 50 μl target cells (10^6 thymocytes plus 10^7 sheep red blood cells (to improve pelleting) in 10% BSA in BBSS). The tubes were shaken immediately and after $\frac{1}{2}$h. After a further $\frac{1}{2}$h, the target cells were washed twice by centrifugation for 10,000 g.min, with 1 ml 0.1% BSA in BBSS. 0.1 ml of "^{125}I-labelled" anti-(IgG) antibody (6×10^5 d.p.m.; specific radioactivity 20 μCi/ μg) in 0.5% BSA in BBSS was added and incubated with the cells for 1h on ice. The cells were washed as before, suspended in 0.6 ml of BBSS and transferred to 1 ml plastic tubes containing 0.1 ml 4M NaOH for gamma counting (efficiency 30%) in a Nuclear Enterprises Counter. To assay soluble fractions in deoxycholate, 0.025M Tris-HCl, 0.14M NaCl, pH 8.0 was used instead of BBSS and for the first incubation, 25 μl of extract was incubated directly with 25 μl of the diluted antisera. For assays of extracts containing small amounts of protein, the dilutions of the extracts were in 0.25% BSA to minimise non-specific losses of protein.

The conditions of the assays were such that the target cells were in excess and the "^{125}I-labelled" antibody was neither limiting

nor saturating (see Morris & Williams, 1975; and Ch.1.4.3). The concentration of the first antiserum was chosen so as to give a reasonable number of specific counts per min. (10,000 - 15, 000 c.p.m.) after the binding of the "^{125}I-labelled" antibody.

2.4.1.1 Assay for Thy-1.1 Balb/c anti-(Wistar rat brain) serum was used at a dilution of 1/60 for the first incubation; the target cells were AKR mouse thymocytes (plus sheep red blood cells as above); and the second antibody was "^{125}I-labelled" rabbit F(ab')$_2$ anti-(mouse IgG). In later experiments, e.g. to test the effect of heat and proteolysis on Thy-1, CBA anti-(Wistar rat brain) serum was used at a dilution of 1/150 in place of the Balb/c anti-(Wistar rat brain) serum.

The specificity of the assay depends on the combination of the first antiserum and the target cells. This assay selects only for antigenic determinants recognised by the antisera and present in both Wistar rat brain and on mouse AKR thymocytes. That only the Thy-1.1 alloantigenic determinant is recognised is shown by absorption analysis with tissues from mouse strains congenic for the Thy-1 locus (Acton et al, 1974). This is shown in Fig. 2.1 where the assay is completely inhibited by brain from A/Thy-1.1 mice but brain from A/Jax mouse gives no inhibition. The only allelic differences demonstrated between these congenic strains are at the Thy-1 locus (John et al, 1972) and thus the assay is specific for the Thy-1.1 alloantigenic determinant.

2.4.1.2 Assay for Thy-1 xenoantigenic activities. The Thy-1 rat specific and rat-mouse cross-reacting xenoantigenic determinants have been clearly shown to be on the same molecule as the Thy-1.1 alloantigenic determinants (Morris et al, 1975; Letarte-Muirhead et al, 1975) and can be used to follow the Thy-1 antigen.

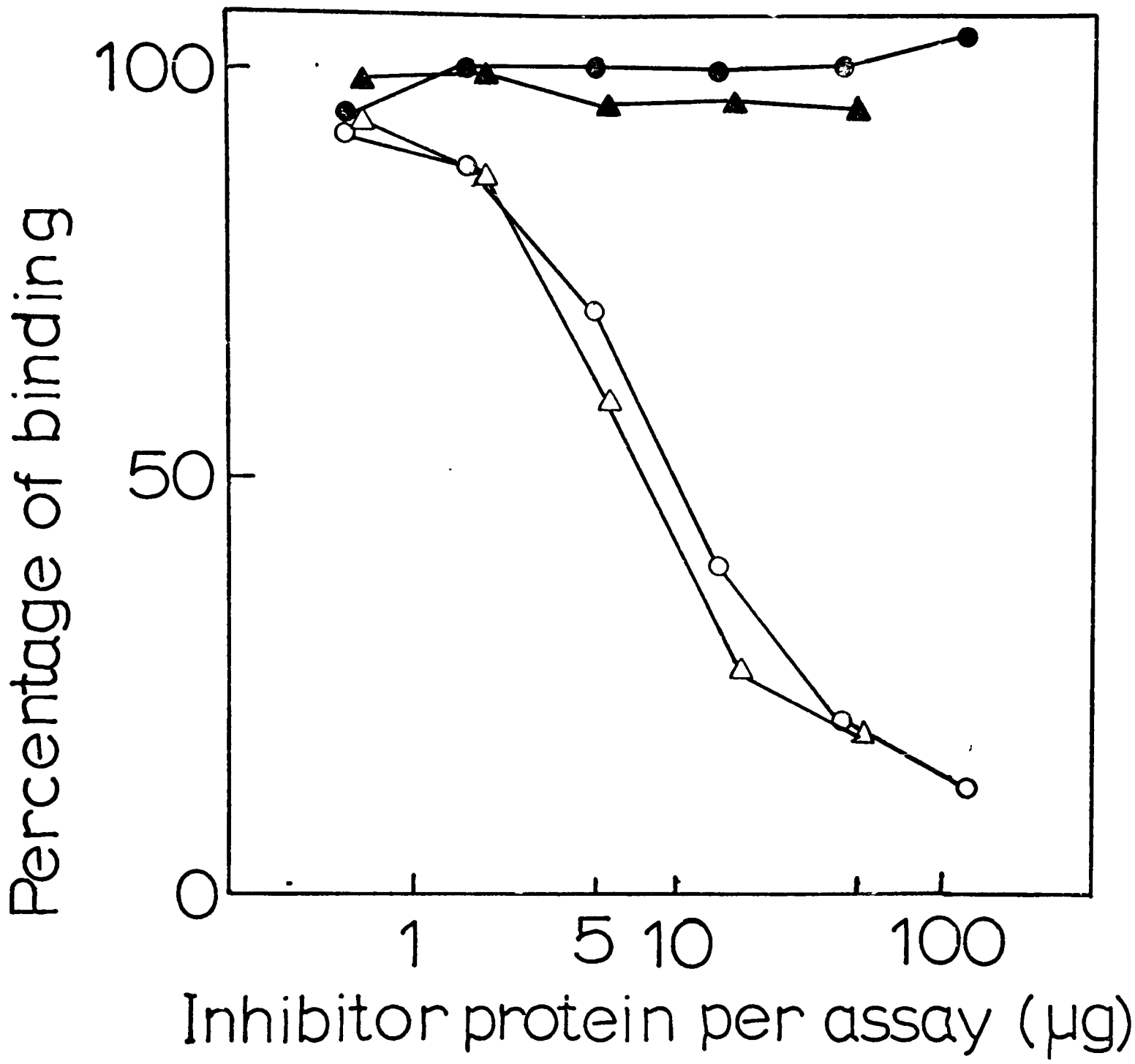


Fig. 2.1 The specificity of a binding assay for Thy-1.1

25 µl aliquots of CBA mouse anti-(Wistar rat brain) serum diluted 1/150 were absorbed with A/Thy-1.1 mouse brain (o), A/Thy-1.1 mouse thymocytes (Δ), A/Jax mouse brain (●), A/Jax mouse thymocytes (▲); the binding of residual antibody to AKR mouse thymocyte target cells was followed with ^{125}I-labelled rabbit anti-(mouse IgG).

The IgG fraction at 47 mg/ml, obtained by precipitation of the rabbit anti-(rat brain Thy-1) serum with 18% Na_2SO_4 (Williams & Chase, 1967) was diluted to 1/6000 in 0.5% BSA in BBSS and used in the first incubation. Rat thymocytes and ^{125}I-horse F(ab')$_2$ anti-(rabbit IgG) were used as target cells and second antibody respectively. The analysis of this antisera is described in Ch.4.5, which shows that only 4% of the anti-(Thy-1) antibodies were against the Thy-1.1 alloantigenic determinant. Thus the assay was used routinely to assay the Thy-1 xenoantigenic determinants.

A second assay for Thy-1 xenoantigenic determinants was used in the antigenic comparison of brain Thy-1 and thymocyte Thy-1. The conditions were exactly as above except that rabbit anti-(thymocyte Thy-1$_{L+}$) serum was used at a dilution of 1/400 (Letarte-Muirhead et al, 1975).

2.4.2 Assay of antibody concentration (Morris & Williams, 1975).

The dilutions of the antiserum rabbit anti-(brain Thy-1) were incubated with 5 x 10^6 thymocytes 10^7 sheep red blood cells, the cells washed and reacted with ^{125}I- horse anti-(rabbit IgG) as above except that saturating conditions of the second antibody were used (20 μg/ml and 0.02 μCi/μg) i.e. conditions of excess target cells and saturating second antibody.

2.5 The purification of Thy-1 from rat brain.

All procedures were at 0-4°C unless stated otherwise.

2.5.1 Solubilisation of Thy-1 from brain membranes.

Rats aged 6-12 weeks were killed with CO_2, whole brains were removed and frozen in liquid N_2 for storage at -70°C until required.

This procedure had no effect on Thy-1.1 antigenic activity compared with fresh material.

Rat brains in 10 vols of 0.32M sucrose were homogenised with either a Potter-Elvehjem homogenizer or a Waring Blender (for larger preparations). The larger debris was removed by centrifugation for 16,000 g.min. and the pellet washed once in 2 vols of 0.32M sucrose. The pooled supernatants were centrifuged for 2,000,000 g.min. and the crude membrane obtained was resuspended in 0.05M NaCl, 0.01M Tris-HCl, pH 7.4 to give 12 mg protein/ml. An equal volume of lubrol PX in the same buffer was added and the mixture stirred on ice for 1h. This extract was centrifuged for 6,000,000 g.min. and the pellet resuspended in 0.01M Tris-HCl, 0.02% (w/v) NaN_3, pH 8.0 to give 12 mg protein/ml. An equal volume of 4% (w/v) sodium deoxycholate in the same buffer was added, the mixture frozen and stored overnight at -20°C. On thawing it was centrifuged at 6,000,000 g.min. and the pellet re-extracted in 2% (w/v) deoxycholate in the above buffer. The supernatants were pooled to give the solubilised Thy-1 fraction.

2.5.2 Affinity chromatography with lentil lectin coupled to Sepharose 4B

Lentil lectin was coupled to Sepharose 4B at 10 mg lectin/ml of swollen beads as described in Ch. 2.3.4. A 35 ml column was used in a typical preparation from 63g of rat brain. It was prewashed with 1M methyl-α-D-glucopyranoside in 1% (w/v) deoxycholate buffer, then extensively with 0.5% (w/v) deoxycholate buffer. The solubilised Thy-1 fraction was passed through the column at 15 ml/h. The column was then washed with 3-4 column volumes of 0.5% (w/v) deoxycholate buffer and eluted with 1M methyl α-D-glucopyranoside in 1% (w/v) deoxycholate buffer at 5 ml/h. Protein was estimated from the E^{1cm}_{280nm} readings and the fractions were assayed for Thy-1.1 antigenic activity.

The column was washed successively with 0.5% (w/v) deoxycholate buffer, 0.01M Tris-HCl pH 8.0 and stored in 0.025M Tris-HCl, 0.005M $CaCl_2$,

0.005M $MnCl_2$, .04% (w/v) NaN_3, pH 7.5.

2.5.3 Gel filtration in deoxycholate.

Upward flowing columns of Sephadex G-200 were eluted in 0.5% (w/v) deoxycholate buffer. The material eluted from the lentil lectin affinity column was concentrated to 60 ml and filtered on a Pharmacia K-50 (5 cm x 90 cm) column at 20 ml/h, collecting 20 ml fractions. For measurement of Stokes radius, 5 ml of eluate from the lentil lectin column was filtered on a Pharmacia K-26 (2.6 cm x 90 cm) G-200 column at 8 ml/h, collecting 5 ml fractions. Protein was followed by the $OD^{1\ cm}_{280\ nm}$ readings and individual fractions were assayed for Thy-1.1 antigenic activity.

The elution characteristics of the column were determined by chromatography of marker proteins which were loaded similarly and their positions of elution determined by measuring the $E^{1\ cm}_{280}$. The total and void volumes were determined with $Na^{125}I$ and blue dextran respectively. The partition coefficients (k_D) of the marker proteins were calculated from the relationship:

$$k_D = \frac{V_e - V_o}{V_t - V_o}$$ (Siegel & Monty, 1966)

k_D = partition coefficient

and V_e = elution volume corresponding to peak concentration of solute

V_o = void volume of the column

V_t = total volume of the column.

A linear relationship was obtained for a plot of the Stokes radius versus the cube root of the partition coefficient (Siegel & Monty, 1966).

2.5.4 Sucrose density centrifugation.

Sedimentation on sucrose gradients was with a SW 50.1 rotor in a Beckman L5-65 centrifuge essentially as described by Letarte-Muirhead et al (1974). 5-20% sucrose gradients of volume 5.1 ml in 1% (w/v)

deoxycholate, 0.01M Tris-HCl, 0.02% (w/v) NaN_3, pH 8.0, were prepared with a device similar to that described by Martin & Ames (1961); gradient centrifugation in ^2H_2O (Meunier et al, 1972) was also carried out and for this all solutions and extracts were prepared in ^2H_2O.

Deoxycholate extract (0.25 ml), to which marker proteins had been added, was applied to the top of the sucrose gradient. The markers used were 30μg each of rat serum albumin, and ovalbumin and trace amounts of "^{125}I-labelled" myoglobin (0.25μCi/μg). The gradients were run at 5°C at 44,000 rev/min for 40h and then fractionated by inserting a long thin needle to the bottom of the tube and with an LKB periflex pump,0.33 ml fractions were collected. The linearity of the gradients was checked by reading the refractive indexes of these fractions with an Abbé refractometer. The position of "^{125}I-labelled" myoglobin was determined by counting the fractions in a Nuclear Enterprises Gamma counter and the position of the other markers was determined with specific antisera and double diffusion micro-analysis (Clausen, 1969). Although only semiquantitative, this method indicated the peak of each marker protein. Each fraction was assayed for Thy-1 antigenic activities.

2.5.5 Marker proteins for gel filtration and sucrose gradient centrifugation.

The purity and size of all marker proteins were tested by polyacrylamide gel electrophoresis in SDS. The molecular parameters of these proteins, used in the calculation of the hydrodynamic parameters of Thy-1 are shown in Table 2.1.

2.6 Miscellaneous Methods

2.6.1 Purification of sodium deoxycholate.

In later experiments on the chemical characterisation of Thy-1, the deoxycholate used in the purification had been recrystallized twice from acetone:water (80:20, v/v). On dissolving the deoxycholate the solution was filtered; this removed a considerable amount of yellowish

Table 2.1 Molecular Parameters of Marker Proteins

Protein	Partial specific volume ($\bar{v}$) (ml/g)	Stokes radius (nm)	Sedimentation coefficient ($S_{20,w} \times 10^{13}$)	Molecular weight (daltons)	References
Myoglobin	0.739	1.7[a]	2.08	17,900	Banaszak et al, 1963 Crumpton & Polson, 1965
Ovalbumin	0.749	3.0	3.55	43,000	Smith, 1970 Tanford et al, 1974
Rat serum albumin	0.720	3.6[a]	4.41	63,600	Smith, 1970
Rabbit IgG	0.72	4.8	6.7	148,000	Ansari & Salahuddin, 1973 Noelken et al, 1965

a) The Stokes radius was calculated from:

Molecular Weight = $6\pi\eta NaS/(1-\bar{v}\rho)$ (Siegel & Monty, 1966)

where η is viscosity; a, Stokes radius; S, $S_{120,w}$; $\bar{v}$, partial specific volume and ρ, density of medium.

material. Two recrystallizations reduced the $E^{1\ cm}_{280\ nm}$ for 1% solution from about 0.15 to 0.03-0.05.

2.6.2 Concentration of fractions.

Deoxycholate solutions were concentrated with an Amicon Diaflo apparatus with a PM10 membrane which had been equilibrated with deoxycholate. When fractions are concentrated by this method, the detergent is also concentrated and this can lead to the formation of a gel. Thus where a large concentration step was required, e.g. after lentil lectin affinity chromatography, a Bio Fibre 80 ultrafiltration beaker (Bio-Rad Labs., Bromley, Kent) was used. The deoxycholate concentration was reduced by simultaneous dialysis with 0.01M Tris-HCl, pH 8.0.

2.6.3 Removal of deoxycholate by ethanol precipitation.

After concentration of the purified Thy-1 obtained after gel filtration, the Thy-1 was at approximately 1 mg/ml in 60 mg deoxycholate/ml. To remove the deoxycholate, absolute ethanol at -20°C was added to a final concentration of 75%. A few drops of a saturated solution of anhydrous sodium acetate in absolute ethanol were added to enhance flocculation and the mixture left at -20°C for 48h. The precipitate was recovered and washed in absolute ethanol by centrifugation at 40,000 g.min at -10°C. The pellet was dissolved in water and centrifuged at 3×10^6 g.min.

2.6.4 Effect of heat on Thy-1 antigenic activities.

Purified Thy-1 after precipitation with ethanol was taken up at 10 μg/ml in 0.1% (w/v) sodium deoxycholate, 0.25% bovine serum albumin, 0.01M Tris-HCl, 0.02% NaN_3, pH 8.0. Aliquots were heated at a variety of temperatures for 10 min., cooled to 0°C and assayed for Thy-1.1 and Thy-1 xenoantigenic activities.

2.6.5 Effect of proteolytic enzymes on Thy-1.

Purified Thy-1 at 400 μg/ml in 0.25% (w/v) deoxycholate, 0.02% NaN_3,

0.01M Tris-HCl pH 8.0 was incubated with various proteolytic enzymes at an enzyme:protein ratio of 1:12, at 37°C for 24h. A control was treated similarly but without the addition of enzyme. An aliquot was taken for analysis by electrophoresis on polyacrylamide gels in SDS, heated to 100°C for 3 min. and stored at -70°C. The Thy-1.1 and Thy-1 xenoantigenic activities were assayed at 0°C in a 1500 fold excess of bovine serum albumin to inhibit residual proteolytic activity. Controls with bovine serum albumin were treated similarly, and tested in the assays to show that the residual enzymes had no effect. These controls were also analysed by polyacrylamide gel electrophoresis which indicated that all these enzyme preparations were active.

The effects of pronase, trypsin, chymotrypsin and papain were tested under these conditions except that papain was added in the presence of 0.04M cysteine-HCl.

2.6.6 Effect of reduction and alkylation on Thy-1 antigenic activities.

2 μg of purified brain Thy-1 after precipitation with ethanol was dried down in vacuo. 5 μl of 7M guanidine-HCl, 0.5M Tris-HCl, 0.007M dithiothreitol, pH 8.1 was added. Two controls were run in which 5 μl of 0.14M NaCl, 0.025M Tris-HCl, pH 8.0 or 5 μl of 7M guanidine-HCl, 0.5M Tris-HCl, pH 8.1 were added to the same amount of Thy-1 to give the "buffer control" and "guanidine control" respectively. The samples were incubated at 37°C for 2½h and 1 μl 0.095M iodoacetamide in 7M guanidine-HCl, 0.5M Tris-HCl, pH 8.1 added. After incubation for ½h at 37°C, the samples were stored at -70°C. The Thy-1.1 and Thy-1 xenoantigenic determinants were assayed after each of the samples had been diluted with 0.5 ml of 0.25% BSA in 0.14M NaCl, 0.025M Tris-HCl, pH 8.0 buffer.

2.7 Equilibrium ultracentrifugation

Sedimentation equilibrium experiments were performed in a Beckman

Spinco Model E ultracentrifuge using an An-D rotor. A 12 mm double-sector cell with carbon-filled epon centrepiece and sapphire windows was used with buffer in the reference channel. The column heights were 3mm. The sedimentation equilibrium experiments were of the meniscus depletion type (see Yphantis, 1964) and the equilibrium distribution was recorded with Rayliegh interference optics.

Thymocyte Thy-1_{L+} at 0.7 mg/ml in 0.1M Tris-HCl, 0.02% NaN_3, pH 8.0 was centrifuged at 16.6°C and 8776 rev/min. The sedimentation was observed at 6 hourly intervals to ensure that equilibrium had been obtained. For sedimentation in 0.5% deoxycholate, the thymocyte Thy-1_{L+} above was diluted with an equal volume of 1.0% deoxycholate in the same buffer and centrifuged at 21°C and 37,020 rev/min.

CHAPTER 3

THE SOLUBILISATION OF THE THY-1 ANTIGEN FROM RAT BRAIN

3.1 Introduction

Rat brain contains large amounts of Thy-1 antigenic activity (Douglas, 1972; Acton et al, 1974) and the availability of this tissue makes it a suitable source of material to attempt to purify this antigen in sufficient quantities for biochemical characterisation (see below). The Thy-1 molecule in the rat is recognised by the Thy-1.1 alloantigenic determinant which can be genetically defined in the mouse (Reif & Allen, 1964) and the specificity of the Thy-1.1 assay used, is described in Ch.2.4.1. The Thy-1 molecule was also followed by an assay for the Thy-1 xenoantigenic determinants as this assay gave a higher specific binding to background ratio and was unaffected by deoxycholate (see Ch. 2.4.1 and below).

As a first step in the purification of Thy-1, the solubilisation of the antigen from rat brain membrane was attempted. This chapter describes the effective solubilisation obtained with deoxycholate and properties of the Thy-1 antigen in this detergent.

3.2 The Quantitation of the Thy-1 antigen in rat brain.

The amount of Thy-1 in rat thymocytes has been estimated to be 2.8 mg per 10^{11} thymocytes (Letarte-Muirhead et al, 1975). This value is based on an estimate of 600,000 Thy-1.1 antigenic sites per thymocyte (Acton et al, 1974) and a molecular weight of 28,000 calculated from hydrodynamic parameters in deoxycholate (Letarte-Muirhead et al, 1974). If the inhibition of the Thy-1.1 assay by rat brain homogenate is compared with that obtained with rat thymocytes (Fig. 3.1) an estimate



Fig. 3.1 Inhibition of assay for Thy-1.1 by rat thymocytes and brain

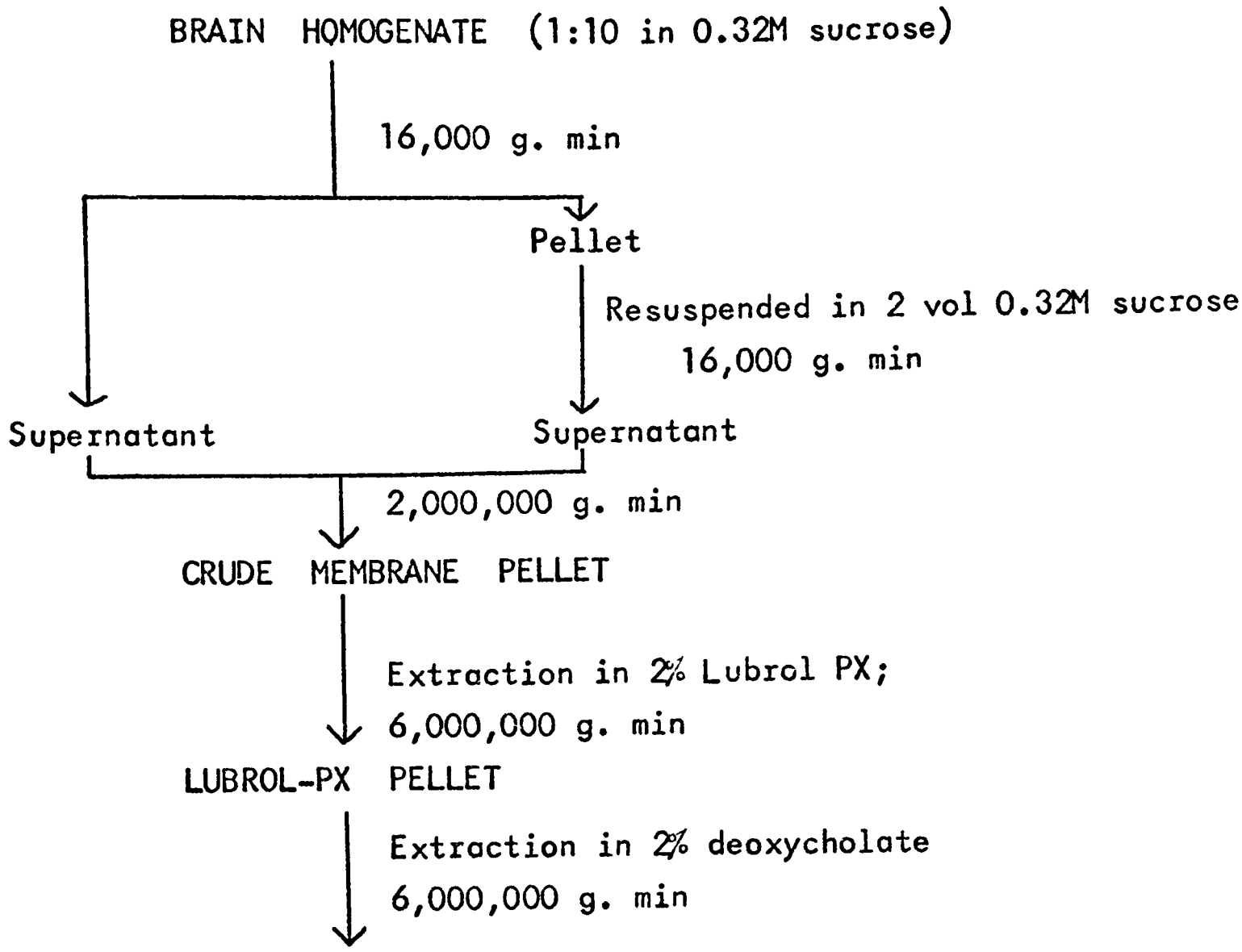
25 µl aliquots of CBA mouse anti-(Wistar rat brain) serum diluted 1/150 were absorbed with rat thymocytes (□) and rat brain homogenate (o); the binding of residual antibody to AKR mouse thymocyte target cells was followed with ^{125}I-labelled rabbit anti-(mouse IgG).

of the amount of Thy-1 in rat brain can also be made. Assuming that brain Thy-1 has a similar molecular weight to that of thymocyte Thy-1 (see below) then there is 95 μg Thy-1 per g wet weight of brain or 145 μg per average rat brain. Thus, on a per rat basis, there is more Thy-1 in brain than thymus which contains about 40 μg per animal (1.5×10^9 thymocytes). On a protein basis a purification of about 1200 fold should result in pure Thy-1.

3.3 The solubilisation of Thy-1 antigenic activities.

A previous attempt to solubilise Thy-1 antigenic activity from rat brain showed that several non-ionic detergents did not inactivate the antigenic activity but nor did they give effective solubilisation (Letarte-Muirhead et al, 1974). However a method for solubilising the Thy-1 antigenic activities was found and is illustrated in Fig. 3.2 with the yields obtained for both Thy-1 assays. A crude membrane was prepared in high yield from which solubilisation was attempted. Deoxycholate gave effective solubilisation if the crude membrane was first extracted with the non-ionic detergent lubrol PX. The yield of solubilised Thy-1 was similar to that obtained if the crude membrane was extracted directly with deoxycholate but the lubrol extraction solubilised about 40% of the protein and phospholipids without loss of Thy-1 antigenic activities and thus gave about a 2-fold purification in Thy-1 (Fig. 3.2). The yield of solubilisation was further increased when the membrane in deoxycholate was frozen and thawed before centrifugation, and about 50% of the Thy-1 antigenic activities was obtained in a 6,000,000 g.min. deoxycholate supernatant. The yields obtained for the two Thy-1 assays were slightly different and an increase in the amount of Thy-1.1 antigenic activity was observed on solubilisation with deoxycholate. It would appear that some of this determinant may be masked before the addition of deoxycholate but this effect is difficult

Fig. 3.2 Solubilisation of brain Thy-1



DEOXYCHOLATE EXTRACT

(supernatant)

Fraction	% Yield of:			
	Protein	Phospho-lipid	Thy-1.1 determinant	Thy-1 xenoantigenic determinants
Brain homogenate	100	100	100	100
Crude membrane pellet	39	69	70	80
Lubrol PX pellet	22	41	43	77
Deoxycholate extract	19	28	70	42

to estimate as the Thy-1.1 inhibition curve has a higher background in the presence of deoxycholate. This is illustrated in Fig. 3.3 which shows the effect on the Thy-1.1 assay by the addition of deoxycholate to the "lubrol pellet" (see Fig. 3.2). This effect is discussed further in Ch. 5.4.

3.4 The hydrodynamic properties of Thy-1 in deoxycholate.

The nature of the solubilised Thy-1 antigen in deoxycholate was studied by zone sedimentation on sucrose gradients and by gel filtration. Such an analysis at an early stage in the purification should show whether the antigenic activity is associated with a single homogeneous component or multiple forms in deoxycholate. Also from the molecular parameters obtained by these methods, the molecular weight and frictional coefficient of the antigen-detergent complex can be estimated.

3.4.1 Sedimentation of brain Thy-1 on sucrose density gradients.

Solubilised rat brain Thy-1 in deoxycholate was sedimented on 5-20% (w/v) sucrose gradients in H_2O (Fig. 3.4a) and ^2H_2O (Fig. 3.4b) assaying for both Thy-1.1 alloantigenic and Thy-1 xenoantigenic determinants. Sedimentation in the ^2H_2O gradients in which the density was 1.118-1.164 g/ml compared to 1.018-1.073 g/ml for the H_2O gradients, was carried out in order to estimate the partial specific volume ($\bar{v}$) which should indicate whether the antigenic activity is associated with low density material, i.e. lipids (Meunier et al, 1972). Fig. 3.4 clearly shows that there is a high recovery of both Thy-1 antigenic activities which ran together as homogenous components on both gradients. The refractive indexes of the gradient fractions after centrifugation were measured (Fig. 3.5) and showed that the density gradients were linear except for the bottom fractions which were not used in the determination of the parameters below. The distances moved by the Thy-1 antigen and

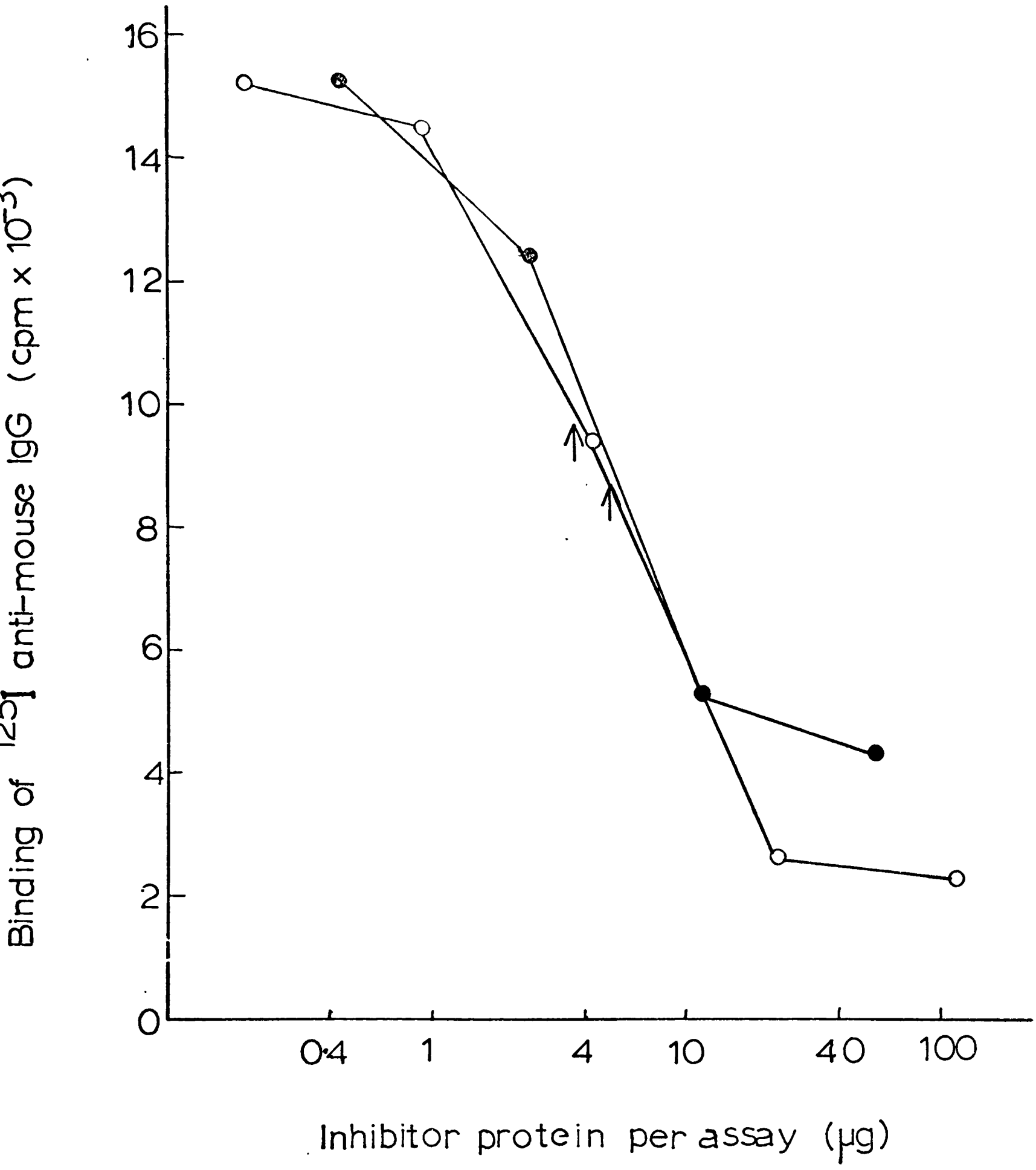


Fig. 3.3 Effect of deoxycholate on the antigenic activity of the "lubrol pellet".

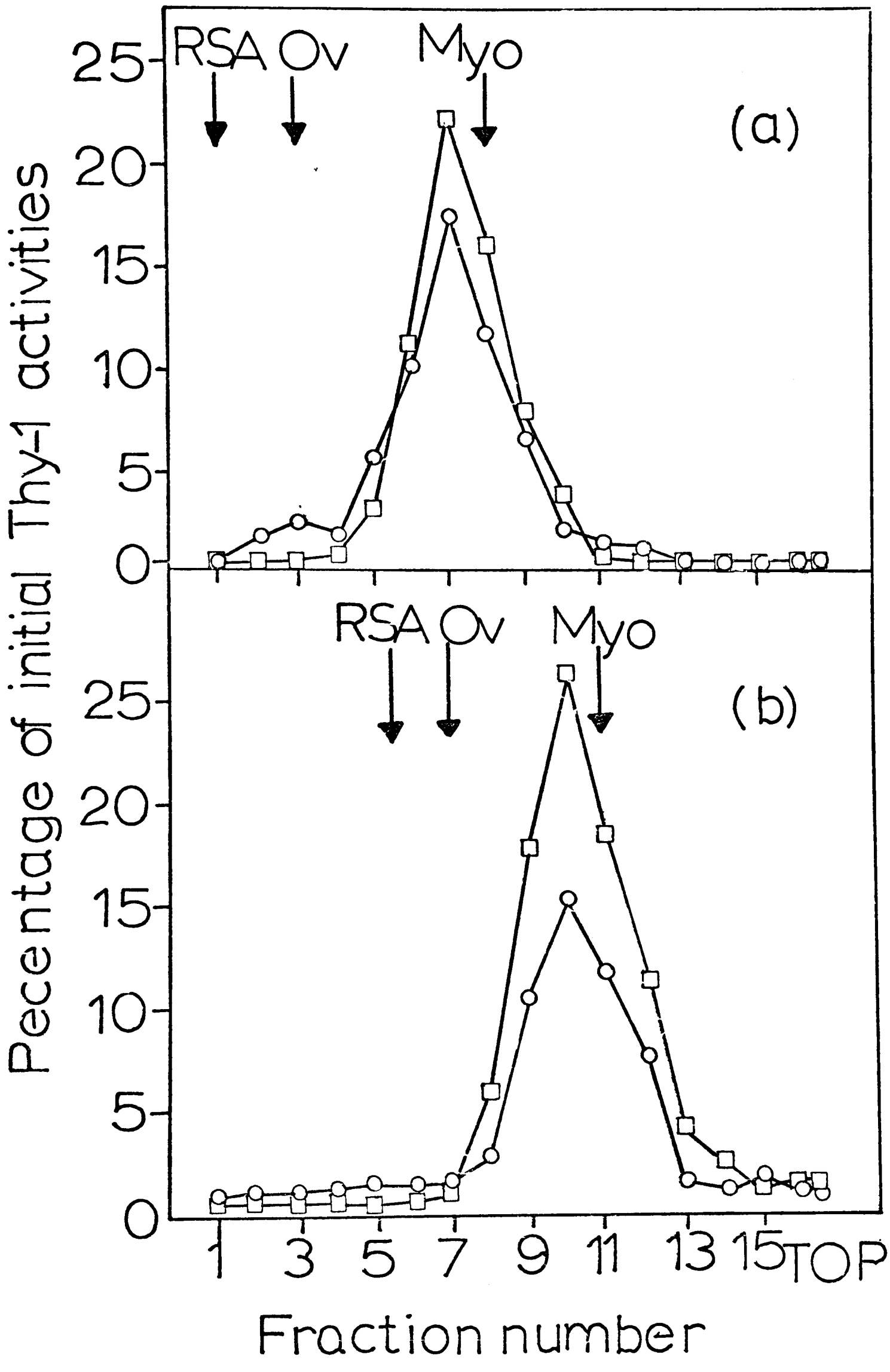
25 µl aliquots of Balb/c anti-(Wistar rat brain) serum diluted 1/50 were absorbed with "lubrol pellet" (see Fig. 3.2) (o) and lubrol pellet after the addition of an equal volume of 4% (w/v) deoxycholate (●); the latter extract was diluted so that the final concentration of deoxycholate was 0.2% in all the dilutions of the extract.

The binding of the residual antibody to AKR mouse thymocyte target cells was followed with ^{125}I-labelled rabbit anti-(mouse IgG). The points of 50% inhibition of the two inhibitions are indicated by arrows.

Fig. 3.4 Sucrose gradient centrifugation of solubilised Thy-1 in deoxycholate

(a) shows the distribution of Thy-1.1 (o) and Thy-1 xenoantigenic (□) activities after centrifugation for 40h at 44,000 rev/min of deoxycholate extract of brain membrane on 5-20% sucrose gradient in 1% deoxycholate buffer in H_2O. The positions of marker proteins sedimented on the same gradient are shown: RSA, rat serum albumin; Ov, ovalbumin; Myo, myoglobin.

(b) as for (a) except gradients and extracts were prepared in ^2H_2O.


RSA
Ov
Myo
(a)
RSA
Ov
Myo
(b)
Pecentage of initial Thy-1 activities
0
5
10
15
20
25
1
3
5
7
9
11
13
15
TOP
Fraction number

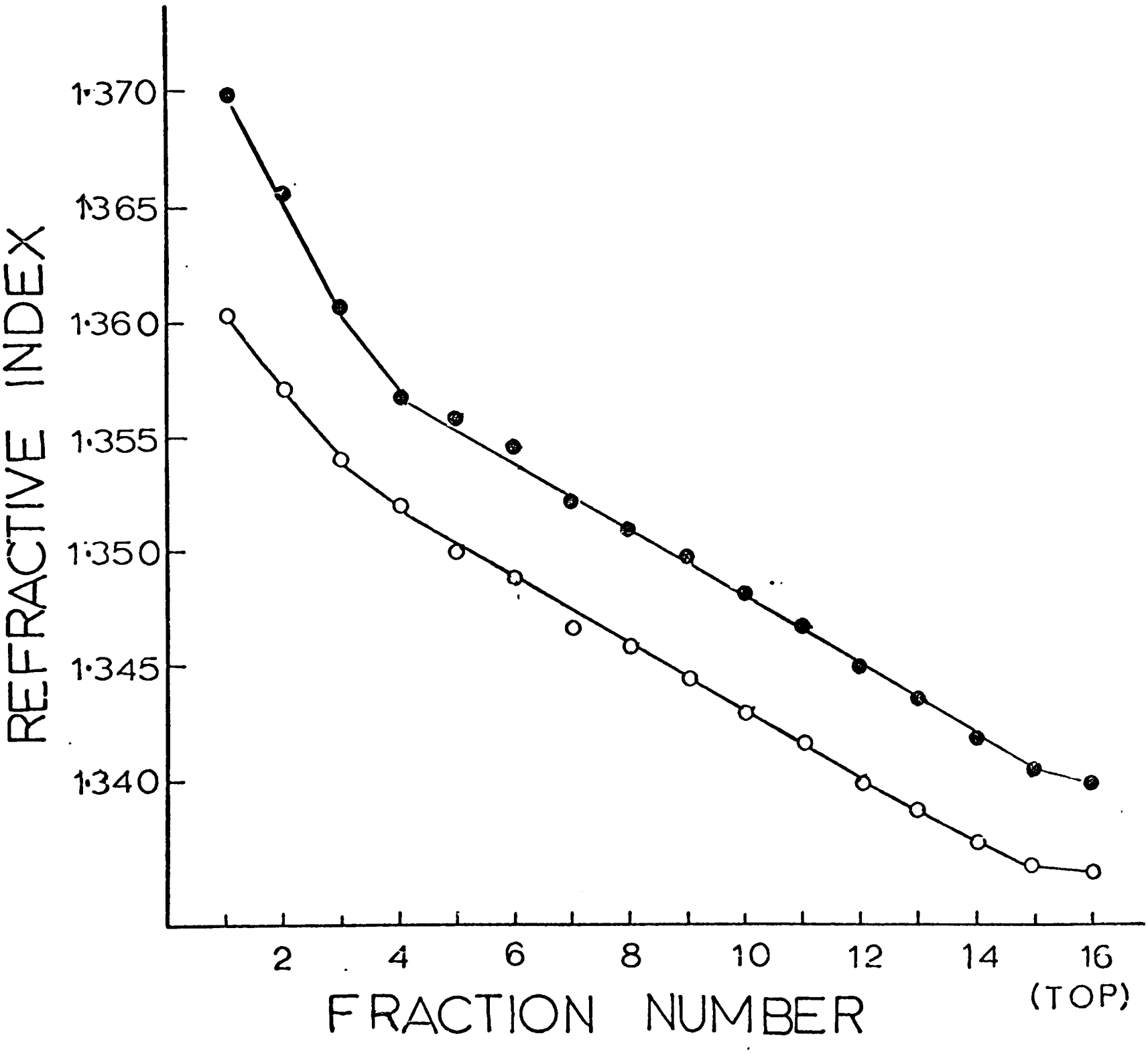


Fig. 3.5 The linearity of sucrose gradients

The refractive index of the sucrose density gradient fractions in Fig. 3.4 were measured with an Abbé refractometer; (o) represents fractions from gradient in H_2O (Fig. 3.4a) and (●) from gradient in ^2H_2O (Fig. 3.4b).

the marker proteins were measured and the partial specific volume ($\bar{v}$) and sedimentation coefficient ($S_{20,w}$) were calculated as described by Meunier et al, (1972) using the parameters of the marker proteins given in Ch. 2.5.5. For Thy-1 solubilised in deoxycholate, a $\bar{v}$ value of 0.72 ml/g and an $S_{,20,w}$ of 2.2s were calculated.

These results showed that Thy-1 was solubilised in a homogenous form in deoxycholate and was not associated with large amounts of lipid.

3.4.2 Gel filtration on Sephadex G-200 in deoxycholate

The elution of a solubilised Thy-1 extract from a Sephadex G-200 column in deoxycholate is shown in Fig. 3.6. (In this experiment the extract loaded was that after lentil lectin affinity chromatography - see Ch. 4.2). This shows that the Thy-1.1 antigenic activity is eluted in a homogeneous peak in the same elution position as ovalbumin. The Stokes radius of the Thy-1 antigen in deoxycholate was calculated from the calibration with marker proteins and the relationship between the Stokes radius and the cube root of the partition coefficient (see Ch.2.5.3), and a value of 3.0 nm was obtained.

3.4.3 Calculation of the hydrodynamic parameters of Thy-1 in deoxycholate.

From the values for $S_{,20,w}$, $\bar{v}$ and Stokes radius determined above the molecular weight of the Thy-1 antigen in deoxycholate was calculated with the relationship used by Siegel & Monty (1966)

$$M = \frac{6\pi\eta\, NaS}{1 - \bar{v}\rho}$$

where M, molecular weight; η, viscosity; N, Avogadro's number; a, Stokes radius; S, $S_{20,w}$; $\bar{v}$, partial specific volume; ρ, density of medium.

From this the molecular weight of Thy-1 in deoxycholate was estimated to be 27,000. Under the conditions used, micelles of deoxycholate should have a molecular weight of about 2,000 (Small, 1968) and thus it is unlikely that the antigenic activity is associated with a small

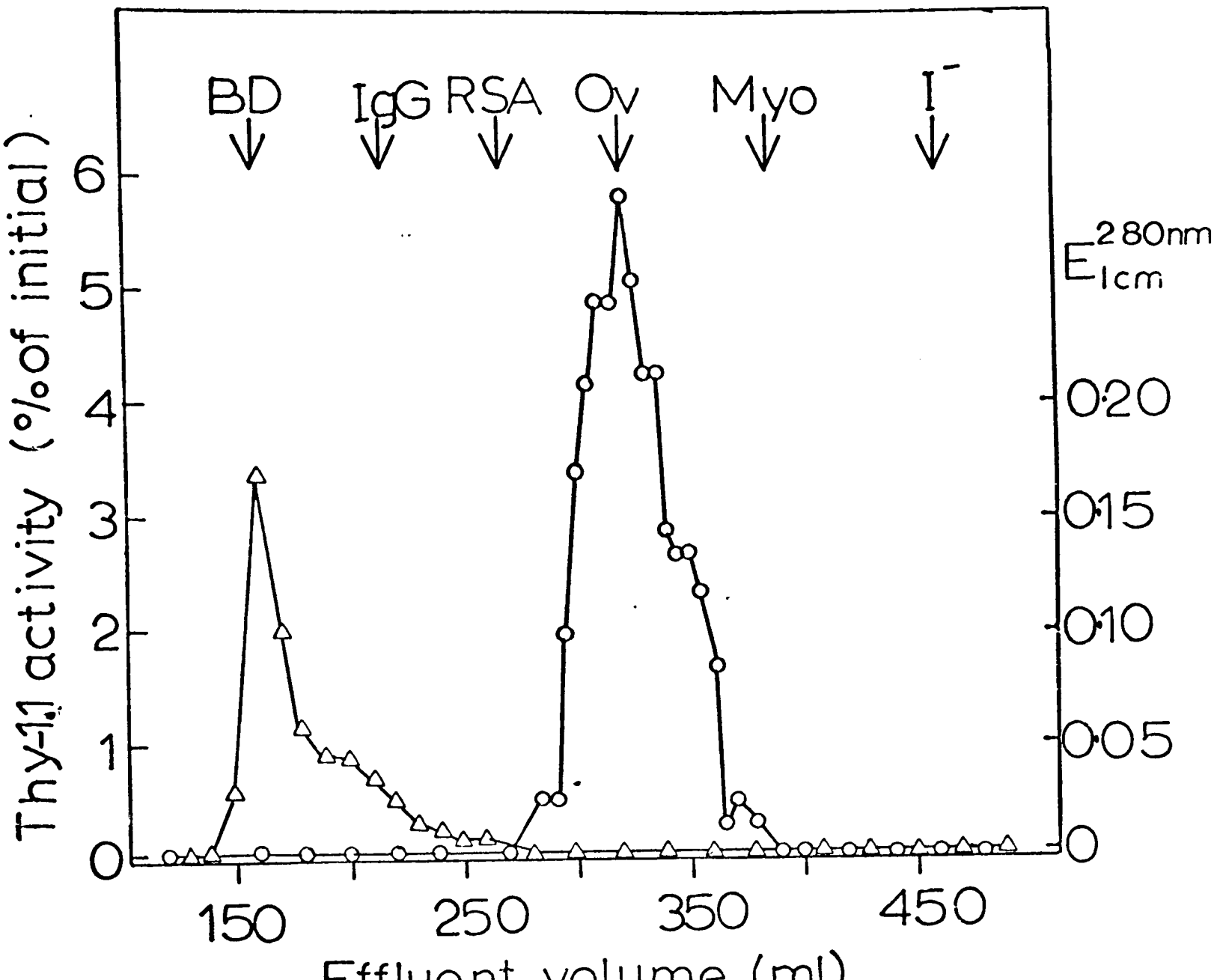


Fig. 3.6 Gel filtration of solubilised Thy-1 on Sephadex G-200 in deoxycholate

5 ml of eluate from lentil lectin column (see Ch. 4.2) was chromatographed on a Sephadex G-200 column (2.6 cm x 90 cm) in 0.5% deoxycholate. The fractions (5 ml) were assayed for Thy-1.1 antigenic activity (o) which is expressed as the percentage of the Thy-1.1 activity of the loaded extract and the $E^{1\ cm}_{280\ nm}$ (Δ) measured.

The elution positions of protein markers run immediately before and after the fractionation are shown: IgG, bovine IgG; RSA, rat serum albumin; Ov, ovalbumin; Myo, myoglobin. The void and total volumes are indicated by Blue Dextran (BD) and Na ^{125}I (I^-).

component, for example a glycolipid, which is incorporated into a detergent micelle; such mixed micelles would not be expected to be large in these conditions where a large excess of deoxycholate was present (Helenius & Simons, 1975).

The frictional coefficient was estimated from the relationship:

$$\text{frictional coefficient } f/f_o = a\left(\frac{4\pi N}{3\bar{v} M}\right)^{\frac{1}{3}}$$

(abbreviations as above)

A value of 1.52 was obtained for the Thy-1 antigenic activity in deoxycholate.

3.5 Discussion.

Decxycholate was the only detergent tested which solubilised the Thy-1 antigen in good yield and the hydrodynamic properties of the antigen in this detergent were studied. The parameters obtained apply to the antigen plus bound deoxycholate and the molecular size is likely to be an overestimate as membrane proteins bind considerably more non-ionic or weakly ionic detergents (up to 120% of the weight of the protein) than the marker proteins (Helenius & Simons, 1975; Clarke, 1975).

The $\bar{v}$ value of 0.72 ml/g obtained for brain Thy-1 is typical of a protein ($\bar{v}$ = 0.70-0.74 ml/g; Cohn & Edsall, 1943; McMeekin & Marshall, 1952) or a glycoprotein (for the sugar residues $\bar{v}$ = 0.63-0.70 ml/g; Gibbons, 1972). The $\bar{v}$ value for proteins and glycoproteins will be affected only slightly by the binding of deoxycholate which has a $\bar{v}$ of 0.76 ml/g (Small, 1968).

The frictional coefficient of 1.52 indicates that the solubilised Thy-1 plus bound deoxycholate is somewhat asymmetric compared with globular proteins, for example urease and haemoglobin (frictional coefficients of 1.19 and 1.14 respectively) but less asymmetric than

non-globular proteins like fibrinogen (frictional coefficient = 2.34; Siegel & Monty, 1966). Several other membrane molecules have similar frictional ciefficients in deoxycholate (1.5-1.8) to solubilised Thy-1 e.g. rat histocompatibility antigen, Ag-B(4) (Letarte-Muirhead et al, 1974); insulin receptor (Cuatrecasas, 1972), gonadotropin receptor (Dufau et al, 1973), acetylcholine receptor (Meunier et al, 1972).

Comparison of the hydrodynamic parameters of brain Thy-1 in deoxycholate with those of thymocyte Thy-1 in Table 3.1 shows that, given the accuracy of the methods used, the Thy-1 antigens from the two tissues cannot be distinguished. In both cases only one homogeneous component was observed.

Table 3.1 The hydrodynamic parameters of Thy-1 solubilised in deoxycholate from rat brain and thymocytes.

	Brain Thy-1	Thymocyte Thy-1
$S_{,20,w}$	2.2	2.4
$\bar{v}$ (ml/g)	0.72	0.70
Stokes radius (nm)	3.0	3.1
Molecular Weight	27,000	28,000
Frictional Coefficient	1.52	1.57

The data for thymocyte Thy-1 are from Letarte-Muirhead et al (1974).

CHAPTER 4

THE PURIFICATION OF THE THY-1 ANTIGEN FROM RAT BRAIN

4.1 Introduction

The solubilisation of Thy-1 by deoxycholate (Ch. 3) allows the application of conventional biochemical techniques to separate Thy-1 from the other membrane components. This chapter describes the purification of Thy-1 by affinity chromatography with lentil lectin and gel filtration in deoxycholate. The properties of the purified antigen are compared to those obtained for thymocyte Thy-1 by Letarte-Muirhead et al (1975).

4.2 Affinity chromatography with lentil lectin

A deoxycholate extract was prepared as in Ch. 3 in a typical preparation from 63g wet weight of rat brain with the yields shown in Table 4.1 for Thy-1 antigenic activities, phospholipid and protein. This extract was passed through a lentil lectin affinity column and Fig. 4.1 shows that the majority of the protein, as indicated by the E^{1cm}_{280nm} values was not retarded by the column whereas the majority of the Thy-1.1 activity was bound and could be eluted with methyl α-D-glucopyranoside in deoxycholate. In this experiment the affinity column was approaching saturation, but under non-saturating conditions (e.g. in the first fractions through the column) more than 80% of the Thy-1 was bound. The yields obtained (Table 4.1) indicated that about 80% of the Thy-1 xenoantigenic activity loaded onto the affinity column was eluted with methyl α-D-glucopyranoside in deoxycholate. In terms of Thy-1.1 antigenic activity there appeared to be a considerable loss of Thy-1 antigen, but this may be more apparent than real since the Thy-1.1

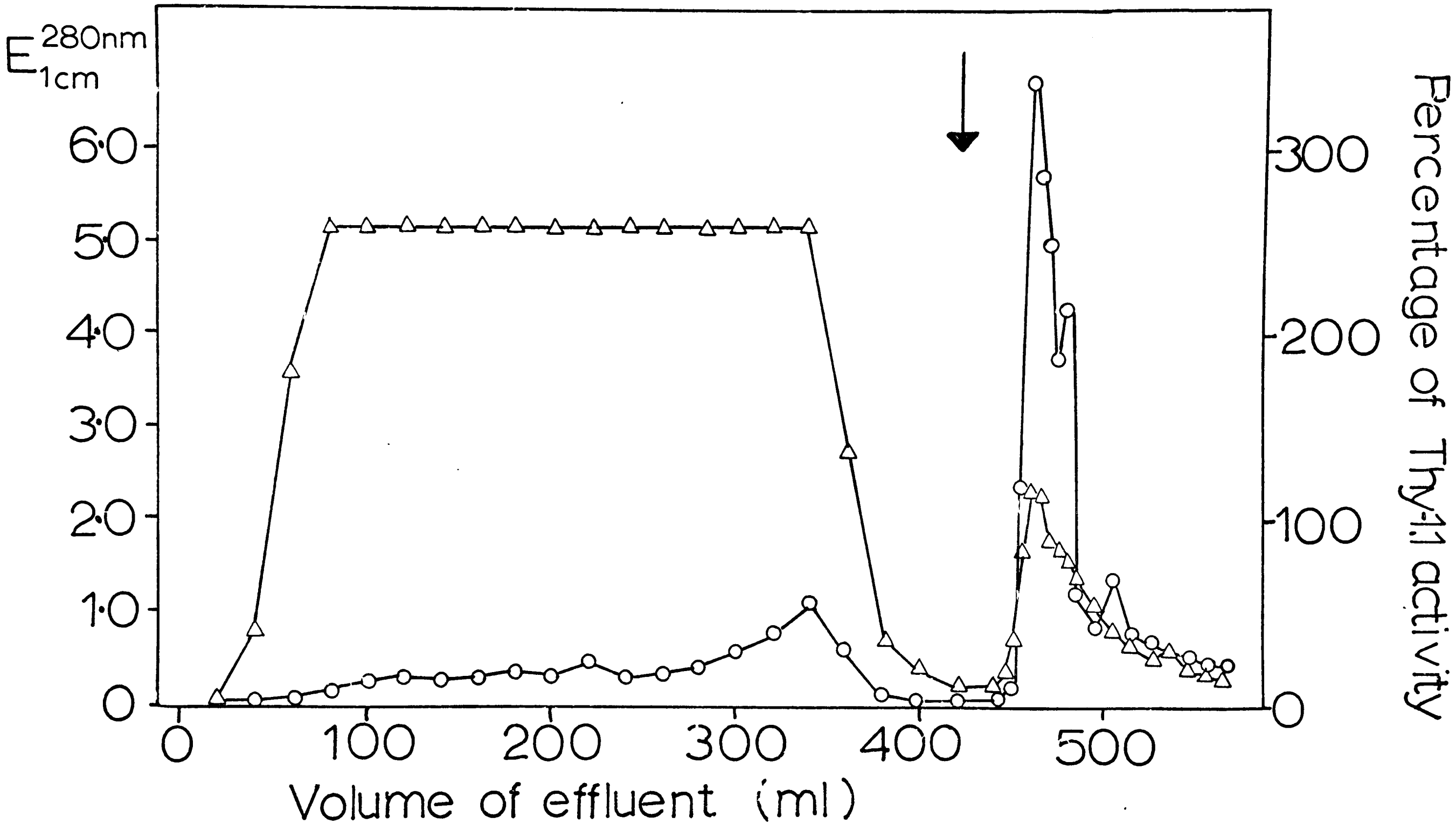
			Thy-1.1 antigenic determinant			Thy-1 xenoantigenic determinants		
Fraction	Protein (mg)	Phospholipid (mg)	10^{-5} x activity (units)	Relative sp. activity	Yield (%)	10^{-5} x activity (units)	Relative sp. activity	Yield (%)
Brain homogenate	7300	2900	18.7	1.0	100	81.5	1.0	100
Crude membrane pellet	2880	2000	13.0	1.8	70	65.5	2.0	80
Lubrol-PX pellet	1600	1200	8.0	2.0	43	63.0	3.5	77
Deoxycholate extract	1440	799	13.0	3.5	70	34.0	2.1	42
Eluted from lentil lectin	74	10	4.5	24	24	28.0	34	34
After Sephadex G-200 chromatography	2.0	< 0.1	2.2	440	12	16.0	720	20

Table 4.1 Purification of brain Thy-1

A unit of activity is defined as the amount of antigen needed to give 50% inhibition of the appropriate binding assay. The specific activity is the number of units of activity per mg of protein and is related to that of brain homogenate.

Fig. 4.1 Affinity chromatography with a lentil lectin column.

325 ml of solubilised Thy-1 in deoxycholate was loaded on to a lentil lectin-Sepharose 4B affinity column (volume 35 ml) and bound material eluted with 1M methyl α-D-glycopyranoside in 1% (w/v) deoxycholate buffer, commencing at the point indicated by the arrow. The fractions were assayed for Thy-1.1 antigenic activity (o) which is expressed as a percentage of that in the starting extract (3,600 units/ml) and the $E^{1\ cm}_{280\ nm}$ was measured (Δ).


E^{280nm}_{1cm}
60
50
40
30
20
10
0
300
200
100
0
0
100
200
300
400
500
Volume of effluent (ml)
Percentage of Thy-1.1 activity

assay is affected to some extent by deoxycholate (see Ch. 3.3). This is probably due to antibodies with low affinities and is discussed further in Ch. 5.4. However the overall yield after the lentil lectin step was in reasonable agreement for the two assays.

The profile in Fig. 4.1 indicated that this was a particularly good purification step and Table 4.1 showed that the majority of the Thy-1 antigenic activity was eluted with only 5% of the protein and less than 2% of the phospholipids loaded onto the affinity column.

4.3 Gel filtration on Sephadex G-200 in deoxycholate

The Thy-1 containing material eluted from the lentil lectin column was concentrated and chromatographed on Sephadex G-200 in deoxycholate. Fig. 4.2 shows that the Thy-1.1 antigenic activity was recovered in high yield as a homogenous peak which was separated from the bulk of the protein (as indicated by the E^{1cm}_{280nm}). This was also a good purification step as shown in Table 4.1.

4.4 Analysis of the purification by polyacrylamide gel electrophoresis in SDS

From the results given in Table 4.1 and the calculation for the purification required in Ch. 3.2, Thy-1 should be a major component in the fraction containing Thy-1 antigenic activity after the gel filtration step; it may also be detectable in the material eluted from the lentil lectin column (Ch. 4.2). Thus samples from these stages were analysed by polyacrylamide gel electrophoresis in SDS and the gels stained for protein and carbohydrate (Fig. 4.3). Several carbohydrate and protein staining bands were present in the eluate from the lentil lectin (Fig. 4.3.a and c). However after the gel filtration only one band remained, staining for both protein and carbohydrate (Fig. 4.3.b and c). After the gel filtration a minor glycoprotein was also sometimes detected, but this could be eliminated by a second step of gel filtration with Sephadex G-200 in deoxycholate, to give the single glycoprotein in the antigenically

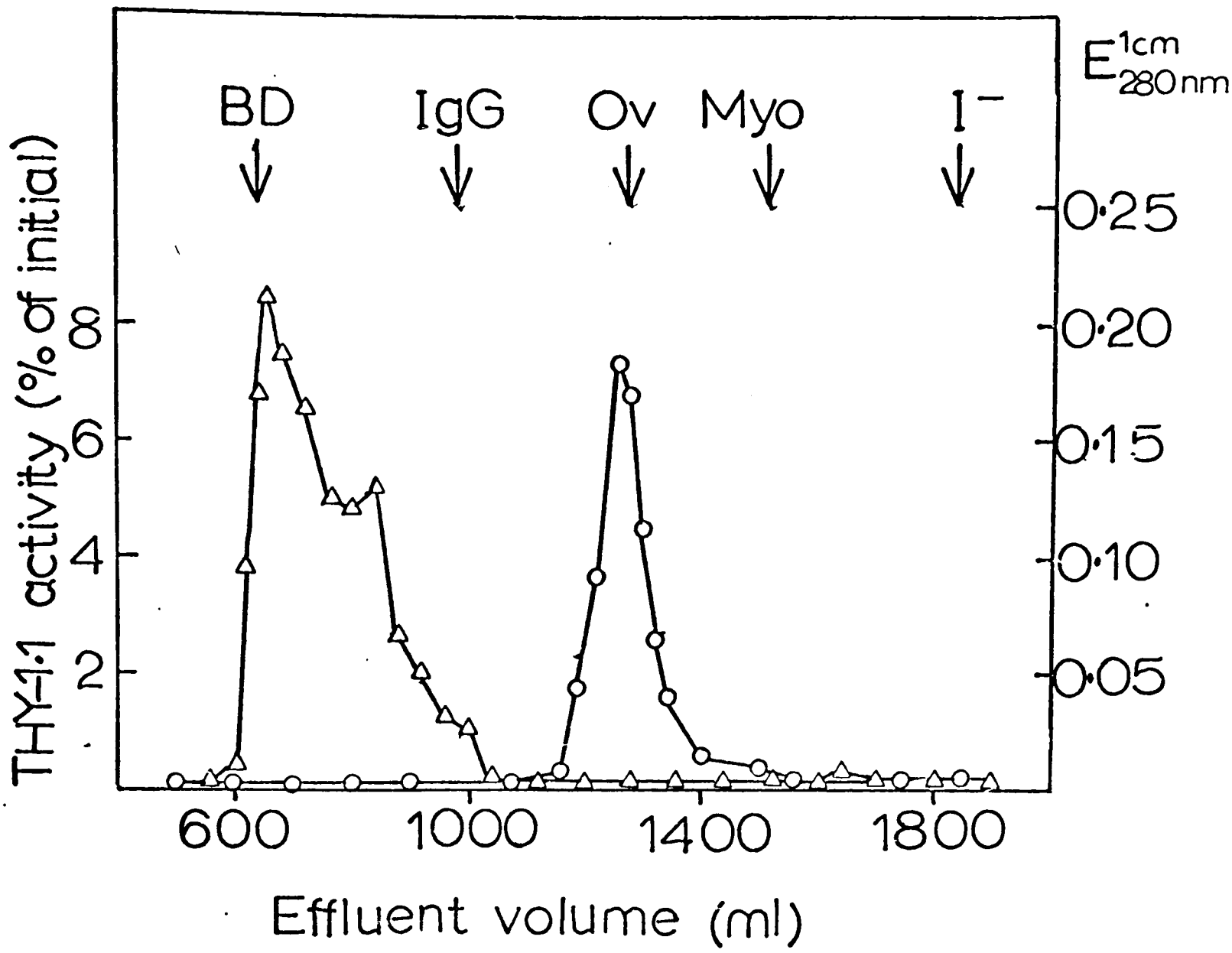


Fig. 4.2 Gel filtration of eluate from the lentil lectin column on Sephadex G-200 in deoxycholate

60 mls of eluate from the lentil lectin affinity column was chromatographed on a Sephadex G-200 column (5 cm x 90 cm) in 0.5% deoxycholate. Fractions (20 ml) were assayed for Thy-1.1 antigenic activity (o) which is expressed as the percentage of the loaded extract, and the $E^{1\ cm}_{280\ nm}$ (Δ) measured.

The elution positions of protein markers run immediately prior to the fractionation are shown: IgG, bovine IgG, Ov, ovalbumin, Myo, myoglobin. The void and total volumes are indicated by BD (Blue Dextran) and I^- ($Na\ ^{125}I$)

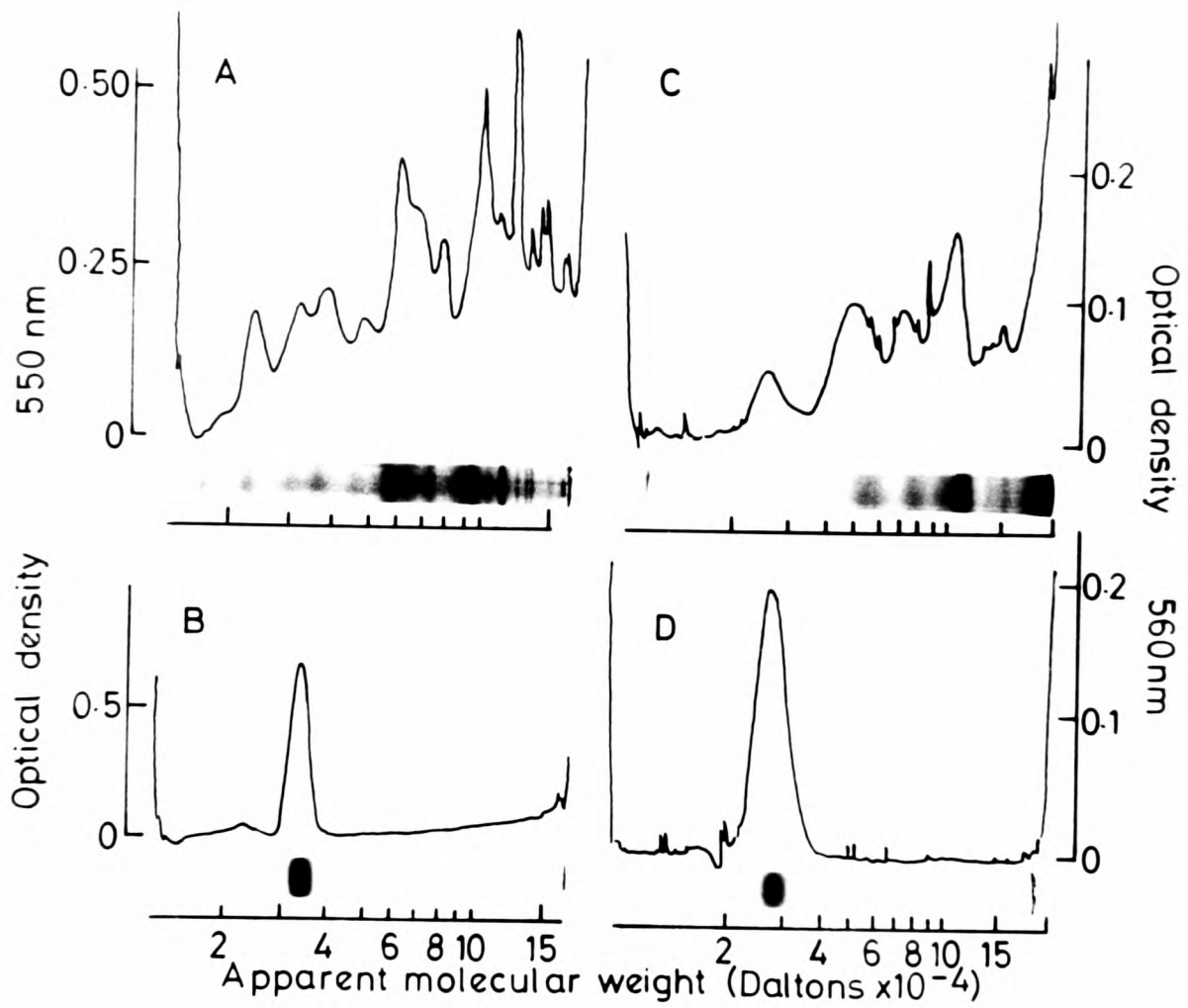


Fig. 4.3 Polyacrylamide gel electrophoresis in SDS of fractions during purification

The following samples were loaded on to 5.6% polyacrylamide gels in SDS: 45 μg (a) and 220 μg (c) of eluate from lentil lectin column; 9 μg (b) and 22 μg (d) of the Thy-1 fraction after rechromatography on Sephadex G-200 in deoxycholate. Gels were stained for protein (a and b) or carbohydrate (c and d) and scanned at 550 nm and 560 nm respectively.

active fraction as in Fig. 4.3.b and d. As Thy-1 was expected to be at least a major component after gel filtration, the most likely explanation is that brain Thy-1 is this glycoprotein.

However to confirm that all the Thy-1 antigenicity was associated with the glycoprotein, the antigenically active fraction after gel filtration was concentrated and electrophoresed on polyacrylamide gels in 0.1% SDS; the gels were sliced, eluted and assayed for Thy-1 antigenic activities as described in Ch. 2.2.2. This SDS treatment caused a small (approximately 30%) loss of antigenic activity compared with that in deoxycholate buffer, but the shapes of the inhibition curves were unaltered and both Thy-1.1 and Thy-1 xenoantigenic activities could be assayed. Fig. 4.4 shows that both antigenic activities ran with a mobility relative to the internal markers, indistinguishable from that of a sample run similarly except stained for protein. Thus this glycoprotein almost certainly carries the Thy-1 antigenic determinants.

Reduction and alkylation of the glycoprotein had no effect on its mobility; thus the molecule consists of only one polypeptide chain. As can be seen from Fig. 4.3, Thy-1 electrophoresed as a single symmetrical band on polyacrylamide gels in SDS.

As many glycoproteins electrophorese anomalously on polyacrylamide gels in SDS, giving different apparent molecular weights with different concentrations of acrylamide (Glossmann & Neville, 1971; Segrest et al, 1971), the apparent molecular weight was measured on gels of different acrylamide concentration. On 5.6% acrylamide gels the apparent molecular weight $\pm$ S.E.M. was 31,300 $\pm$ 1,000 and on 12.5% gels, 24,100 $\pm$ 150. These values are discussed below (Ch. 4.7).

4.5 Immunogenicity of brain Thy-1

To obtain further evidence that the glycoprotein purified from rat brain was identical to the Thy-1 antigen, antisera was raised in two rabbits against purified brain Thy-1 and analysed for binding to rat

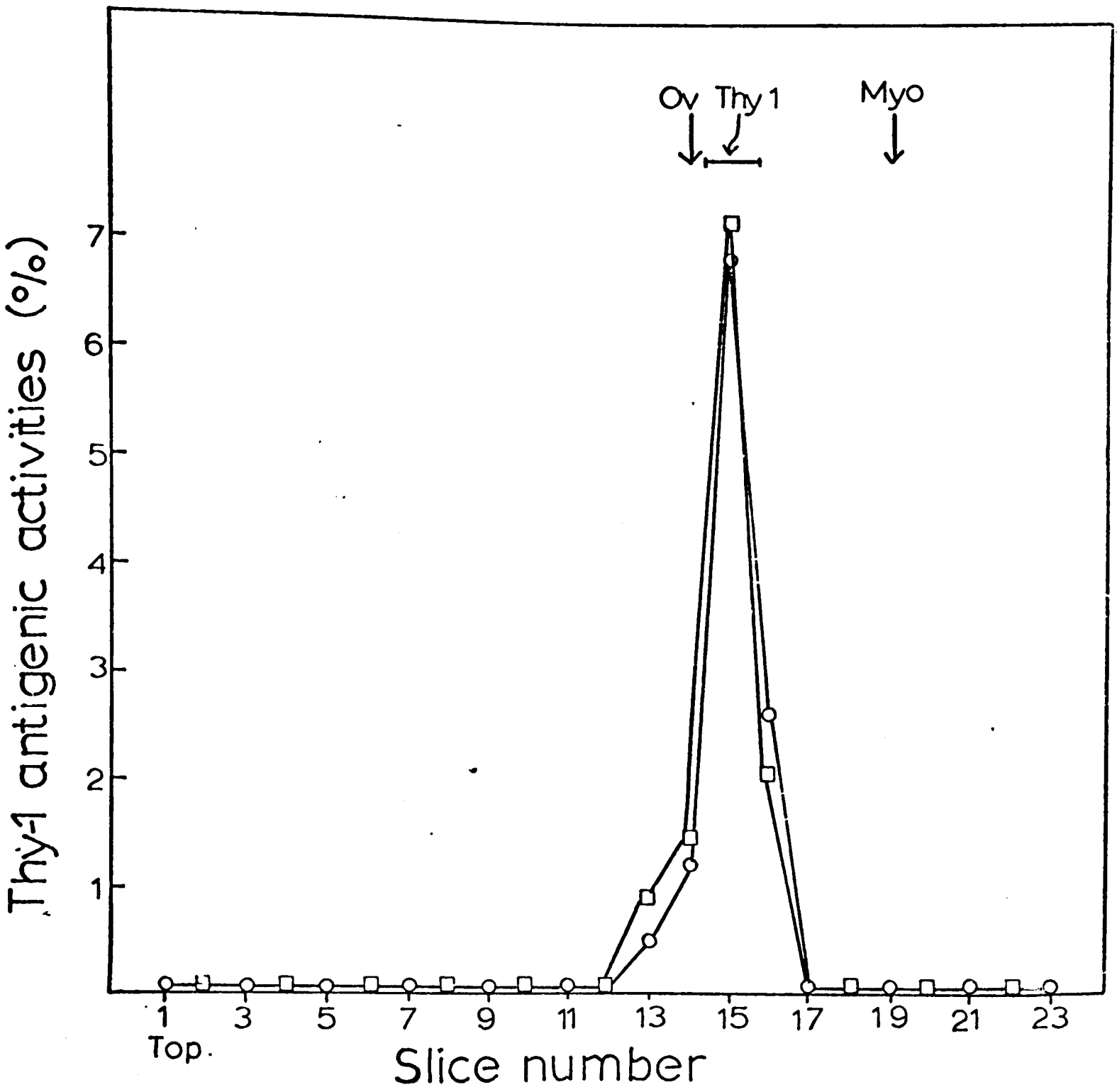


Fig. 4.4 Assay for Thy-1 antigenic activities following electrophoresis on polyacrylamide gels in SDS

3 μg of brain Thy-1 was electrophoresed on 5.6% polyacrylamide gels in 0.1% SDS, the gels sliced and assayed for Thy-1.1 (o) and Thy-1 xeno-antigenic determinants (□). Ov and Myo represent the positions of ovalbumin and myoglobin respectively, which were co-run with the Thy-1 sample. 8 μg of Thy-1 was also electrophoresed on a separate gel and stained for protein. The position of the Thy-1 band was determined relative to the ovalbumin and myoglobin markers and indicated by (-) and (Thy-1).

thymocytes. From previous studies (Morris & Williams, 1975), such sera should have antibodies against a component absent from liver, but found in large amounts on brain and thymocytes and in much smaller amounts on spleen and lymph-node cells. This characteristic distribution was found for both of the rabbit and anti-(brain Thy-1) sera and is shown for one serum (Fig. 4.5). Thus antisera raised against this glycoprotein recognised antigens with the same tissue distribution as Thy-1.

The antisera were further analysed for their specificities against Thy-1 by absorption with A/Jax (Thy-1.2) and A/Thy-1.1 mouse brain, and Wistar rat brain. These gave absorptions of 26, 30 and 100% respectively for one serum (Fig. 4.6). Thus 70% of the anti-(Thy-1) antibodies were against the rat-specific xenoantigenic determinant, 26% against the rat-mouse cross-reacting xenoantigenic determinant and 4% against the Thy-1.1 alloantigenic determinant. As only 4% was against Thy-1.1 this antiserum was routinely used to assay Thy-1 xenoantigenic determinants (the rat specific and rat-mouse cross-reacting determinants were not distinguished; see Ch. 2.4.1.2).

The concentration of specific anti-(Thy-1) antibodies in this serum was 0.3 mg/ml in the strongest bleeds as estimated by the methods of Morris & Williams, (1975) (see Ch. 2.4.2.) This antiserum was also used in antibody affinity columns in the purification of thymocyte Thy-1 (Letarte-Muirhead et al, 1975). Thymocyte Thy-1 differs from brain Thy-1 in that only 50% of the antigen can be purified by binding to, and elution, from lentil lectin affinity columns; this form is called $Thy\text{-}1_{L+}$. The remaining Thy-1 which was not bound by lentil lectin (called $Thy\text{-}1_{L-}$), could be purified by the anti-(brain Thy-1) affinity column as shown in the scheme for the purifications of Thy-1 in Fig. 4.7. This antibody affinity column could also be applied directly to a deoxycholate extract of thymocyte membranes; all the Thy-1 antigenic activities were bound, and after elution a mixture of $Thy\text{-}1_{L+}$ and $Thy\text{-}1_{L-}$ (called $Thy\text{-}1_{total}$)

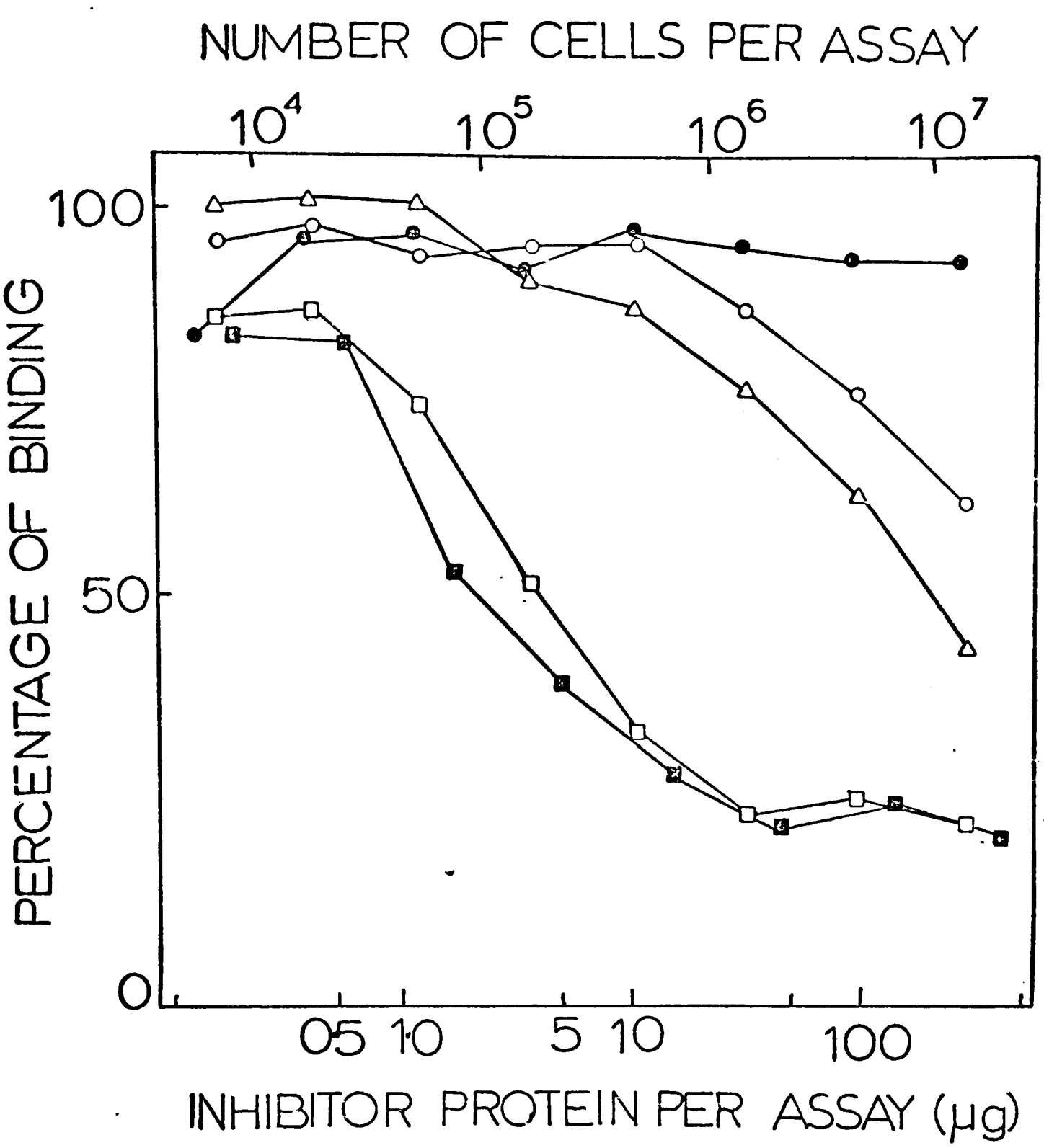


Fig. 4.5 Inhibition of the binding of rabbit anti-(brain Thy-1) serum to rat thymocytes

The IgG fraction of rabbit anti (brain Thy-1) serum (see Ch. 2.1.5) diluted 1/130 was absorbed with rat tissues and the binding of residual antibody to rat thymocytes followed with ^{125}I-labelled horse anti-(rabbit IgG) antibodies under saturating conditions (20 μg/ml). The inhibitions obtained for brain homogenate (▣), liver homogenate (●), spleen cells (△), thymocytes (□) and lymph node cells (o) are shown. One abscissa defines protein and applies to ▣, ●, □, and the other defines cell numbers and applies to □, △, o.

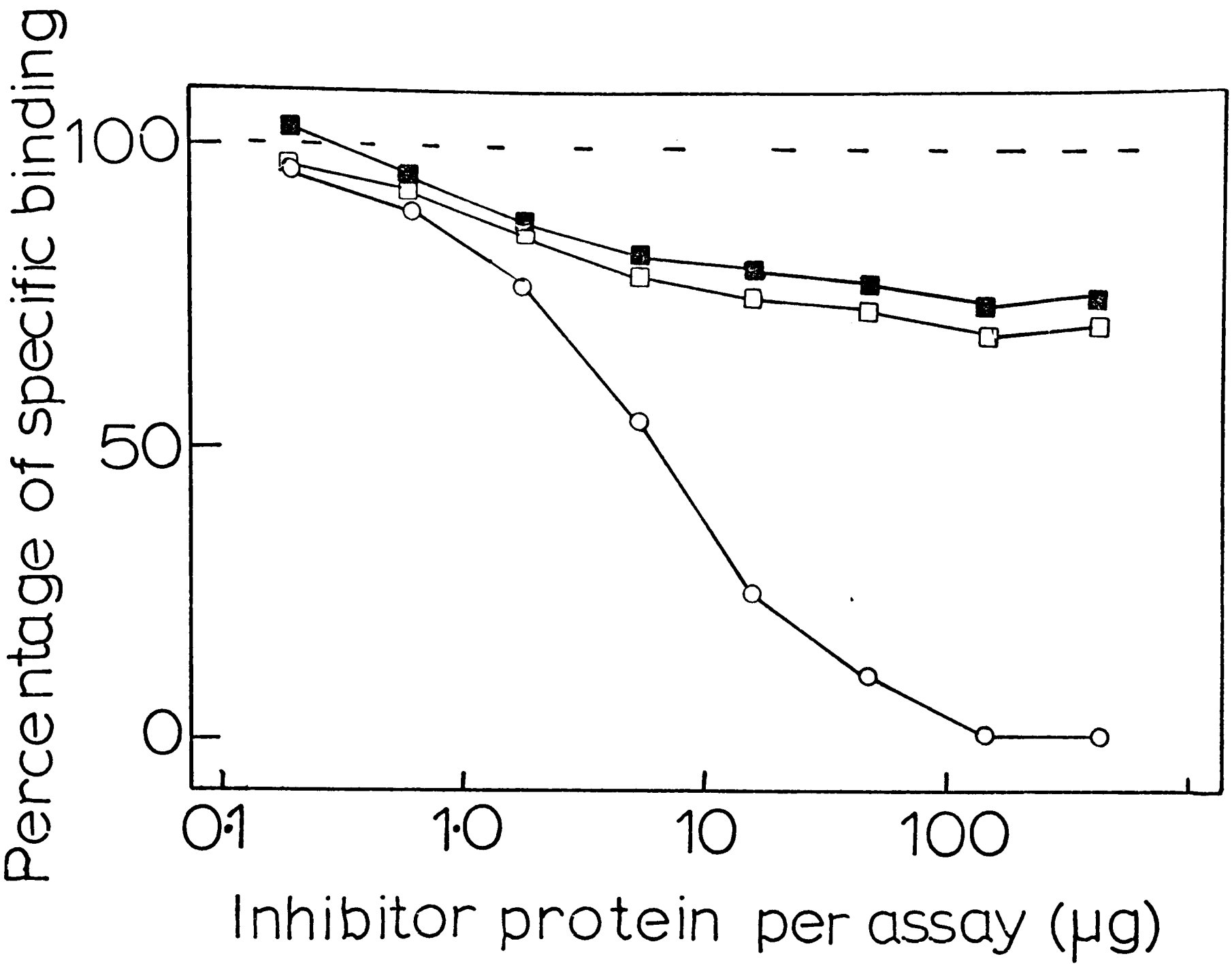


Fig. 4.6 Analysis of the specificities of rabbit anti-(brain Thy-1) serum

25 µl aliquots of the rabbit anti-(brain Thy-1) serum diluted 1/150 were absorbed with brain homogenate from Wistar rat (o), A/Thy-1.1 mouse (□) or A/Jax mouse (■) brain. The binding of residual antibody to rat thymocyte target cells was followed with ^{125}I-labelled horse anti-(rabbit IgG) antibodies under saturating conditions (20 µg/ml).

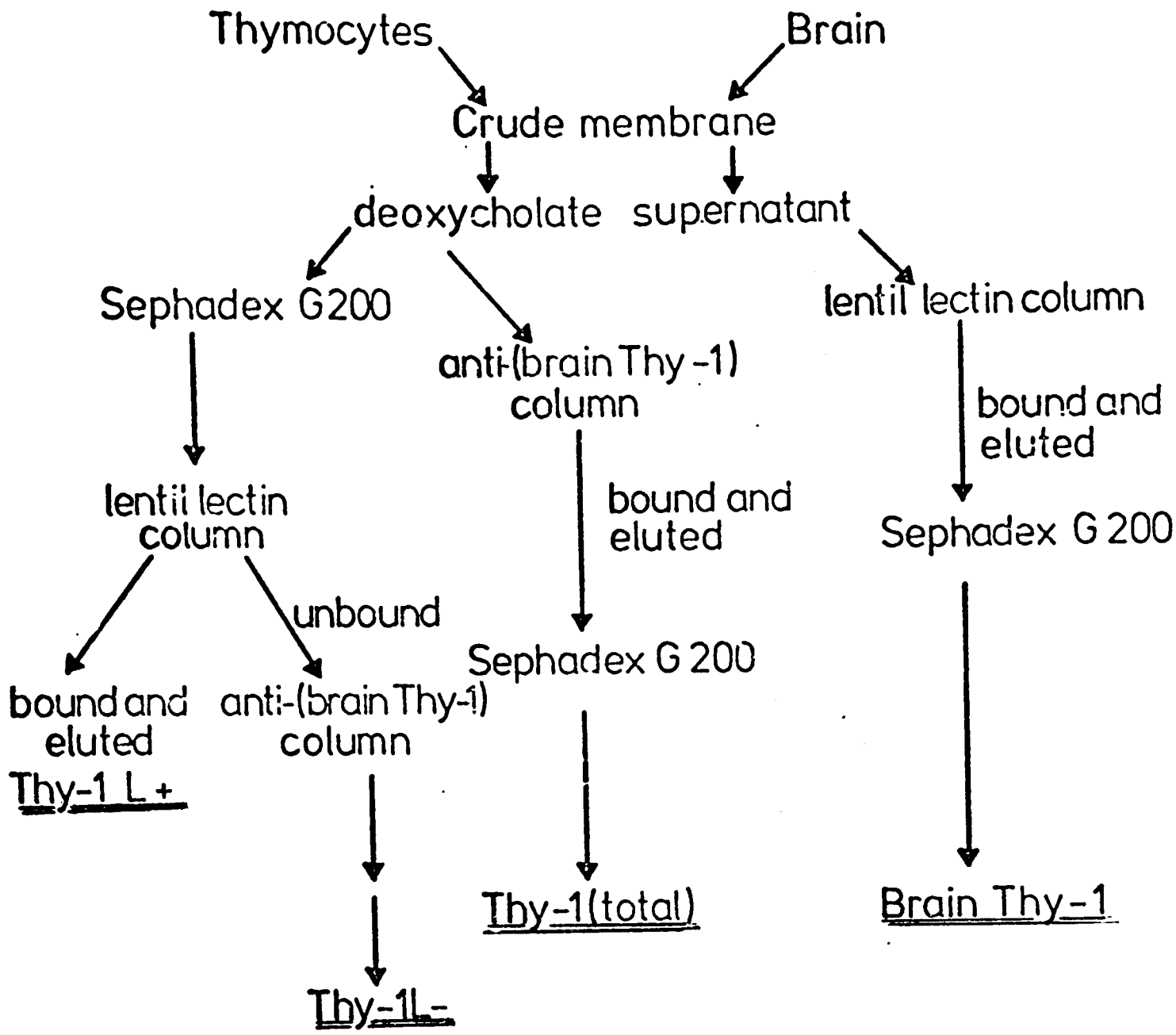


Fig. 4.7 Scheme for the purifications of Thy-1

was purified (Fig. 4.7). Thymocyte Thy-1 was clearly shown to be a glycoprotein similar to brain Thy-1 (Letarte-Muirhead et al, 1975) and their properties are discussed below (Ch. 4.7).

The second anti-(brain Thy-1) serum contained 0.1 mg/ml specific anti-(Thy-1) antibody in the strongest bleeds and analysis as in Fig. 4.6, showed that 45% of the antibodies were against the rat specific xenoantigenic determinant, 37% against the rat-mouse cross-reacting xenoantigenic determinant and 18% against the Thy-1.1 alloantigenic determinant. This antiserum has been used in studies on the nature of mouse thymocyte Thy-1 (Trowbridge et al, 1975; Trowbridge & Hyman, 1975) and these results are discussed further in Ch. 7.

4.6 Antigenic properties of brain Thy-1

The inhibition of the Thy-1.1 assay by both rat thymocytes and brain showed that these tissues both carried the Thy-1.1 antigenic determinant in similar quantities (Ch. 3.2). The antigenicities of the purified brain and thymocyte Thy-1 preparations were compared by studying the inhibition of binding of rabbit anti-(brain Thy-1) serum and rabbit anti-(thymocyte $Thy\text{-}1_{L+}$) serum to rat thymocytes. This analysis should show if thymocyte Thy-1 displayed antigenic determinants not present on brain Thy-1. The results (Fig. 4.8) showed that the two forms of Thy-1 gave identical inhibitions for each assay. The two assays differed in their sensitivities to inhibition and this was probably due to differences in antibody affinity rather than antigenic specificity. In both these assays and also the Thy-1.1 assay, similar amounts of the purified Thy-1 antigen from brain and thymocytes were needed to give 50% inhibition as summarised in Table 4.2. Thus brain and thymocyte Thy-1 could not be distinguished antigenically. In a similar manner the three thymocyte Thy-1 forms ($Thy\text{-}1_{L+}$, $Thy\text{-}1_{L-}$ and $Thy\text{-}1_{Total}$) could also not be distinguished antigenically (Letarte-Muirhead et al, 1975).

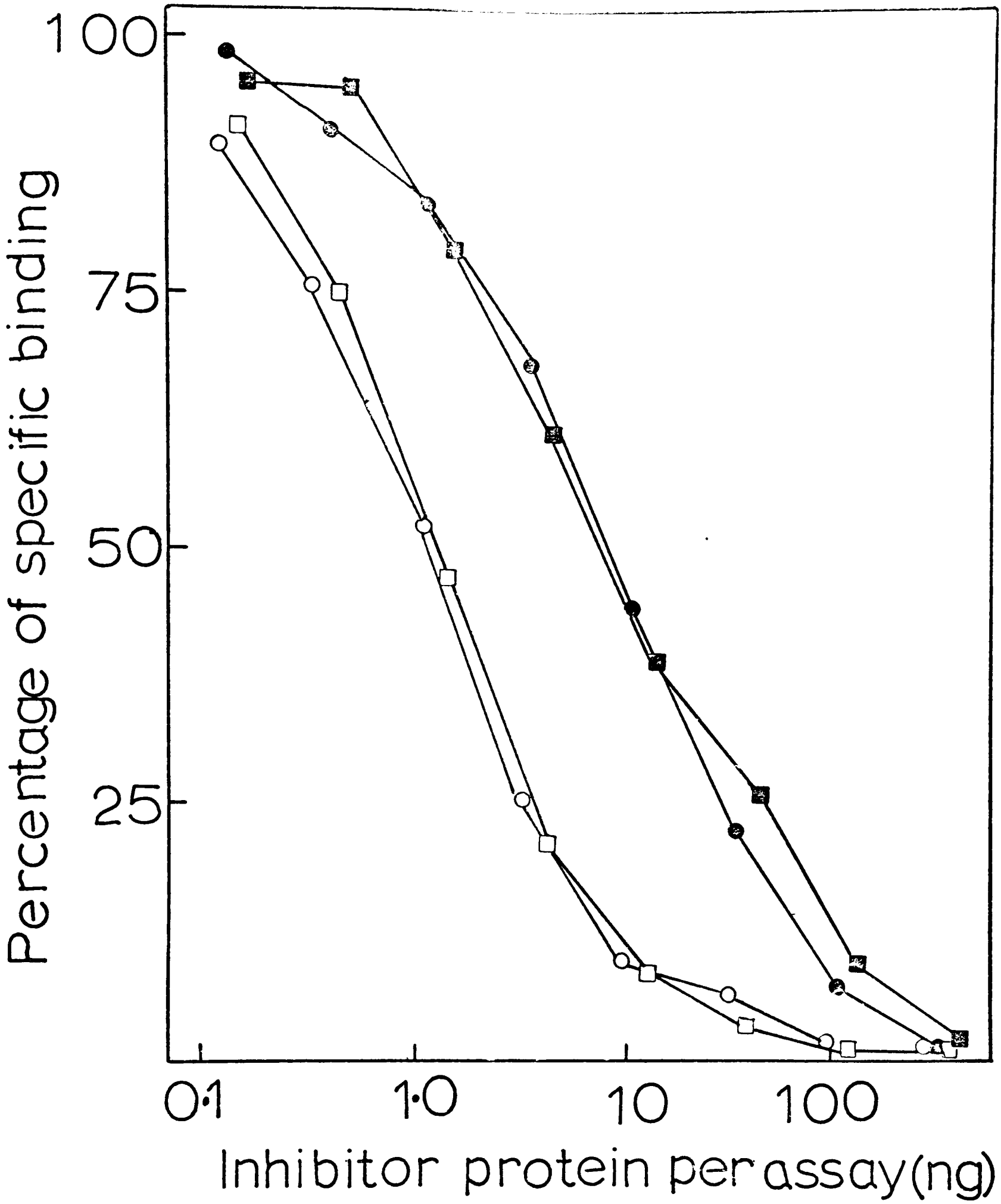


Fig. 4.8 Inhibition of assays for Thy-1 xenoantigenic determinants

25 μl aliquots of rabbit anti-(brain Thy-1) serum diluted 1/6000 were incubated with 25 μl dilutions of purified brain Thy-1 (o) and thymocyte Thy-1_{L+}(□). Similarly rabbit anti-(thymocyte Thy-1) serum at a dilution of 1/400 was incubated with brain Thy-1 (●) and thymocyte Thy-1_{L+} (■). The binding of residual antibody to rat thymocyte target cells was followed with ^{125}I-labelled horse anti-(rabbit IgG) under trace conditions (.2 μg/ml) and is expressed as the percentage of specific cpm obtained for each assay.

Table 4.2 Specific activities of Thy-1 preparations

	Amount of fraction (ng of protein) need for 50% inhibition of assay for:		
	Thy-1.1	Thy-1 xenoantigenic determinants	
		(a)	(b)
Thy-1 (brain)	8.5 ± 1.1(4)	1.4 ± 0.1(5)	5.4 ± 2.0(3)
$Thy\text{-}1_{L+}$ (thymocyte)	7.8 ± 2.3(3)	1.9 ± 0.3(4)	8.0 ± 3.3(3)

Values are given as the means ± S.E.M., with the number of determinations in parentheses.

The two assays for the xenoantigenic determinants differ in the antiserum used in the first incubation:

(a) rabbit anti-(brain Thy-1) serum

(b) rabbit anti-(thymocyte $Thy\text{-}1_{L+}$) serum

4.7 Discussion

The Thy-1 antigen has been purified from rat brain and shown to be a glycoprotein. The apparent molecular weight of the glycoprotein from electrophoresis on 5.6% polyacrylamide gels was higher than that on 12.5% gels; this pattern was also found for Thy-1 from thymocytes (Table 4.3). This anomalous behaviour is common to several glycoproteins and the values obtained on 12.5% gels are generally closer to the correct molecular weight (Segrest et al, 1971; Letarte-Muirhead et al, 1975). This value for brain Thy-1 (24,100) is in good agreement to the value for brain Thy-1 plus bound deoxycholate (27,000) determined from hydrodynamic studies (Ch. 3.4.3). No evidence was obtained for the association of Thy-1 with another polypeptide as for histocompatibility antigen and β_2 microglobulin (Tanigaki & Pressman, 1974). The accurate molecular weights of the forms of Thy-1 are currently being determined according to the methods of Tanford et al (1974); that is to estimate the molecular weight in deoxycholate by sedimentation equilibrium analysis and to determine the weight of bound deoxycholate by equilibrium dialysis.

When the apparent molecular weight of brain Thy-1 is compared to those obtained for thymocyte Thy-1 (Table 4.3) it can be seen that brain Thy-1 appears to be slightly smaller than thymocyte Thy-1_{L+} and considerably smaller than Thy-1_{L-}. Brain Thy-1 and thymocyte Thy-1_{L+} electrophorese as symmetrical bands whilst the Thy-1_{L-} band trailed towards higher molecular weights (see Fig. 5.1). Thus brain Thy-1 was more similar to thymocyte Thy-1_{L+} than Thy-1_{L-} but a form equivalent to Thy-1_{L-} could exist in the 10-20% of brain Thy-1 which fails to bind to lentil lectin. The reason for the small differences in electrophoretic mobility between the three forms of Thy-1 will be studied by chemical analysis (Ch. 5).

Rabbit antisera raised against thymocyte Thy-1_{L+} has been shown to recognise antigens with the same tissue distribution as Thy-1.1 (Letarte-Muirhead et al, 1975) in the same manner as found for rabbit anti-(rat

Table 4.3 The apparent molecular weights of Thy-1 preparations

% of polyacrylamide in gels	Brain	Thymocyte	
	Thy-1	$Thy\text{-}1_{L+}$	$Thy\text{-}1_{L-}$
5.6%	31,300 ± 1000	31,600 ± 400	34,800 ± 380
12.5%	24,100 ± 150	25,300 ± 240	27,200 ± 390

The values are expressed as the mean of 4-7 determinations ± S.E.M.

The data for thymocyte Thy-1 are from Letarte-Muirhead et al (1975).

brain Thy-1) serum (Ch. 4.5). This immunogenic similarity, together with the antigenic similarities between the forms of Thy-1 (Ch. 4.6) and the basic characteristics of the molecules (see above) indicate that there must be considerable structural similarity between brain Thy-1 and thymocyte Thy-1_{L+} and Thy-1_{L-}.

CHAPTER 5

THE CHEMICAL CHARACTERISATION OF THE THY-1 GLYCOPROTEINS

5.1 Introduction

Brain Thy-1 and the two forms of thymocyte Thy-1 have several common antigenic and structural features but can be distinguished by their electrophoretic behaviour on polyacrylamide gels in SDS (see Ch.4). Chemical analysis should indicate the basis for these differences and give structural data relevant to Thy-1 as a major membrane glycoprotein. This analysis may also indicate why Thy-1_{L-} fails to be bound by lentil lectin. However before analysis was possible the large excess of deoxycholate present after purification of the Thy-1 glycoproteins had to be removed.

This chapter describes the effective removal of deoxycholate and the properties of Thy-1 in its absence, and the determination of the amino acid and carbohydrate compositions of the three forms of Thy-1.

5.2 Purification of the Thy-1 glycoproteins

Brain Thy-1 was purified as described above (Chs. 2 and 4) and thymocyte Thy-1_{L+} and Thy-1_{L-} had been purified as described in Letarte-Muirhead et al (1975). The minor impurities in Thy-1_{L-} were removed by gel filtration on Sephadex G-200 in deoxycholate. This deoxycholate had been twice recrystallised and the Thy-1_{L+} was also rechromatographed on Sephadex G-200 with this deoxycholate.

5.3 Removal of deoxycholate by ethanol precipitation

After concentration by ultrafiltration the preparations were at approximately 1 mg Thy-1/ml in 50 mg deoxycholate/ml. The deoxycholate was removed by precipitating the Thy-1 glycoproteins at -20°C with ethanol in which deoxycholate is very soluble. The Thy-1 glycoproteins could then be dissolved in water and some slight turbidity removed by centrifugation. The resulting pellet contained less than 5% of the initial Thy-1 by antigenic activity or amino acid and carbohydrate analysis and was probably due to impurities in the deoxycholate.

The yield of precipitation was estimated from the protein content before and after precipitation and values of 70% for the thymocyte forms and 40% for brain Thy-1 were obtained. As a lower yield was obtained for brain Thy-1, one cannot preclude the presence of other forms of brain Thy-1 which are not precipitable. To investigate this, the ethanol extractions were dialysed against 20% ethanol and then 10mM Tris-HCl pH 8.0 to remove the deoxycholate and then the ethanol; analysis of this material failed to account for all the loss of protein and only small amounts of Thy-1 antigenic activity and glycoprotein (from polyacrylamide gel electrophoreses in SDS) were detected. Thus the yield of the precipitation of brain Thy-1 may in fact be somewhat higher than that indicated by the protein estimation.

The ethanol precipitation procedure had no effect on the electrophoretic behaviour of the Thy-1 glycoproteins on polyacrylamide gels in SDS and no contaminant bands staining for protein or carbohydrate were detected before or after precipitation (Fig. 5.1).

5.4 The properties of Thy-1 in the absence of deoxycholate

5.4.1 Molecular weight

The molecular weight of Thy-1 in the absence of deoxycholate

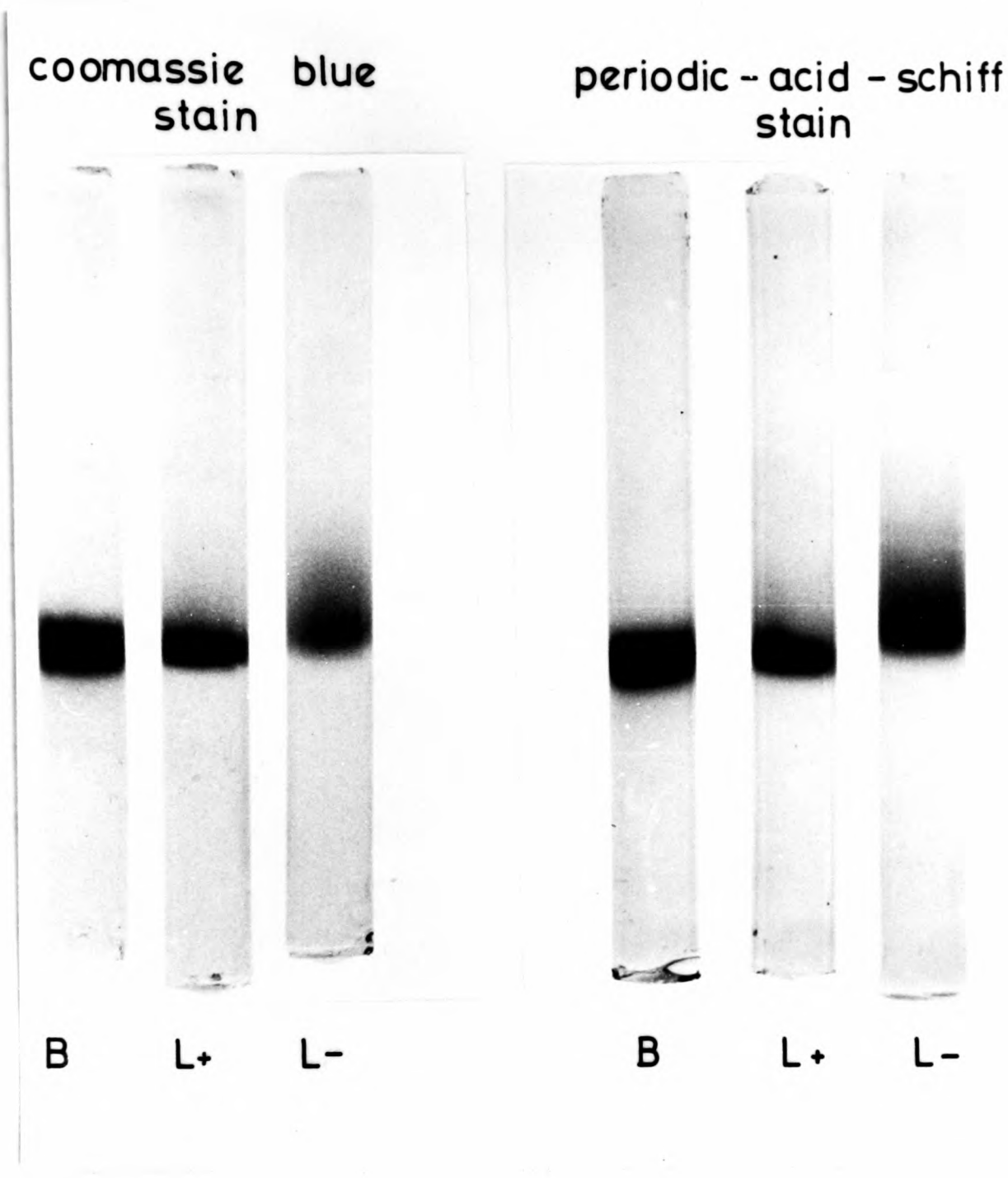


Fig. 5.1 The purity of Thy-1 fractions analysed by electrophoresis on 7.5% polyacrylamide gels in SDS.

B, L+, L- denote Thy-1 purified from brain, and Thy-1_{L+} and Thy-1_{L-} from thymocytes respectively. 14 μg and 22 μg of the glycoproteins were loaded on to the gels stained for protein with Coomassie Blue and for carbohydrate with periodic acid-Schiff stain respectively.

was determined by equilibrium ultracentrifugation (Yphantis, 1964). Thy-1_{L+} was sedimented to equilibrium and Fig. 5.2 shows that the plot of ln (fringe displacement) versus r^2 was non-linear, indicating heterogeneity in the molecular weights of the Thy-1. The weight average molecular weight was calculated at the two positions shown in Fig. 5.2 by measuring the gradient at these positions and using the relation

$$\frac{d(\ln\ c)}{dr^2} = \frac{M\omega^2(1 - \bar{v}\rho)}{2RT}$$

where c is concentration of solute; r, distance from centre of rotor; M, molecular weight; ω, angular velocity; $\bar{v}$, partial specific volume; ρ, density of medium; R, universal gas constant; T, temperature (^{o} Absolute); the fringe displacement is directly proportional to the concentration (one fringe displacement = 0.25 mg protein/ml)

Values of 230,000 and 370,000 were obtained for the weight average molecular weights using a $\bar{v}$ value of 0.691 calculated from the amino acid and carbohydrate compositions (see below) as described by Cohn & Edsall (1943) and Gibbons (1972). Thus the Thy-1_{L+} was present in an aggregated form in the absence of deoxycholate and the majority had a weight average molecular weight between 230,000 and 370,000.

Readdition of deoxycholate to the Thy-1_{L+} caused a different sedimentation pattern when analysed similarly. A linear relationship between ln (fringe displacement) versus r^2 (Fig. 5.3) was obtained indicating that the Thy-1 in deoxycholate was present as a homogeneous component whose molecular weight was estimated to be 29,000 using a $\bar{v}$ of 0.70 ml/g for the Thy-1 deoxycholate complex (Letarte-Muirhead et al, 1974). This molecular weight is in close agreement with that obtained from sedimentation velocity studies on sucrose density gradients for thymocyte Thy-1 (28,000; Letarte-Muirhead et al, 1974) and for brain Thy-1 (27,000; Ch. 3).

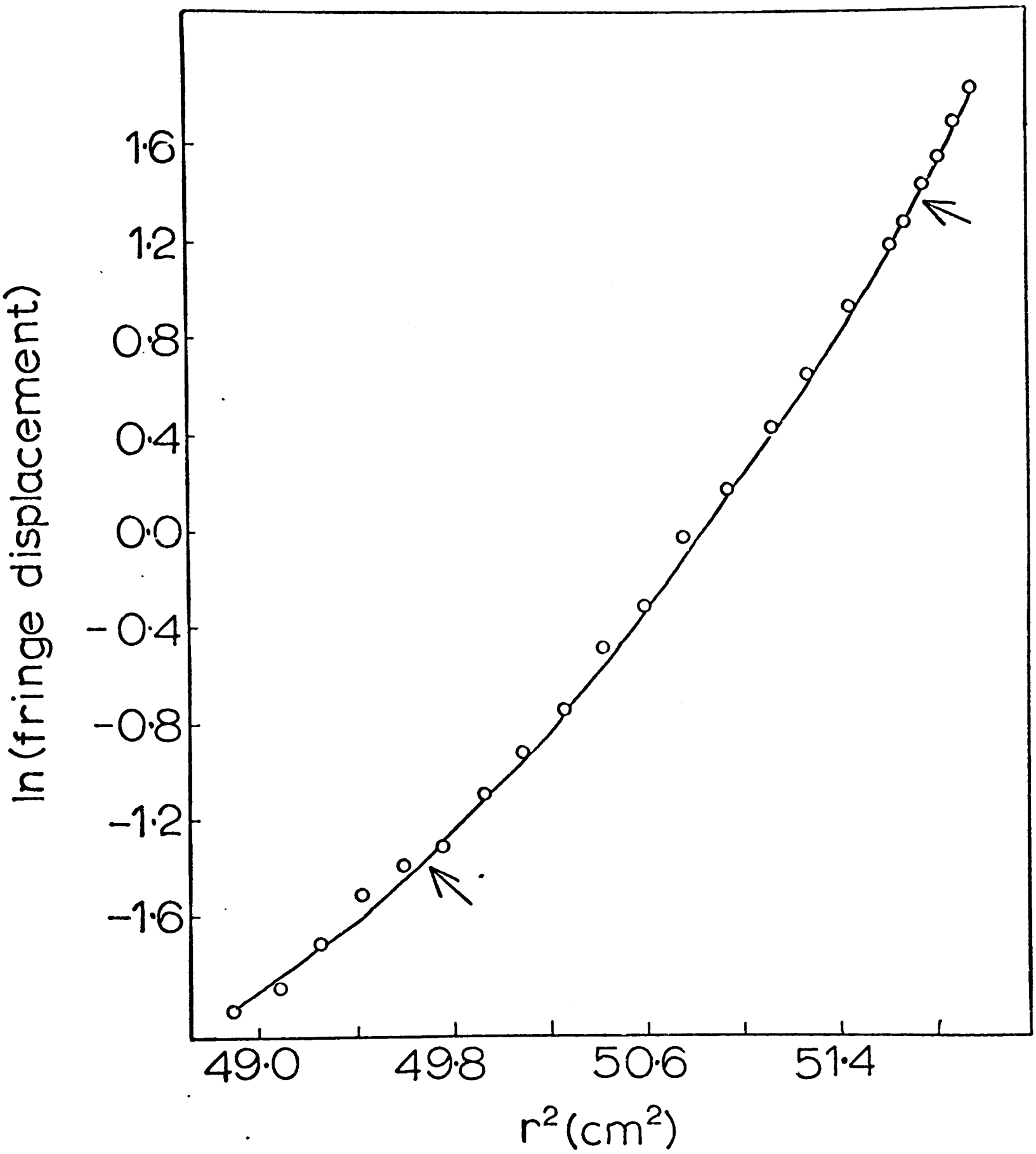
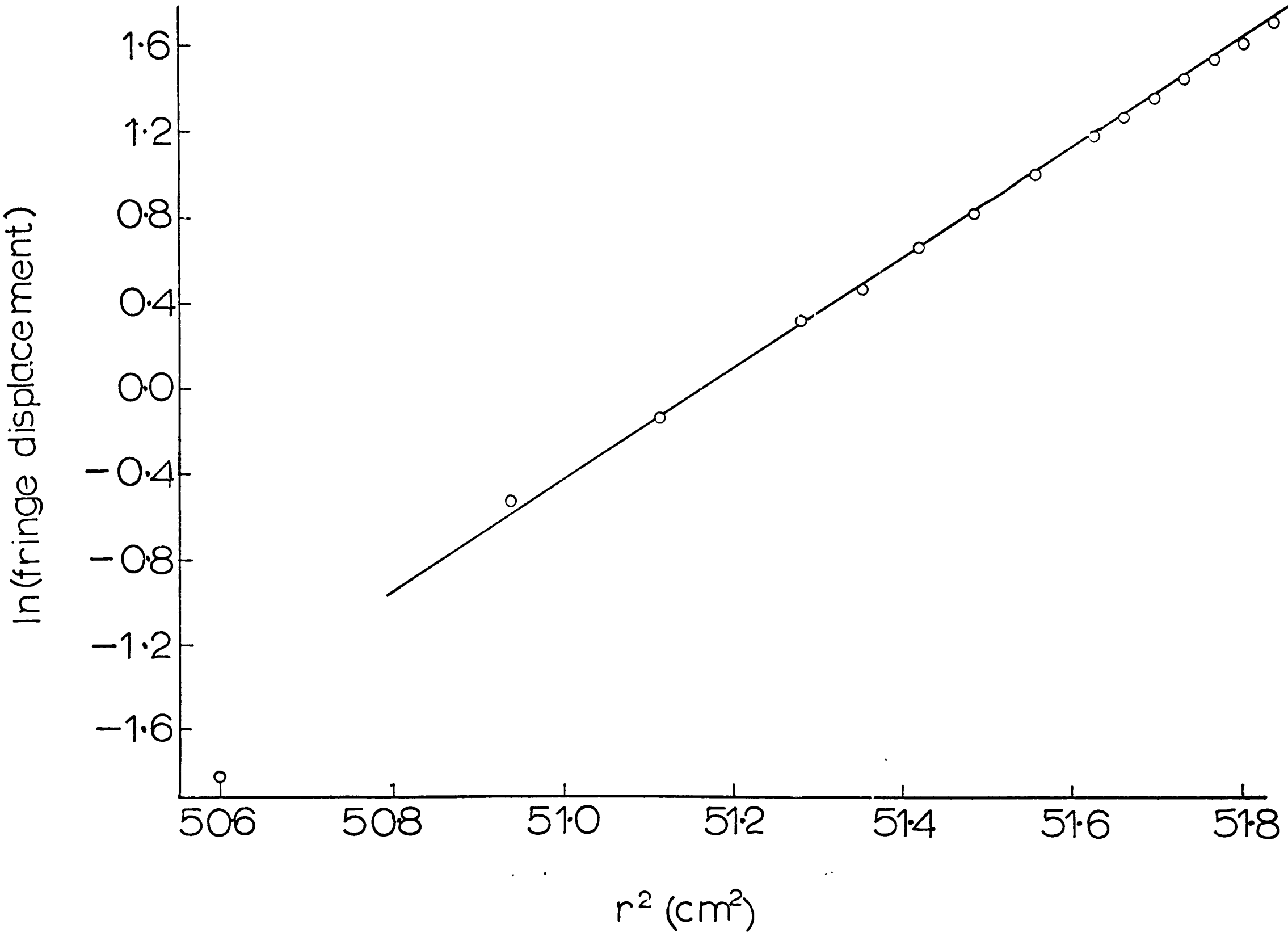


Fig. 5.2 Equilibrium ultracentrifugation of thymocyte Thy-1_{L+} in the absence of deoxycholate

Thymocyte Thy-1_{L+} at 0.7 mg/ml in .01 M Tris-HCl, 0.02% NaN_3 pH 8.0 was centrifuged at 8776 rev/min until equilibrium when the distribution was recorded by interference optics and where r is the distance from the axis of ratation.

Fig. 5.3 Equilibrium ultracentrifugation of thymocyte Thy-1_{L+} in deoxycholate

Thymocyte Thy-1_{L+} at 0.35 mg/ml in 0.5% (w/v) deoxycholate buffer was centrifuged at 37,000 rev/min until equilibrium, when the distribution was recorded by interference optics and where r is the distance from the axis of rotation.


1·6
1·2
0·8
0·4
0·0
−0·4
−0·8
−1·2
−1·6
ln(fringe displacement)
506
508
510
512
514
516
518
r² (cm²)

5.4.2 The physical properties of Thy-1 in the absence of deoxycholate

The removal of the large amounts of deoxycholate (which contained material which absorbed in the ultra violet spectrum), facilitated the determination of the absorption spectrum of Thy-1 as shown for brain Thy-1 in Fig. 5.4. The spectrum is notable for its E_{280nm}/E_{260nm} of 1.1 which is low in comparison with typical proteins (1.75) and the maximal absorption in the near ultraviolet is at 275nm. This characteristic absorption curve was also found for thymocyte $Thy\text{-}1_{L+}$ and $Thy\text{-}1_{L-}$.

The protein concentration was determined by the Hartree method and equated to that from the amino acid analysis (see below) and the E^{1cm}_{280nm} (Table 5.1). This shows that the Hartree method gives an overestimate of the true protein concentration but is quite close to the weight of glycoprotein calculated from the summation of amino acid and carbohydrate compositions (see below).

5.4.3 The antigenic properties of Thy-1 in absence of deoxycholate

The ethanol precipitation procedure did not destroy the antigenic determinants of Thy-1 and their specific activities were determined in the absence of deoxycholate (Table 5.2). This shows that, the specific activities of Thy-1 xenoantigenic determinants were very similar to those obtained prior to ethanol precipitation (Ch. 4.6), whilst there appeared to be a gain in the specific activity of the Thy-1.1 determinant, on removal of the deoxycholate. This effect was thought to be due to the effect of deoxycholate on the nature of the antigen. Thus the inhibition of the Thy-1 assays was observed after the readdition of deoxycholate to the pure Thy-1. Fig. 5.5 shows that the heterologous type of Thy-1.1 assay used to assay Thy-1.1 in this thesis, with the CBA mouse anti-(Wistar rat brain) serum, was inhibited less effectively by Thy-1 in the presence of deoxycholate. This gave a

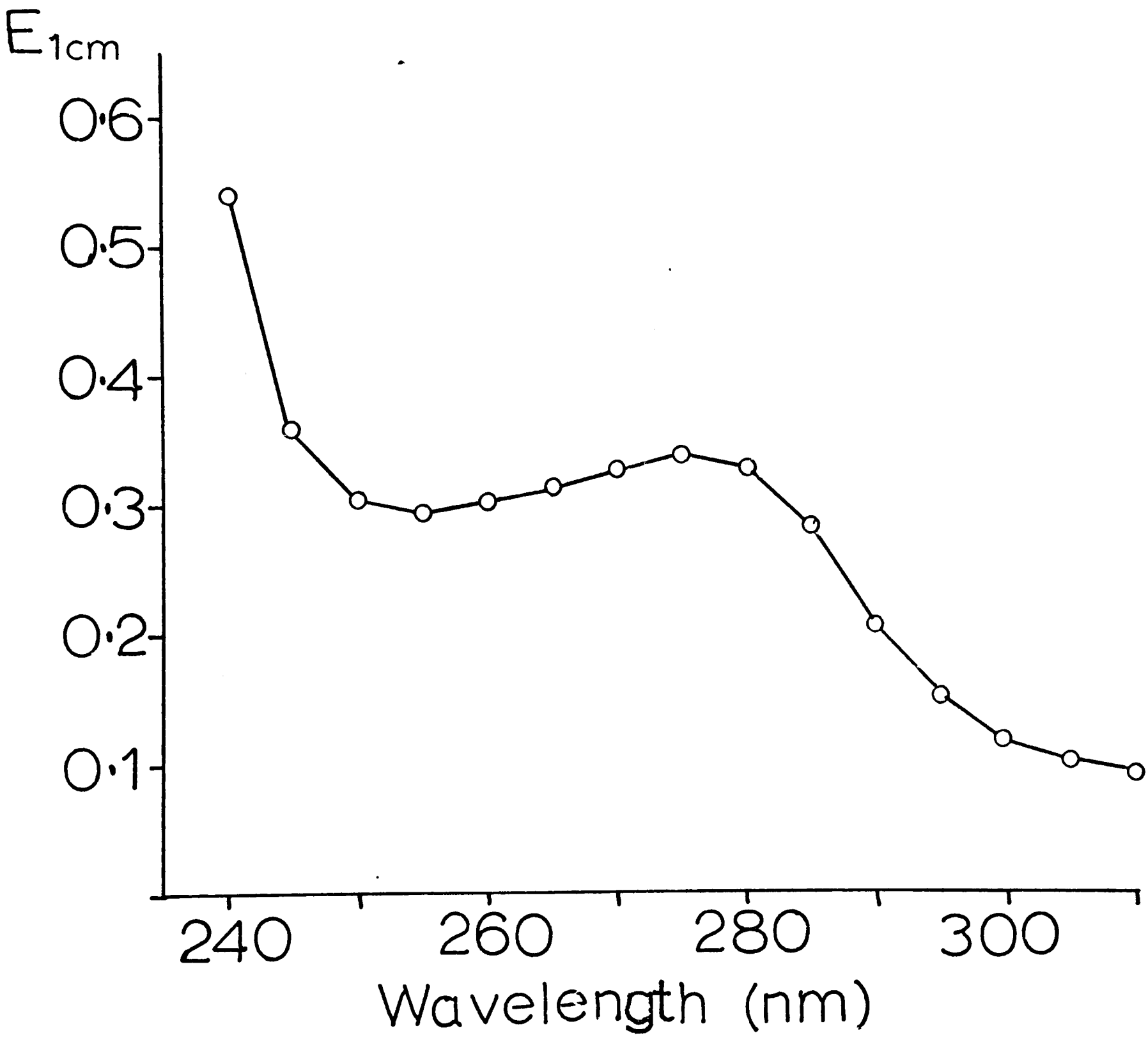


Fig. 5.4 The absorption spectrum of brain Thy-1 in the absence of deoxycholate

The absorption spectrum was determined for brain Thy-1 at 0.6 mg protein/ml in 0.01M Tris-HCl, 0.02% NaN_3, pH 8.0.

Table 5.1 Comparison of Thy-1 glycoproteins - physical properties

	Amount of Thy-1 estimated in terms of: protein[a]	amino acids[b]	glycoprotein[c]	E^{1cm}_{280nm}
Thy-1 (brain)	1.33	1.00	1.37	0.68
$Thy\text{-}1_{L+}$ (thymocytes)	1.50	1.00	1.45	0.88
$Thy\text{-}1_{L-}$ (thymocytes)	1.49	1.00	1.49	1.20[d]

a) protein determined by Hartree method

b) calculated from amino acid analysis

c) calculated from combined amino acid and carbohydrate analyses

d) from only one determination where some residual turbidity remained.

Table 5.2 Specific activities of Thy-1 preparations after precipitation with ethanol

	Thy-1.1 determinant	Thy-1 xenoantigenic determinants
Thy-1 (brain)	5.8 (5)	1.5 (7)
$Thy\text{-}1_{L+}$ (thymocyte)	4.6 (2)	1.6 (5)
$Thy\text{-}1_{L-}$ (thymocyte)	5.0 (1)	1.9 (4)

The specific activities are expressed as the ng of Thy-1 (from the amino acid and carbohydrate compositions) needed to give 50% inhibition of the respective assays with the number of determinations in parentheses.

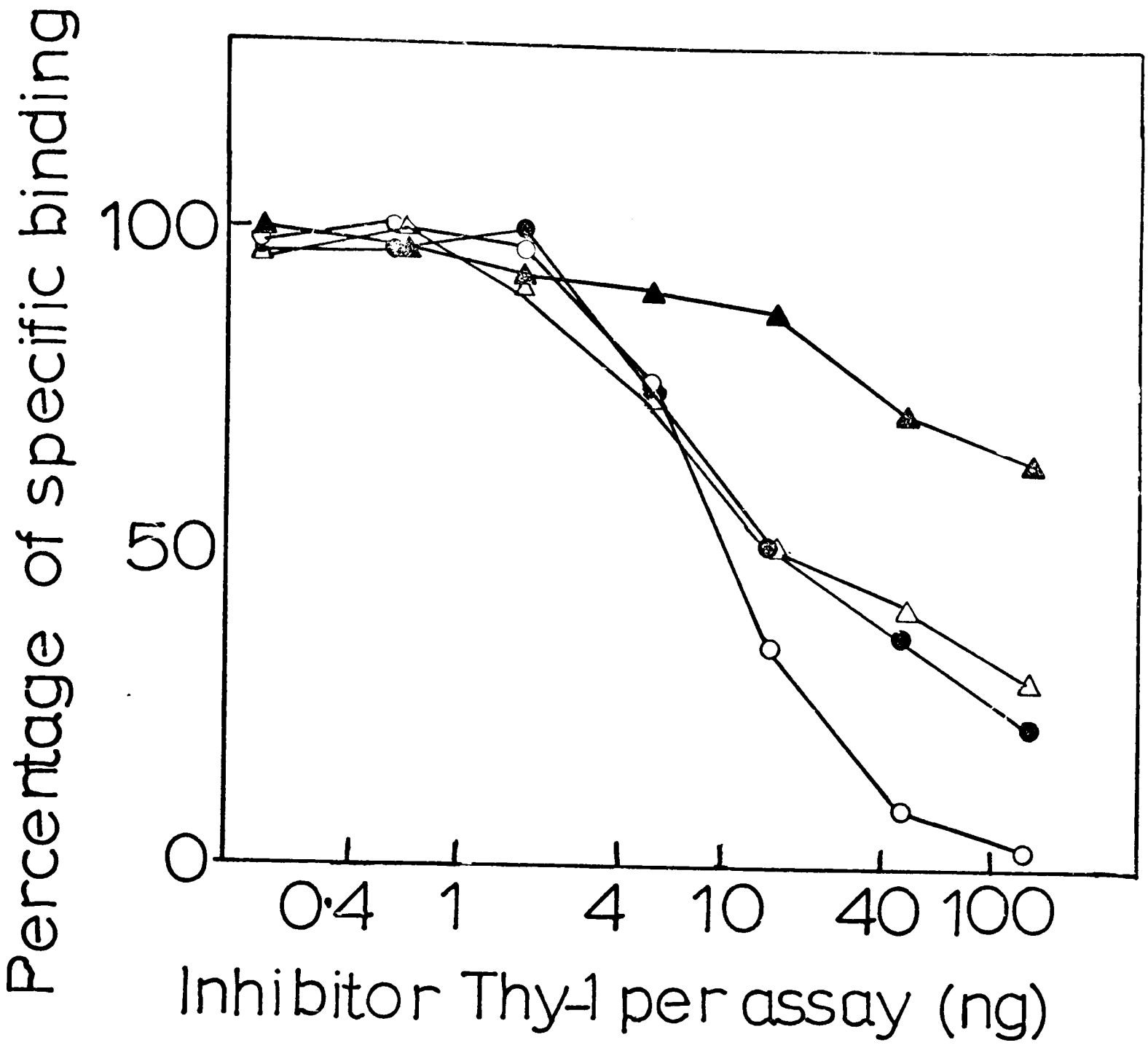


Fig. 5.5 The effect of deoxycholate on inhibition assays for Thy-1.1

25 μl aliquots of CBA mouse anti-(rat brain) serum diluted 1/200 (o, ●) or CBA mouse anti-(AKR mouse thymocyte) serum diluted 1/200 (△, ▲) were absorbed with purified brain Thy-1 in the absence (o, △) of deoxycholate or in the presence of (●, ▲) of 0.2% deoxycholate. The binding of residual antibody to AKR mouse thymocyte or Wistar rat thymocyte target cells, for the two assays respectively, was followed by the binding of ^{125}I-labelled rabbit anti-(mouse IgG). This is expressed as the percentage of specific cpm for each assay.

much larger effect on the other assay using CBA mouse anti-(mouse AKR thymocyte) serum as reported previously for a similar assay (Letarte-Muirhead et al, 1974). Both these assays are specific for the same Thy-1.1 antigenic determinants as defined by absorptions with tissues of mouse strains (Ch. 2.4.1.1). As the only change in Thy-1 caused by the addition of deoxycholate is from a polymeric to a monomeric form (see above) the most likely explanation is that this effect is due to low affinity antibodies in the anti-(Thy-1.1) sera. Thus for effective inhibition of these assays, the binding of inhibitor antigen to antibody must be irreversible under the conditions used and monomeric antigen will not be able to compete as effectively for the antibody, with the polymeric target cell unless the antibodies are of sufficient affinity. Thus the CBA mouse anti-(AKR mouse thymocyte) serum presumably contains a larger proportion of lower affinity antibodies than the mouse anti-(Wistar rat brain) serum to explain the effects observed in Fig. 5.5. As the assay for the xenoantigenic determinants was not affected by deoxycholate, this rabbit anti-(rat brain Thy-1) serum presumably contains far fewer low affinity antibodies than the mouse antisera, as would be expected as the rabbit serum was hyperimmune. Thus the ethanol precipitation had no detectable effect on the antigenic determinants of Thy-1.

5.5 The amino acid analysis of the Thy-1 glycoproteins

The three preparations of Thy-1 shown in Fig. 5.1 were analysed for their amino acid compositions by standard techniques. A second completely independent preparation of Thy-1_{L+} and of brain Thy-1 were also analysed and the average amino acid compositions from at least four analyses of each preparation are shown in Table 5.3. The compositions of the three forms were very similar and the only differences which were statistically significant were those between the leucine and glycine contents of brain Thy-1 and thymocyte Thy-1.

Table 5.3 Amino acid compositions of the Thy-1 glycoproteins

	Residues of each amino acid per 100 amino acids		
	Brain Thy-1	Thymocyte Thy-1_{L+}	Thymocyte Thy-1_{L-}
Asx	12.6 ± 0.1	12.5 ± 0.3	13.0 ± 0.2
Glx	9.0 ± 0.2	9.0 ± 0.1	9.1 ± 0.3
His	4.1 ± 0.2	3.8 ± 0.1	4.0 ± 0.3
Lys	6.9 ± 0.2	7.2 ± 0.1	7.0 ± 0.2
Arg	7.4 ± 0.1	7.4 ± 0.1	7.2 ± 0.1
Thr[a]	7.6 ± 0.2	8.5 ± 0.2	8.2 ± 0.4
Ser[a]	7.4 ± 0.3	7.1 ± 0.2	7.8 ± 0.4
Pro	3.6 ± 0.4	3.8 ± 0.4	2.9 ± 0.1
Ala	3.2 ± 0.1	2.9 ± 0.1	3.1 ± 0.1
Cys[b]	3.1 ± 0.1	3.2 ± 0.1	3.1
Gly	6.0 ± 0.1	4.8 ± 0.2	5.0 ± 0.1
Tyr	2.0 ± 0.2	2.0 ± 0.1	2.0
Val	7.2 ± 0.1	7.3 ± 0.1	7.4 ± 0.1
Ile	3.9 ± 0.1	4.1 ± 0.1	4.0 ± 0.1
Leu	10.3 ± 0.2	11.1 ± 0.1	11.0 ± 0.1
Phe	4.0 ± 0.1	3.7 ± 0.1	3.7 ± 0.1
Met	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Trp[c]	0.7	0.7	0.7

The values are expressed as the mean ± S.E.M. except where two or less determinations were available, and the amino acids are grouped into polar, intermediate and hydrophobic as in Steck & Fox (1972) from the data of Nozaki & Tanford (1971)

a) corrected for loss during hydrolysis as calculated for brain Thy-1 after 24h and 72h hydrolyses

b) estimated as cysteic acid

c) see text

An attempt to estimate tryptophan in brain Thy-1 and thymocyte Thy-1_{L+} after hydrolysis with p-toluene sulphonic acid (Liu & Chang, 1971) gave inconclusive results with either no tryptophan or very low amounts ($<1\%$ of total amino acids) being detected. However a value of 0.7% for the tryptophan content was calculated from the absorption spectra of brain Thy-1 (Fig. 5.4) and the calculated contribution due to the tyrosine content (Table 5.3).

5.6 The carbohydrate analysis of the Thy-1 glycoproteins

The two independent preparations of both brain Thy-1 and thymocyte Thy-1_{L+} and one of Thy-1_{L-} analysed for amino acid compositions (see above) were analysed for carbohydrate residues by three methods as indicated in Table 5.4. This showed that there were marked differences in carbohydrate compositions between brain and thymocyte Thy-1 with smaller differences between thymocyte Thy-1_{L+} and Thy-1_{L-} e.g. galactosamine was found only in brain samples whereas sialic acid was present in very low amounts in brain Thy-1 and in much larger amounts in thymocyte Thy-1. The amounts of fucose, glucose and galactose also differed considerably between brain and thymocyte Thy-1 with smaller differences in mannose and glucosamine contents. The differences between thymocyte Thy-1_{L+} and Thy-1_{L-} were much less marked but differences in the amounts of galactose, mannose, glucosamine and sialic acid may be significant. These differences do not provide an obvious explanation as to why Thy-1_{L-} fails to bind to lentil lectin.

Some minor peaks were observed in the gas liquid chromatography analysis; one co-ran with a glucosamine peak and could be extracted with hexane (see methods). Gas liquid chromatography of the hexane extracts

Table 5.4 Carbohydrate compositions of the Thy-1 glycoproteins

Moles of carbohydrate per 100 moles of amino acids.

1) Analysis by gas liquid chromatography

	Brain Thy-1	Thymocyte Thy-1_{L+}	Thymocyte Thy-1_{L-}
fucose	1.8 ± 0.1	1.0 ± 0.1	0.9 (0.9, 0.9)
mannose	11.9 ± 0.7	10.6 ± 0.6	9.4 (9.6, 9.2)
galactose	1.8 ± 0.2	5.5 ± 0.3	6.9 (7.1, 6.6)
glucose	0.6 ± 0.1	1.3 ± 0.1	1.1 (1.1, 1.0)
glucosamine	6.3 ± 0.4	7.0 ± 0.6	8.3 ± 0.5
galactosamine	1.0 ± 0.1	0.0	0.0 (0, 0)
sialic acid	0.2 (0.2)[a]	1.8 ± 0.3	2.2 (2.1, 2.3)

2) Amino sugars by ion-exchange chromatography

glucosamine	8.3 ± 0.3	9.4 ± 0.4	11.7 ± 0.6
galactosamine	1.1 (1.1)		

3) Sialic acid by fluorescent method

sialic acid	0.3 (0.4, 0.1)	2.1 (1.9, 2.3)	2.9 (3.0, 2.8)

4) Percentage of carbohydrate by weight[b]

28.6	32.3	34.8

The values are expressed as the mean ± S.E.M. except where two or less determinations were available when the values obtained are given in parentheses

a) the amount of sialic acid present was close to the limit of detection

b) calculated from the values obtained by gas liquid chromatography and ion-exchange chromatography (glucosamine, galactosamine); the amino sugars were presumed to be present as the N-acetyl derivative and sialic acid as N-acetyl neuraminic acid.

confirmed the presence of a fatty acid which ran in an identical manner as stearic acid, and in brain Thy-1 small amounts of another lipid, probably oleic acid, were detected. A maximum limit of lipids present was estimated to be 0.9, 1.3, 1.6 lipid residues per 100 amino acid residues for brain Thy-1, thymocyte Thy-1_{L+} and Thy-1_{L-} preparations respectively. The use of deoxycholate and ethanol in the purification procedures would be expected to remove non-covalently bound lipids, so the significance of this lipid remains to be explained. The remaining unidentified peaks on the gas liquid chromatography analysis amounted to 3% of the total peak area of the sugars.

Sephadex was used in the final step of the purifications and it is possible that glucose could be leached from the column and be present in the Thy-1. However the gel filtration conditions were relatively mild and the concentration and ethanol precipitation steps would be expected to remove free glucose. Furthermore the values from different preparations were consistent so that it is probable that the glucose is a constituent of Thy-1.

The values for the amino sugars obtained from gas liquid chromatography analysis were consistently lower than those obtained by ion-exchange chromatography after hydrolysis with p-toluene sulphonic acid. The yield of this hydrolysis was taken to be 70% as this was found for ovomucoid treated similarly, but different glycoproteins could give different yields for either technique and some variations in yields have been reported (Snary et al, 1976; Allen & Neuberger, 1975). However the differences between the forms of Thy-1 are reflected in both methods of analysis.

From a summation of the amino acid and carbohydrate analyses, the percentage compositions by weight of the Thy-1 glycoproteins were calculated (Table 5.4). The amino sugars were assumed to be present as the N-acetyl derivative and the sialic acid as N-acetyl-neuraminic acid.

The values were all about 30% but Thy-1_{L-} contained more carbohydrate than Thy-1_{L+} which in turn had more than brain Thy-1. This may explain the different electrophoretic mobilities of the 3 forms on polyacrylamide gels in SDS (Ch. 4.7).

5.7 Discussion

5.7.1 Removal of deoxycholate by ethanol precipitation

This procedure provides a simple method to remove the large excess of deoxycholate from the purified Thy-1 glycoproteins and render them water soluble without loss of antigenic activity. In the absence of deoxycholate, the Thy-1 is in an aggregated form of about 10-15 molecules per aggregate; this aggregation is probably caused by the association of hydrophobic regions of the Thy-1 molecules (normally inserted into the lipid bilayer; see below) to form the core of a micellar-like structure with the hydrophilic groups at the outside. The redissociation of the aggregates into monomers would then be interpreted by the binding of deoxycholate to these hydrophobic regions and hence solubilising them (see below).

The absorption spectrum of Thy-1 in the absence of deoxycholate is unusual for a protein because of the low E_{280nm}/E_{260nm}. The value of 1.1 is more typical of a protein in the presence of about 2-3% by weight of nucleic acids (Dawson et al, 1969). The chemical analysis did not give any evidence concerning the E_{260nm} absorbing material, the basis of which remains to be resolved.

Deoxycholate could also be removed from Thy-1 by precipitation with trichloroacetic acid and extraction with acetone as in the concentration of samples for electrophoresis on polyacrylamide gels in SDS. However this form of Thy-1 could only be dissolved in strong dissociating conditions, for example, formic acid, 50% acetic acid or SDS. The ethanol precipitation procedure provides Thy-1 in the absence of

deoxycholate by mild procedures suitable for further analysis.

The Thy-1 in the absence of deoxycholate is also suitable for physiological experiments where the presence of deoxycholate would cause cell lysis. For instance Williams (1976) used Thy-1 in this form to inhibit the binding of anti-(Thy-1) antibodies to bone marrow cells and lymphocytes in controls in autoradiography experiments on Thy-1 bearing cells. This Thy-1 form will also be useful in experiments to test for the function of Thy-1 (see Ch. 7).

The explanation of the effect of deoxycholate on the Thy-1.1 assay in terms of polymeric and monomeric forms of the antigen may provide the reason for some of the experimental confusion about the nature of Thy-1 (discussed in Ch. 7). Also this effect explains why a lower purification factor was obtained for Thy-1.1 than for the Thy-1 xenoantigenic determinants, i.e. 440 fold and 720 fold respectively (Table 4.1). However both factors were somewhat lower than that predicted (1200 fold). The amount of Thy-1 in rat brain can now be estimated more accurately with the assay for Thy-1 xenoantigenic determinants calibrated with a known weight of Thy-1 as determined from the chemical analysis. A value of 180 μg/g wet weight of brain was obtained compared with the previous estimate of 95 μg/g (Ch. 3.2).

5.7.2 Amino acid compositions

The amino acid compositions of the three forms of Thy-1 were very similar and if there are any differences in the polypeptide chains, they will only be clearly resolved by amino acid sequence analysis. When the amino acid composition is compared on the basis of the percentage of polar, intermediate and hydrophobic residues as in Table 5.3, with a selection of other typical membrane proteins (Table 5.5) it can be seen that Thy-1 is not particularly hydrophobic and in fact contains a large proportion of the more hydrophilic residues. However some other

Table 5.5 Comparison of the hydrophobicity of some membrane proteins

Membrane Protein	% of residues that are: polar	intermediate	hydrophobic	Reference
Brain Thy-1	40.1	30.8	29.1	
Bovine retinal rhodopsin	23.9	35.2	40.8	1
Bovine myelin proteolipid	19.3	41.6	39.7	1
Human erythrocyte "band 3"	29.7	31.3	38.9	2
Cholinergic receptor protein from Electrophorus Electricus	31.2	30.3	38.5	3
Cytochrome b_5	37.5	29.9	32.6	1
Brush border peptidase F from rat intestine	35.9	34.8	29.3	4
Major human erythrocyte glycoprotein	31.0	44.7	24.3	5

The amino acids are grouped as in Table 5.3.

References

1) Steck & Fox (1972)

2) Yu & Steck (1975a)

3) Meunier et al (1974)

4) Kim & Brophy (1976)

5) Grefrath & Reynolds (1974).

membrane proteins are rich in the more hydrophilic residues and it is thought that they are anchored in the lipid bilayer of the membrane by a short hydrophobic region of the polypeptide chain. Evidence for this has been found in cytochrome b_5 (Spatz & Strittmatter, 1971), and intestinal hydrolases (Maroux & Louvard, 1976) where peptides enriched in the more hydrophobic residues have been analysed and in the major human erythrocyte glycoprotein which contains a short sequence (23 residues) of mainly hydrophobic amino acids which is believed to span the lipid bilayer (Tomita & Marchesi, 1975). In cytochrome b_5 the removal of deoxycholate caused aggregation in a similar manner to Thy-1 and binding studies with deoxycholate have shown that the detergent only binds to the hydrophobic region which is necessary for insertion into the membrane (Spatz & Strittmatter, 1971; Robinson & Tanford, 1975). This finding suggests that the deoxycholate solubilizes the protein from the membrane by replacing hydrophobic interactions between the protein and other membrane components with hydrophobic interactions between the detergent and the hydrophobic region of the protein. In view of the amino acid composition and solubilisation data on Thy-1 by deoxycholate (see above) a similar method of insertion of Thy-1 into the membrane is likely.

5.7.3 Carbohydrate analysis

The three forms of Thy-1 all contained large amounts of carbohydrate (30% by weight). However the compositions of brain and thymocyte Thy-1 were quite different and smaller differences were observed between thymocyte Thy-1_{L+} and Thy-1_{L-}. The latter differences probably represent the microheterogeneity of carbohydrate residues observed for many glycoproteins (Montgomery, 1972). This variation is caused by variable amounts of the terminal sugars (especially fucose and sialic acid) and no evidence of heterogeneity based on the substitution of one sugar for another has yet to be obtained (Spiro, 1973). Presumably

the sugar structures which are bound by lentil lectin are either absent or masked by other sugar residues in the Thy-1_L- form. In contrast the differences between brain and thymocyte Thy-1 are striking and would appear to represent a different order of heterogeneity. Given that differences in composition are likely to be reflected in larger differences in structure, the carbohydrate chains of brain and thymocyte Thy-1 may be unrelated. The only other reported glycoprotein where the same polypeptide chain is associated with different carbohydrate structures (other than those in the microheterogeneity class) is in hen transferrins (Williams, 1968). Ovotransferrin from hen egg contains no galactose or sialic acid whereas serum transferrin contains approximately 1.8 moles of each per mole of glycoprotein (molecular weight, 80,000). The other carbohydrate residues are present in the same amounts in each glycoprotein and the majority of the carbohydrate is associated with a single peptide with the same amino acid sequence in each glycoprotein. The differences in carbohydrate compositions between brain and thymocyte Thy-1 could represent far greater structural heterogeneity than this, e.g. the galactosamine present only in brain Thy-1 may be bound to serine or threonine and provide a carbohydrate-protein linkage absent from thymocyte Thy-1; an additional chain here must be compensated by less of another chain(s) as the total amount of carbohydrate in the 3 forms is very similar.

Glucose was found in both brain and thymocyte Thy-1 and is a rare constituent of glycoproteins except those containing collagenous structures e.g. basement membrane glycoproteins, complement component C1q (see Spiro, 1973). In these, glucose is present in a characteristic sequence "hydroxylysine-galactose-glucose" which is not present in Thy-1 as no hydroxylysine was detected. However glucose has been found in glycoprotein fractions obtained after lipid extraction from pig lymphocyte membranes (Snary et al, 1976), intestinal membranes (Kim &

Perdoma, 1974), and rat brain membranes (Margolis et al, 1975). Some of this glucose may not be present as an integral part of the glycoproteins as a glycolipid containing glucose (dolichol phosphate bound to a small polysaccharide) has been found in the protein/glycoprotein fraction after similar lipid extractions (Parodi et al, 1972). However glucose has been identified in glycopeptides obtained after papain digestion of chicken lymphocytes (Droege et al, 1975) and also in a few purified glycoproteins e.g. a glycoprotein from calf brain (van Niew Amerongen et al, 1972) rat intestinal brush border membrane peptidases (Kim & Brophy, 1976) and a cardiovascular glycoprotein (Wagh & Roberts, 1972). In the latter case a structure for the carbohydrate residues, including glucose, has been proposed (Roberts & Wagh, 1976).

The presence of very low amounts of sialic acid in brain Thy-1 is interesting as this tissue is particularly rich in sialic acid. It is present both in gangliosides which account for 0.1-0.3% of the wet weight of rat brain (Spence & Wolfe, 1967), and in glycoproteins which are found in all regions of the brain but predominate in areas rich in neuronal bodies; about 90% is membrane bound and found mainly in microsomal and synaptosomal plasma membrane fractions (Brunngraber et al, 1969; Dekirmenjian & Brunngraber, 1969; Roukema & Heijlman, 1970). Presumably the lack of sialic acid must be due to the lack of glycosyl transferases of the correct specificity. Although neuraminidase has been reported on brain membranes (Tettamanti et al, 1972), the purification of Thy-1 was carried out at 0-4^{o}C which should minimise any enzymatic loss of sialic acid, and this type of procedure did not prevent the identification of the brain sialoglycoproteins as above. Thus either brain Thy-1 has particularly labile sialic acid residues or as is more likely, it is present in very low amounts.

A possible role for the carbohydrate of Thy-1 is discussed in Ch. 7.

CHAPTER 6

THE NATURE OF THE THY-1 ANTIGENIC DETERMINANTS

6.1 Introduction

Three sets of Thy-1 antigenic determinants have been defined (Morris & Williams, 1975). These have been used to follow the Thy-1 molecule in purification studies and shown to be present on the purified glycoproteins (see Ch. 4). If these determinants are carried by the carbohydrate structures, it is possible that similar carbohydrate structures are present on other proteins or lipids as for example the ABO blood group antigenic determinants. These are associated with mucopolysaccharides, intestinal membrane dissacharidases and glycolipids (Hakomori & Kobata, 1974; Kelly & Alpers, 1973). Thus the nature of the Thy-1 antigenic determinants is important in understanding whether one is following the polypeptide or carbohydrate structures in distribution studies of Thy-1.

This chapter describes experiments to determine the effect of heating, proteolytic digestion, and reduction and alkylation on the Thy-1 antigenic determinants. These procedures should destroy protein antigenic determinants which are usually dependent on conformation (Crumpton, 1967) but leave carbohydrate antigenic determinants intact (Watkins, 1972).

6.2 The effect of heat on the Thy-1 antigenic activities

Samples of pure brain Thy-1 were heated for 10 min at various temperatures, cooled to $0^{o}C$ and assayed for Thy-1.1 and Thy-1 xeno-antigenic determinants. Fig. 6.1 shows that all the antigenic determinants of brain Thy-1 were destroyed in the temperature range 70-$80^{o}C$. Similar results were also obtained for thymocyte Thy-1_{L+}.

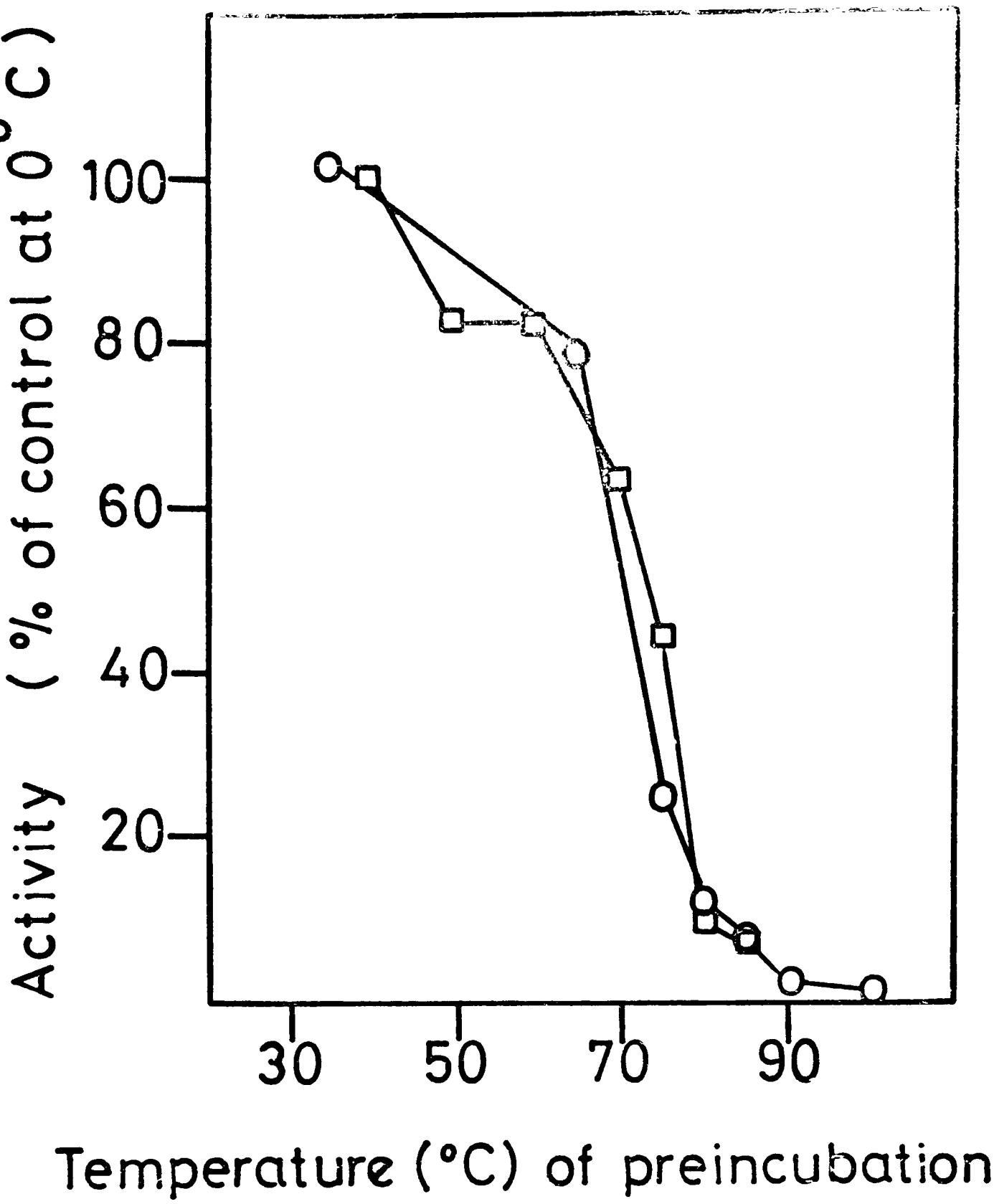


Fig. 6.1 The effect of heat on the Thy-1 antigenic determinants

Purified brain Thy-1 was heated for 10 mins at the temperature shown and cooled to 0°C to assay antigenic determinants. The figure shows the results of two experiments assaying for Thy-1.1 (o) and Thy-1 xenoantigenic activities (□) which are expressed as the percentage activity compared with a sample kept at 0°C throughout (100%).

6.3 The effect of proteolytic digestion on the Thy-1 antigenic activities

The effects of proteolytic digestion were evaluated by measuring the loss of antigenic activity with the inhibition assays for the Thy-1.1 and Thy-1 xenoantigenic determinants and following the destruction of the polypeptide chain by electrophoresis on polyacrylamide gels in SDS. Thy-1 was quite resistent to all the proteolytic enzymes but after incubation with pronase at a high enzyme substrate ratio (1:12) for 24h at 37°C, 95% of the Thy-1 antigenic determinants were destroyed (Table 6.1). Analysis by polyacrylamide gel electrophoresis in SDS showed that the polypeptide chain was almost completely destroyed (Fig. 6.2a). Digestion for 3h with pronase gave only partial loss of antigenic activity (Table 6.1) and of the polypeptide chain (Fig. 6.2a). Thymocyte Thy-1_{L+} could also be degraded by pronase but it appeared to be slightly more resistant than brain Thy-1 (Table 6.1, Fig. 6.2b).

When brain or thymocyte Thy-1 were incubated with trypsin, chymotrypsin or papain under similar conditions then only partial destruction of the antigenic activities (Table 6.1) and the polypeptide chain occurred (Fig. 6.3). The proteolytic enzymes themselves were not detectable on the polyacrylamide gels after similar treatment. In these digestions the amount of Thy-1 antigenic activity remaining (Table 6.1) correlated quite well with the polypeptide chain remaining (Figs. 6.2 & 6.3). Thus there is no evidence contrary to the conclusion that the antigenic determinants were destroyed as the polypeptide chain was digested. Also no large peptides were detected after proteolysis unlike those observed in some membrane proteins e.g. cytochrome b_5 (Spatz & Strittmatter, 1971) and histocompatibility antigen (Nathenson & Cullen, 1974).

6.4 The effect of reduction and alkylation on the Thy-1 antigenic activities

Purified brain Thy-1 was reduced with dithiothreitol in 7M guanidine-HCl. Fig. 6.4 shows that following treatment with 7M guanidine

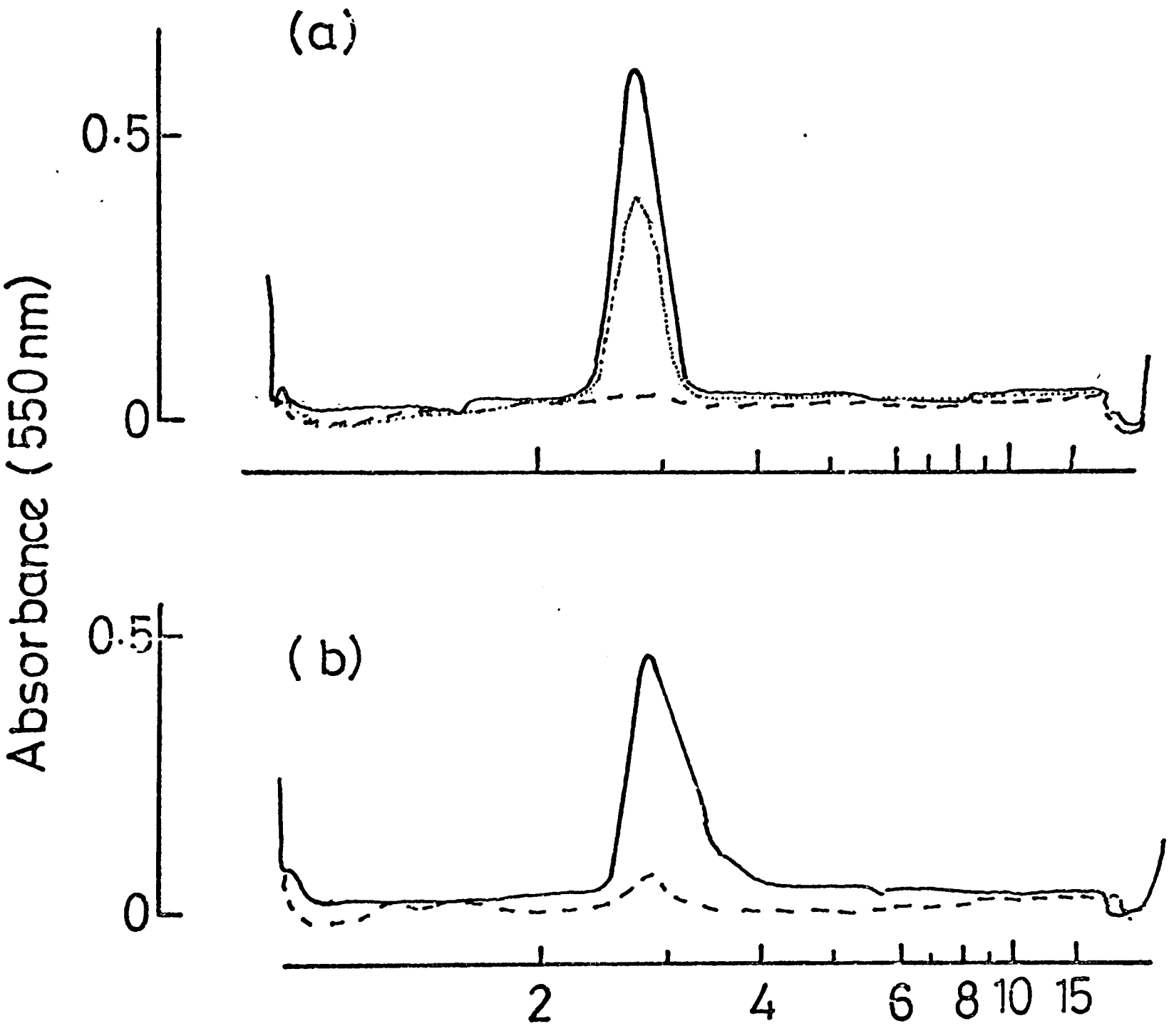
Table 6.1 Effects of proteolytic enzymes on Thy-1 antigenic activities

	Residual antigenic activities (%)	
	Thy-1.1 antigenic activity	Thy-1 xenoantigenic activity
Brain Thy-1		
digested with:		
pronase (3h)	68	66
pronase (24h)	7	6
trypsin	65	48
papain	44	43
chymotrypsin	35	32
Thymocyte Thy-1_{L+}		
digested with:		
pronase	16	14

The values given are related to controls treated similarly except in the absence of the enzyme and are of a typical digestion which was also analysed by electrophoresis on polyacrylamide gels in SDS (Figs. 6.2 and 6.3). Proteolysis was for 24h except as indicated for pronase on brain Thy-1.

Fig. 6.2 Effect of pronase on brain Thy-1 and thymocyte Thy-1_{L+}

The purified Thy-1 glycoproteins were digested with pronase and the effects analysed by electrophoresis on 7.5% polyacrylamide gels in SDS. Equal amounts of protein from each digest and its control (about 5 μg) were electrophoresed and after staining for protein with Coomassie Blue, the gels were scanned at 550 nm. a) shows the scans of brain Thy-1 control (—), and brain Thy-1 after digestion with pronase for 3h (.....) and 24h (-----). b) shows the scans for thymocyte Thy-1_{L+} control (—) and Thy-1_{L+} after digestion with pronase for 24h (----).


(a)
0.5
0
Absorbance (550nm)
(b)
0.5
0
2
4
6
8
10
15
10^{-4} x apparent molecular weight.

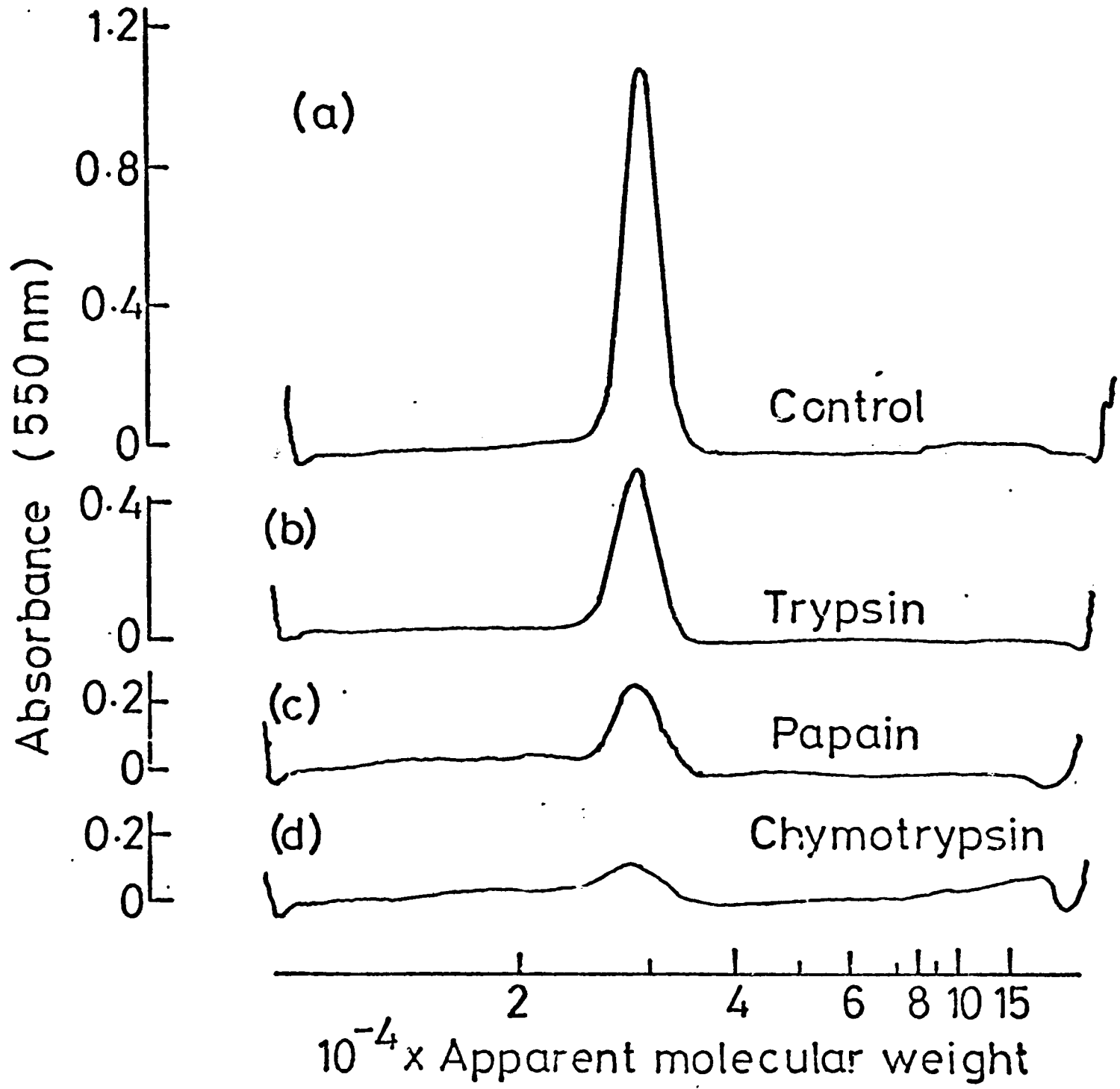


Fig. 6.3 The effects of proteolytic digestion on brain Thy-1

Brain Thy-1 was digested with trypsin, papain or chymotrypsin (see Table 6.1) and the digests analysed by electrophoresis on 7.5% polyacrylamide gels in SDS as in Fig. 6.2. (a) shows a scan for brain Thy-1 control and after digestion of similar amounts of Thy-1 with (b) trypsin, (c) papain, (d) chymotrypsin.

Fig. 6.4 Effect of reduction and alkylation on the Thy-1 antigenic activities

(a) shows the inhibition of the assay for Thy-1.1 by pure brain Thy-1 after treatment with: 0.14M NaCl, 0.025M Tris-HCl, pH 8.0, the buffer control (□), 7M guanidine-HCl, the guanidine control (o), and dithiothreitol in 7M guanidine-HCl (reduced and alkylated sample) (●). All samples were alkylated with iodoacetamide (see Ch. 2.6.6).

(b) shows the inhibition of the assay for Thy-1 xenoantigenic determinants as for (a).

a) assay for Thy-1.1

b) assay for Thy-1 xenoantigenic determinants

Binding of ^{125}I-anti-IgG (cpm×10^{-3})

20
16
12
8
4
0

0·4 1 4 10 40 100

Inhibitor protein per assay (ng)

alone about 60-70% of the Thy-1 antigenic determinants could be recovered after dilution of the guanidine to less than 0.09M, but no Thy-1 antigenic activity was detectable after reduction with dithiothreitol. Thus the loss of antigenic activity parallels the presumed irreversible denaturation of the protein caused by reduction and alkylation whereas guanidine-HCl alone causes little irreversible denaturation .

6.5 Discussion

There are four lines of evidence which suggest that all the antigenic determinants recognised on the Thy-1 molecule are on the polypeptide chain rather than the carbohydrate structures. Three different treatments, heating, proteolytic digestion and reduction and alkylation, all caused destruction of Thy-1 antigenic determinants. These treatments should destroy the conformation of the protein and hence protein based antigenic determinants but not carbohydrate antigenic determinants which are usually dependent on configuration and often stable in strong (protein) denaturing conditions e.g. at 100°C or in 90% phenol (Watkins, 1972). Fourthly, the two forms of thymocyte Thy-1 and brain Thy-1 were antigenically similar (Ch. 4) and must contain some common structural features; from the chemical analysis (Ch. 5) these features are more likely to be associated with the polypeptide chain rather than the carbohydrate structures. Thus these results strongly suggest that all the antigenic determinants recognised on rat Thy-1 are expressed on the protein part of the molecule. The failure to detect antibodies against carbohydrate determinants in the rabbit anti-(Thy-1) serum is presumably because the rabbits are tolerant to these carbohydrate configurations.

Kucich et al (1975) have also suggested that the Thy-1.2 alloantigenic determinant of mouse Thy-1 is protein in nature because digestion of whole cells of a Thy-1.2 mouse line with papain resulted

in the loss of Thy-1.2 antigenic activity. From complementation studies with mutated cell lines deficient in Thy-1, Trowbridge & Hyman (1975) suggested that the Thy-1 antigenic determinants were carried by carbohydrate structures. However these data could be interpreted equally well with the antigenic determinants being carried by the protein part of the molecule.

As the Thy-1 antigenic determinants are most likely to be on the protein part of Thy-1, the distribution of Thy-1 within rat and mouse tissues (see Ch.1.4.6) represents the distribution of the Thy-1 polypeptide chain and one can eliminate the possibility that one is following carbohydrate determinants associated with different proteins or lipids. Another example where the knowledge of the nature of the Thy-1 antigenic determinants is important is in studies on the expression of Thy-1 in various neurological mutants (Schachner, 1973b). These mice have an abnormal arrangement of cells in the cerebellum apparently caused by cell surface alterations (De Long & Sidman, 1970) but no differences in their Thy-1 expression were found compared to controls. However in view of the above studies, the Thy-1 molecule, although present, could have altered carbohydrate structures and it is possible that these mediate the cell recognition processes which are altered in the mutants.

CHAPTER 7

GENERAL DISCUSSION

7.1 Confusion about the nature of Thy-1

The Thy-1 antigen was purified from rat brain and shown to be a glycoprotein of about 24,000 molecular weight. A similar glycoprotein has been characterised as the Thy-1 antigen from rat thymocytes (Letarte-Muirhead et al, 1975). As previous suggestions as to the nature of Thy-1 have been diverse and confused (discussed below), it is important to verify that these glycoproteins are the molecules which carry the Thy-1 antigenic determinants. This was established for both brain Thy-1 (this study) and thymocyte Thy-1 (Letarte-Muirhead et al, 1975) by several criteria, e.g.

a) The glycoprotein fractions contained the Thy-1 antigenic determinants at the high specific activity predicted for the purified antigen, including the genetically defined Thy-1.1 alloantigenic determinant.

b) The antigenic activities were associated with the glycoprotein band after electrophoresis on polyacrylamide gels in SDS.

c) Analysis at an early stage in the purifications showed the antigenic activity was associated with only one homogenous component whose hydrodynamic parameters were compatible with the molecular features of the purified glycoproteins.

d) Antisera raised against either of the purified glycoproteins recognised antigens with the same distinctive tissue distribution as found for Thy-1.1. Also, the antisera raised against the brain Thy-1 glycoprotein could be used in

an affinity column to remove all Thy-1.1 antigenic activity from solubilised rat thymocyte membranes and this activity was associated with the thymocyte glycoproteins.

e) The chemical analysis of the Thy-1 glycoproteins showed a similarity in amino acid compcsitions between thymocyte Thy-1 and brain Thy-1 compatible with their antigenic similarities.

The majority of studies on the nature of Thy-1 have been on mouse Thy-1 and in particular that from thymocytes. However the basic characteristics of the molecule are likely to be similar in both mice and rats. For instance they both carry the rat-mouse cross reacting xenoantigenic determinants and the mouse Thy-1.1 determinant is also present on rat Thy-1. Mouse Thy-1.1 antigenic activity can be solubilised by deoxycholate and the antigen elutes at the same position as rat Thy-1 after gel filtration in deoxycholate (Letarte-Muirhead et al, 1975). Electrophoresis of mouse thymocyte membrane on polyacrylamide gels in SDS results in a similar profile of glycoprotein bands, including one corresponding to rat Thy-1, as found for rat thymocyte membrane (Williams et al, 1977). Trowbridge et al (1975) have used hyperimmune rabbit anti-(rat brain Thy-1) serum (the second antiserum in Ch. 4.5) to immunoprecipitate a glycoprotein of about 25,000 molecular weight from mouse thymocytes which had been surface labelled with ^{125}I and solubilised in detergent. This glycoprotein could also be identified on the membrane of cell culture lines which expressed Thy-1 antigenic activity but absent from mutants which no longer expressed the antigen (Trowbridge & Hyman, 1975). In similar experiments on mouse thymocytes, Vitetta et al (1973) and Trowbridge et al (1975) could not obtain immunoprecipitation with conventional alloantisera. This is probably due to these antisera containing too large a proportion of low affinity antibodies (see Ch. 5.3) to precipitate solubilised

Thy-1 molecules, whereas the rabbit anti-(Thy-1) serum, made specific for the Thy-1 alloantigenic determinant by absorption, contains antibodies of higher affinity to give the specific precipitation observed by Trowbridge et al (1975).

Vitetta et al (1973) were able to immunoprecipitate material with congenic anti-(Thy-1) alloantisera if the thymocytes were "solubilised" by freezing and thawing. From radioactive labelling studies and analysis on polyacrylamide gels in SDS, they concluded that the Thy-1 antigen was a glycolipid complex with a molecular weight of about 35,000. In view of the method of extraction and the above findings, this complex probably contains some Thy-1 glycoprotein but associated with other components in a large low density aggregate. Atwell et al (1973) labelled thymocyte membranes with ^{125}I by the lactoperoxidase method and with a urea/acetic acid extraction, obtained immunoprecipitates with non-congenic anti-(Thy-1) alloantiserum which indicated that Thy-1 was a protein with a molecular weight of 60,000. This result has not been confirmed by others and may have been due to other specificities in the antiserum.

In purification studies on the mouse Thy-1.2 Sauser et al (1974) obtained a fraction containing a protein of 40,000 molecular weight with a 60 fold purification for the Thy-1.1 determinant compared to the starting thymocytes. As the rat Thy-1 glycoprotein was pure when the Thy-1.1 determinant was 260 fold purified (Letarte-Muirhead et al, 1975), it is probable that this protein of Sauser et al (1974) is a major contaminant in a fraction which also contains the Thy-1 glycoprotein.

Esselman and Miller (1974) suggested that mouse Thy-1 is a ganglioside and more specifically that the Thy-1.2 antigenic determinant was on G_{M1} ganglioside (Miller & Esselman, 1975). These conclusions were based on inhibition studies of cytotoxicity assays with purified

gangliosides from mouse brain, reconstituted with lipids, and are obviously in complete disagreement to the present findings for rat brain Thy-1. However these results can be criticised on several grounds. In particular the inhibitions of the assays did not show the high degree of specificity expected for the Thy-1 alloantigens as they were defined by Reif & Allen (1964). For instance, they suggest that G_{M1} ganglioside carries the Thy-1.2 determinant, yet G_{M1} purified from a Thy-1.1 mouse strain inhibits their cytotoxicity assay for Thy-1.2, 50% as well as G_{M1} from a Thy-1.2 mouse. Furthermore very large amounts of ganglioside (500ng) were needed to inhibit the Thy-1.2 cytotoxicity assay compared with less than 10ng of Thy-1 glycoprotein needed for 50% inhibition of radioactive binding assays for Thy-1.1 (see Ch. 4.6). Taking the molecular weights of the ganglioside and the Thy-1 glycoprotein as 1500 and 24,000 respectively, this represents a difference of 800 fold on a molar basis, which cannot be accounted for by the sensitivities of the assays since these should not differ by more than 10 fold (Acton et al, 1974; Morris & Williams, 1975). Esselman & Miller do not discuss such problems as how G_{M1} purified from one strain of mice might carry determinants different from that purified from a different strain and the tissue distribution of the ganglioside which they admit is not the same as for Thy-1? Thus it is clear that Miller & Esselman (1975) are not assaying the Thy-1 antigen and recent studies (Kucich et al, 1975; Arndt et al, 1976) have not been able to confirm these results. Furthermore co-capping experiments with anti-(Thy-1) sera and anti-(ganglioside) sera showed that these components co-capped independently (D. Marcus, personal communication).

In conclusion it seems very likely that the Thy-1 antigens in both rat and mouse are glycoproteins with molecular weights of around 25,000.

7.2 The structure of the Thy-1 glycoproteins

The exact molecular weight of Thy-1 is currently being determined by the methods of Tanford et al (1974) but the value of 24,000 obtained by electrophoresis on polyacrylamide gels is likely to be reasonably close to the correct molecular weight (Ch. 4.4.). Taking this value, the molecular weight in deoxycholate (29,000; Ch. 5.4.1) indicates that about 12 molecules of deoxycholate (molecular weight, 393) may be bound; this amount is comparable to the binding of 20 molecules of deoxycholate to the hydrophobic region of cytochrome b_5 (Robinson & Tanford, 1975) which is only slightly more hydrophobic than Thy-1 (Ch. 5.7.2). The Thy-1 polypeptide chain can be estimated to consist of 150 amino acids from the percentage of carbohydrate (Ch. 5.6) and assuming a molecular weight of 24,000; possible compositions for the Thy-1 glycoproteins are given in Table 7.1. The question of the identity of the polypeptide chains of the forms of Thy-1 should be resolved by amino acid sequence analysis. If they are identical they must be coded for by the same structural gene. This will also show whether Thy-1 contains a region of the more hydrophobic residues (see Ch. 5.7.2 and below). Carbohydrate structure analysis is necessary to confirm the proposed differences between the carbohydrate structures of brain and thymocyte Thy-1 based on the carbohydrate analyses (Ch. 5.6).

7.3 The Thy-1 antigen as a membrane glycoprotein

It is clear that Thy-1 is a major component of rat thymocyte membranes (Ch. 1.4.6 and Ch. 7.1), and must also be a major component of some brain membranes as it is present in similar amounts as in thymocytes (Ch. 3.2). When Thy-1 is compared to other characterised membrane glycoproteins,(Table 7.2), it can be seen that it contains a higher percentage of carbohydrate than many other glycoproteins.

Table 7.1 The chemical composition of the Thy-1 glycoproteins

Carbohydrate[b]	Number of residues per molecule of Thy-1[a]		
	Brain Thy-1	Thymocyte	
		$Thy\text{-}1_{L+}$	$Thy\text{-}1_{L-}$
fucose	2.7	1.4	1.4
mannose	17.9	15.9	14.2
galactose	2.6	8.2	10.3
glucose	0.8	1.9	1.8
glucosamine	12.4	14.1	17.1
galactosamine	1.6	0.0	0.0
sialic acid	0.3	2.7	3.3

Amino acids[c]	All 3 forms of Thy-1
Asx	19.0
Thr	12.2
Ser	11.0
Glx	13.6
Pro	5.6
Gly	8.2
Ala	4.6
Val	11.0
Ile	6.1
Leu	16.2
Tyr	3.1
Phe	5.8
His	5.9
Lys	10.6
Arg	11.2
Cys	4.7
Met	1.5
Trp	1.0

a) based on a molecular weight of 24,000 for brain Thy-1

b) the carbohydrate values are from the gas liquid chromatography and ion exchange chromatography (glucosamine and galactosamine) see Ch.5.

c) amino acid analyses are the mean of the analyses for Thy-1 from brain and $Thy\text{-}1_{L+}$

Table 7.2 The compositions of membrane glycoproteins

Glycoprotein	Molecular weight	% carbohydrate	Reference
Thy-1	24,000	30	see Ch. 5
human erythrocyte glycoprotein	29,000	55	Grefrath & Reynolds (1974
rat intestinal peptidase F	280,000	23	Kim &
S	280,000	29	Brophy (1976)
bovine rhodopsin	38,000	7	Plantner & Kean (1976)
sperm glycoprotein I	28,000	44	Hagopian et al (1976)
alkaline phosphatase (calf intestinal)	140,000	12	Fosset et al (1974)
alkaline phosphatase (bovine kidney)	172,000	15	Cathala et al (1975)
acetyl choline receptor (electrophorus electricus)	300,000	+	Meunier et al, 1972,1974
human erythrocyte "band 3"	90,000	< 5	Yu & Steck, 1975a
human histocompatibility antigen	45,000	7	Terhorst et al, 1976
mitochondrial calcium binding glycoprotein	33,000	13	Hughes,1976
Basement membrane glycoprotein (bovine renal)	70,000	9	Ohno et al., 1975

However the membrane glycoprotein which shows several similarities to Thy-1 is the much studied major glycoprotein of human erythrocytes which is known to span the erythrocyte membrane via a short hydrophobic sequence of 23 amino acids (Winzler, 1969; Marchesi et al, 1972; Bretscher, 1971; Steck, 1974; Tomita & Marchesi, 1975). Table 7.3 shows the similarity in basic characteristics in Thy-1 and this glycoprotein, but in detail the amino acid and carbohydrate compositions are quite dissimilar.

Based on the data available on membrane proteins and in particular, the major glycoprotein of human erythrocytes (see above), cytochrome b_5 (Spatz & Strittmatter, 1971; Robinson & Tanford, 1975) and the results obtained for the Thy-1 glycoprotein, the following may be predicted for the properties of the Thy-1 molecule in membranes.

a) The molecule is inserted into the lipid bilayer of the membrane via a short sequence of the more hydrophobic amino acids (see Ch. 5.7.2).

b) The carbohydrate structures will be arranged on the outside of the membrane. This appears to be the general rule for membrane glycoproteins (Steck, 1974; Hughes, 1975).

c) Most of the polypeptide chain will be on the outside of the membrane. As Thy-1 contains a high percentage of the more hydrophilic amino acids (Ch. 5.7.2) these would be expected to be exposed to the aqueous environment. As three sets of antigenic determinants can be recognised at the cell surface, most of the molecule (including the carbohydrate; see (b)) is presumably at the outside of the membrane.

These points are summarised in a model of the proposed structure of the Thy-1 membrane glycoprotein (Fig. 7.1). In this, the Thy-1 antigenic specificities are illustrated as single determinants for simplicity although there is no evidence as to the size of these determinants or whether they overlap. Three carbohydrate chains are

Table 7.3 Comparison of rat thymocyte Thy-1 and the major glycoprotein of human erythrocytes

	Thymocyte Thy-1	Erythrocyte Glycoprotein
Number of molecules per cell	600,000	500,000
Molecular weight	25,000(a)	29,000
Size of polypeptide chain (number of amino acid residues)	150(b)	130
Amino acid composition		
Percentage of:		
hydrophilic residues	40.1	31.0
intermediate residues	30.8	44.7
hydrophobic residues	29.1	24.3
Carbohydrate composition		
Percentage by weight of carbohydrate residues:		
fucose	1.0	1.3
hexoses	17.1	12.2
hexosamines	10.7	16.2
sialic acid	3.5	25.2
total carbohydrate	32.3	54.9

Data for thymocyte Thy-1 are from Acton et al, 1974; Letarte-Muirhead et al, 1975 and the chemical data is for the Thy-1_{L+} form of thymocyte Thy-1 from Ch. 4. The data for the erythrocyte glycoprotein are from Grefrath and Reynolds (1974), and Steck (1974).

a) This is the apparent molecular weight of Thy-1_{L+} obtained from electrophoresis on 12.5% polyacrylamide gels in SDS and is probably close to the correct molecular weight.

b) based on the apparent molecular weight (see (a)).

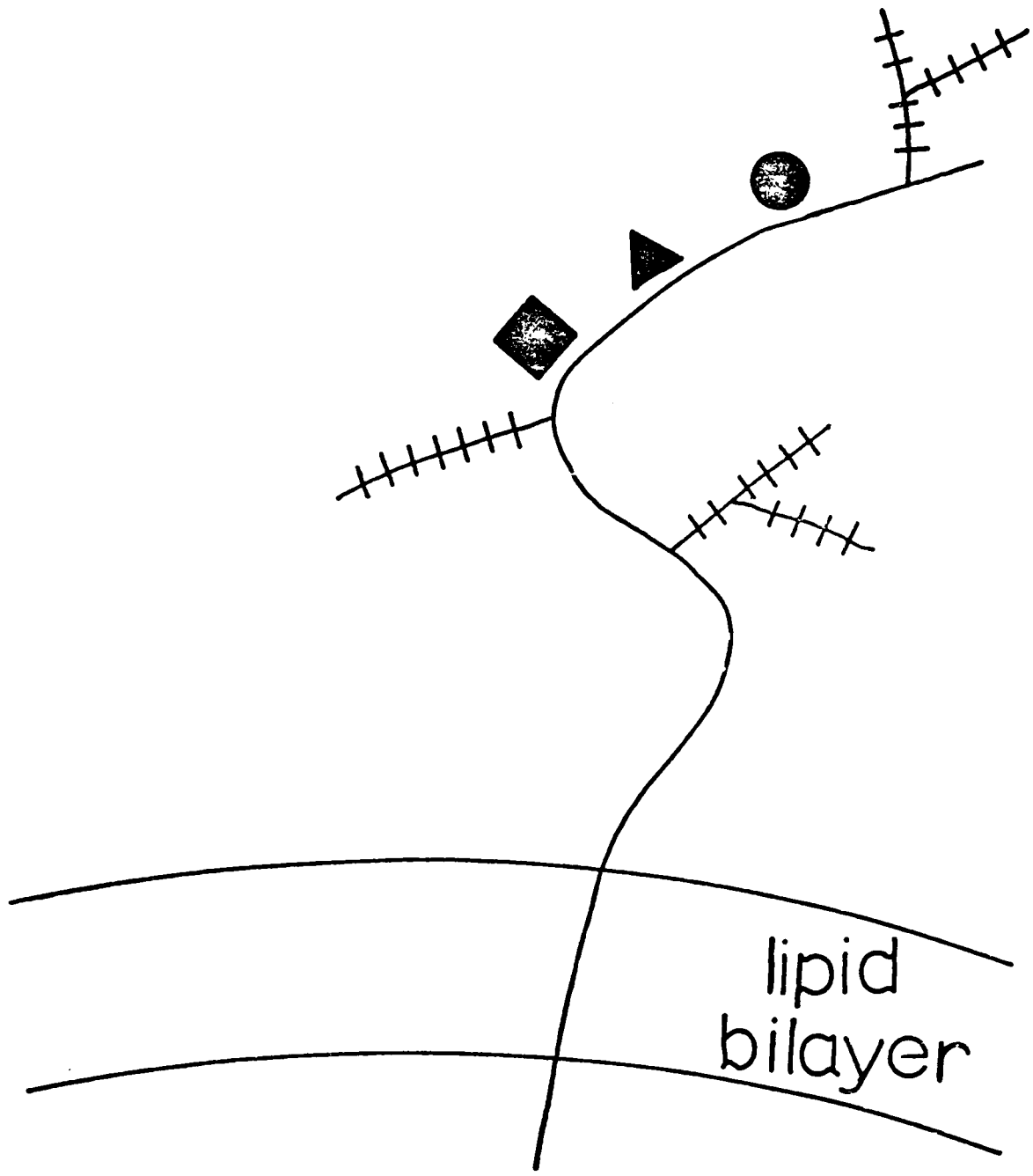


Fig. 7.1 The proposed structure of the Thy-1 molecule

polypeptide chain

carbohydrate chain

the three antigenic determinants of Thy-1, i.e. Thy-1 alloantigenic, and Thy-1 rat-mouse and rat-specific xenoantigenic determinants.

illustrated as a likely number considering the size of commonly found carbohydrate structures and the polypeptide chain is shown to traverse the lipid bilayer. These points will be clarified when more information concerning the structure of Thy-1 and its orientation are available, for example by determining whether Thy-1 is associated ... in an oligomeric form or with any other membrane proteins like some of the erythrocyte membrane proteins (Wang & Richards, 1974; Yu & Steck, 1975b; Nicolson, 1976). One advantage Thy-1 should have compared to the erythrocyte glycoproteins, is that it is synthesised on lymphoid and neuroblastoma cell lines (Zwerner & Acton, 1975; Pfeiffer & Acton, personal communication). These would provide a suitable system to study the biosynthesis of an integral membrane glycoprotein with the advantages that Thy-1 is present in large amounts and good reagents are available e.g. strong specific antisera.

7.4 The function of the Thy-1 molecule

7.4.1 General considerations

Firstly, there is no evidence as to the function of Thy-1. However it is reasonable to assume it has a function and probably in the two tissues where it is abundant in both rat and mouse, i.e. thymus and brain. It may also play a role in some bone marrow and spleen cells in the rat and T-lymphocytes in the mouse and possibly epidermal cells and fibroblasts where Thy-1 is also present in rat and mouse.

Thy-1 has been clearly shown to be a glycoprotein containing a relatively large (30%) amount of carbohydrate but there is very little information concerning the function of these highly glycosylated membrane glycoproteins apart from some enzymes from specialised membranes such as rat intestinal brush border peptidases (Kim & Brophy, 1976). For example there is no known function for the much studied major

glycoprotein of human erythrocytes although the cleavage of sialic acid residues from this molecule may be involved in the removal of aged erythrocytes in vivo (Kay, 1975). Indeed this glycoprotein, normally present at 500,000 molecules per cell (Table 7.3) has been shown to be absent in a human erythrocyte variant - the homozygous En(a-) erythrocyte - without apparent clinical abnormalities (Tanner & Anstee, 1976); although, the other major glycoprotein, "band 3", does have an altered carbohydrate content, these differences do not compensate for the loss of carbohydrate due to the lack of the major glycoprotein (Tanner et al, 1976). This lack of evidence for the function of other higher glycosylated membrane glycoproteins precludes any simple suggestions as to the function of Thy-1 but the structural data obtained for Thy-1 does pose some limitations on possible functions e.g. depending on which part(s) of the molecule mediates the function:
a) If the specific function of Thy-1 is determined by the protein part of Thy-1, then this function is presumably the same in both brain and thymus. In this case the differences in carbohydrate would not be of significance and may simply reflect the presence of glycosyl transferases of different specificities in brain and thymus.
b) The specific function of Thy-1 may be determined by the carbohydrate and the different carbohydrate structures of Thy-1 in brain and thymus could mediate different functions in the two tissues. In this case the protein would serve to display the carbohydrate structures, integrate them onto the membrane surface and possibly to transmit the effect of the carbohydrate-mediated reaction.

The latter alternative is the more attractive in view of the molecular features of Thy-1, i.e. the large amount of carbohydrate and the likelihood that it is arranged in different structures in brain and thymus. The possibility that carbohydrate structures at the cell surface are involved in cell-cell interactions has been receiving

considerable attention (Hughes, 1975) and many examples of specific cell-cell recognition have been studied, e.g. the species specific reaggregation of dissociated marine sponge cells (Wilson, 1907; Humphreys, 1963), the aggregation of cellular slime moulds induced by starvation (Gerisch, 1968), the tissue specific aggregation of embryonic and neural cells (Moscona, 1962 a & b; Roth, 1968; Garber & Moscona, 1972), and glycoproteins or at least carbohydrates have been implicated in several of these e.g. a large highly glycosylated glycoprotein (molecular weight, 2,000,000) has been purified and can mediate the aggregation of sponge cells (Henkart et al, 1974; Cauldwell et al, 1974); the carbohydrate moieties of glycoproteins have been implicated in the adhesion of teratoma cells and chick embryonic neural cells (Oppenheimer, 1974; Balsamo & Lilien, 1975) and more recently a partially purified fraction of glycopeptides has been shown to specifically inhibit the aggregation of fibroblasts (Vicker, 1976). However the clearest example is in the cellular slime mould Dictyostelium discoideum where a lectin, that is a protein which has the ability to bind specific sugar structures, has been purified; it is developmentally regulated (i.e. it appears only after starvation) and is present at the cell surface and is implicated in the aggregation of the slime mould (Rosen et al, 1973; Chang et al, 1975). Different lectin activities have been observed in six different strains of cellular slime moulds and purified from two (Rosen et al, 1975, 1974). Thus it appears that a specific recognition process during the aggregation of these cellular slime moulds is mediated by cell surface lectins which recognise sugar structures at the surface of other cells.

In higher organisms the recognition processes are likely to be more complex but lectins at the cell surface could be involved, and recently a developmentally regulated lectin specificity has been recognised on embryonic chick muscle and a myogenic cell line (Nowak

et al, 1976). A mechanism for cell-cell interactions has been proposed in which carbohydrate structures are recognised and modified by glycosidases or glycosyl transferases (Roseman, 1970; Roth et al, 1971); however the presence of the latter enzymes at the cell surface is still controversial (Hughes, 1975). The properties of Thy-1 are well suited to those expected of a molecule involved in cellular recognition e.g. it could provide a large number of carbohydrate ligands at the cell surface for recognition by lectins, glycosidases or glycosyl transferases present at the surface of other cells.

7.4.2 The function of Thy-1 in the lymphoid system.

Possible speculation about the function of Thy-1 in the lymphoid system is not helped by the general lack of information concerning the function of any glycoproteins at the cell surface. The only lymphocyte specific molecule for which a function is known is the immunoglobulin on B-lymphocytes which is generally accepted to be a receptor for antigen (Warner, 1974). However the class of highly glycosylated glycoproteins of the cell surface, which includes Thy-1, could mediate reactions with other cells or factors as discussed above.

From the distribution of Thy-1 (see Ch. 1.4.6), it is reasonable to assume that Thy-1 has a role on thymocytes where it is expressed at high levels in mice and rats. If it has a function in rat bone marrow cells or murine T-lymphocytes, then this function must differ between the two species. Thus Thy-1 is associated with the precursors of mature lymphocytes, except in the case of murine T-lymphocytes which express Thy-1 in lower amounts than in thymocytes, so that Thy-1 could have a role in the development of lymphocytes. It is now possible to devise experiments to test for this. For example it should be possible to put a radioactive label (e.g. ^{125}I) on purified Thy-1 (after removal of deoxycholate) and look for binding to thymocytes. The occurrence of

specific binding would suggest that Thy-1 is involved in interactions between thymocytes. If this binding was mediated by specific carbohydrate structures then brain Thy-1 may be found not to bind. One could test for binding to thymus epithelial cells in a similar way. Another approach would be to see if purified Thy-1 or anti-(Thy-1) antibodies had any effect on the in vitro passage of selected cells through perfused organ cultures (see Sedgley & Ford, 1976). Although this type of experiment is unlikely to provide clear evidence of the function, it may give indications as to the function which would permit more definitive experiments to be devised.

7.4.3 The function of Thy-1 in the brain

The distribution of Thy-1 amongst the different cell types in the brain is obviously important in understanding the role of Thy-1 and in devising experiments to test for its function. The antigenic studies discussed in Chapter 1.4.6 have shown that Thy-1 is present in all regions in adult brain and is absent at birth, but a clear cellular distribution of Thy-1 has not been obtained although there was some evidence that Thy-1 was associated with neuronal rather than glial cells. The failure to obtain a clear localisation (Moore et al, 1971) was probably due to the weak, low affinity antisera then available. It should now be possible to obtain definitive results with the strong, high affinity anti-(Thy-1) sera now available.

As an alternative to studying antigenicity one could examine brain membranes by polyacrylamide gel electrophoresis in sodium dodecyl sulphate to see if the characteristic glycoprotein band of Thy-1 was present. Many workers have used this technique but examination of their results for a Thy-1 band is complicated by the different gel systems used and frequently the gels were not stained for carbohydrate nor any indication of the apparent molecular weights of the bands given. However a band which could be Thy-1 was found in some preparations of

synaptosomal plasma membranes (Banker et al, 1972; Morgan et al, 1973; Zanetta et al, 1975) but not in others (Waehneldt et al, 1971; Gurd et al, 1974; Susz et al, 1973; Gurd & Mahler, 1974); however it was not observed in preparations of brain myelin or mitochondria nor in post synaptic densities isolated from synaptosomes (Banker et al, 1974). If Thy-1 was present only on synaptosomal plasma membranes it would be expected to be a major component and should be readily identifiable. For instance there is approximately 0.18mg Thy-1/g brain and the amount of synaptosomal plasma membrane protein has been estimated as 0.5 mg/g brain (Gurd et al, 1974). Despite the approximate nature of this estimate and in view of the findings on polyacrylamide gel electrophoresis (see above) it is probable that Thy-1 is present on different types of brain membrane which may include synaptosomal plasma membranes. It should be possible to clarify this by following the antigenicity during purification of brain membrane fractions.

It has been suggested that cell surface glycoproteins are involved in the formation of specific interneuronal connections and establishing correct neuronal relationships although the evidence is at best indirect (Brunngraber, 1969; Barondes, 1970). The molecular properties of Thy-1, discussed above, are suitable for a role in cellular recognition in the brain and especially in its post-natal development. Functional studies with brain are complicated by the complex structure of the tissue. However cell lines with different differentiated properties are available (Schubert et al, 1974) and various cell types can be separated from new born rodent brain (Barkley et al, 1973); these would be more amenable for analysis than the intact brain.

A simple functional test for Thy-1 would be to see if purified Thy-1 bound preferentially to any structural features in the brain. One could use radioactively labelled Thy-1 and follow binding to tissue

sections, cell lines or dissociated cell cultures by autoradiography. Any correlation with a particular cell type should help in devising more definitive experiments.

An alternative approach is to apply purified Thy-1 or anti-(Thy-1) antibodies to in vitro systems which fall into four main types:

a) the normal reassociation of dissociated embryonic or new born neural cells (Gurber & Moscona, 1972; De Long & Sidman, 1970).

b) adhesion assays where the binding of separated populations of neural cells to a monolayer population of neural cells is followed (Walther et al, 1973; Gottlieb & Glaser, 1975).

c) cell lines where differentiation events have been observed and induced (Seeds et al, 1970; Schubert et al, 1969, 1971), including the formation of recognisable synapses (Kidokoro et al, 1976).

d) brain cultures of dissociated neural cells where many normal developmental events have been observed (Seeds & Vatter, 1971; Seeds, 1975).

Of these (d) may be the most suitable to study as the post-natal increase in Thy-1 from birth (Ch.1.4.6) correlates with many developmental events and it is attractive to assume Thy-1 is involved in these.

Thus there are a number of ways the function of Thy-1 could be investigated and more definitive experiments should be possible once a clear localisation of Thy-1 in brain is available.

7.5 Conclusion

The purification of Thy-1 from rat brain and thymocytes (Letarte-Muirhead et al, 1975) has shown that Thy-1 is a major membrane glycoprotein in these tissues. The chemical analysis shows that it belongs to the class of highly glycosylated membrane glycoproteins,

whose functions are unknown. However Thy-1 is likely to be a suitable molecule to investigate possible functions and in particular the cell surface interactions involving carbohydrate structures which are currently of considerable interest. The differences in carbohydrate between brain and thymocyte Thy-1 provide weight for this type of function and may provide useful controls for functional studies in thymus and brain. Although very little can be inferred about the function of Thy-1, it is very amenable to further analysis in many directions, not only in the direct functional sense, but also as an integral membrane glycoprotein.

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Purification of the Thy-1 Molecule, a Major Cell-Surface Glycoprotein of Rat Thymocytes

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The Thy-1 molecule, which was identified by its antigenic activities, has been purified from rat thymocytes. The purification involved preparation of crude membranes and solubilization in deoxycholate, followed by gel filtration and affinity chromatography on antibody or lectin columns. In all cases the purified molecule was a glycoprotein that did not form higher polymers and was not associated with other polypeptide chains. The Thy-1 glycoprotein could be found in two forms, one binding to lentil lectin, the other not. Both forms had the same detectable antigens and were of a similar but not identical size. After sodium dodecyl sulphate–polyacrylamide-gel electrophoresis the apparent molecular weight of Thy-1 binding to lentil lectin was 25000, whereas that not binding to the lectin was 27000, with heterogeneity towards forms of apparently higher molecular weight.

A study of cell-surface antigens provides one method of identifying tissue-specific membrane molecules (Bennett *et al.*, 1972; Morris & Williams, 1975). Antigens which are restricted to particular differentiated cell types may be carried by molecules with functions related to the specific role of the cells which display them. The Thy-1 (θ) alloantigens are of this type, being found in large amounts in brain and thymocytes of rodents (Reif & Allen, 1964, 1966; Douglas, 1972; Acton *et al.*, 1974). In the mouse, the Thy-1 molecule occurs in two allelic forms resulting in two alloantigens Thy-1.1 (θ-AKR) and Thy-1.2 (θ-C_3H) (Reif & Allen, 1964; Snell & Cherry, 1972). In the rat, Thy-1.1 antigen, identified with mouse alloantiserum, is present in the thymus and brain of all strains which have been studied, but the Thy-1.2 antigen has not been found (Douglas, 1972; Acton *et al.*, 1974). It was also established that the rat Thy-1 molecule carries two other antigenic determinants which can be recognized by xenogeneic antiserum, in this case a rabbit anti-(rat brain) serum (Morris & Williams, 1975; Morris *et al.*, 1975). One antigen is found on rat but not on mouse tissues and is referred to as the 'rat-specific Thy-1 xenoantigen', whereas the other is common to rat and mouse and is called the 'rat–mouse cross-reacting xenoantigen'. Quantitative studies using antisera against Thy-1.1 antigen and Thy-1 xenoantigens have shown that there are about 600000 antigenic sites per rat thymocyte, and this suggests that the Thy-1 molecule is a major membrane component (Acton *et al.*, 1974; Morris & Williams, 1975).

To elucidate the function of the Thy-1 molecule an understanding of its molecular structure is required. In preliminary investigations (Letarte-Muirhead *et al.*, 1974; Morris *et al.*, 1975) it was established that rat Thy-1 antigens are not inactivated by detergents, and that the Thy-1 molecule can be solubilized in deoxycholate. In this detergent Thy-1 was found to have a sedimentation coefficient of 2.4S, a Stokes radius of 3.0nm and a partial specific volume of 0.70ml/g. The mol.wt. of the Thy-1–detergent complex was calculated to be 28000, and it seemed likely that Thy-1 was a protein, carbohydrate or glycoprotein, rather than a glycolipid as suggested by others (Esselman & Miller, 1974; Vitetta *et al.*, 1973).

The present paper reports the continuation of these studies, which resulted in the purification of the Thy-1 molecule from deoxycholate extracts of rat thymocyte membrane. Purification was achieved by two independent methods, one involving gel filtration and affinity chromatography with lectins (Hayman & Crumpton, 1972), the other involving antibody affinity chromatography against the brain Thy-1 molecule (Barclay *et al.*, 1975). The results show that rat Thy-1 is a glycoprotein with an apparent mol.wt. of about 25000 and consists of a single polypeptide

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chain. Evidence for possible heterogeneity in the carbohydrate part of the molecule was obtained.

Materials and Methods

Materials

Animals. These were as described by Acton *et al.* (1974) except for Wistar rats which were obtained from the M.R.C. Radiobiology Unit, Harwell, Oxon, U.K.

Buffers. Deoxycholate buffer used for gel filtration and affinity chromatography contained 0.5% deoxycholate, 0.02% NaN_3, and 0.01 M-Tris–HCl, pH 8.0. The Tris was neutralized at 20°C, and at 4°C the pH was 8.4.

Chemicals. Avidin, fetuin, deoxycholate and Tween-40 were from Sigma Chemical Co., London S.W.6, U.K.; acrylamide, bisacrylamide and sodium dodecyl sulphate were from BDH Chemicals Ltd., Poole, Dorset, U.K.; Schiff reagent was from Difco Labs., Surrey, U.K.; Coomassie Blue was from Michrome, Edward Gurr Ltd., London S.W.14, U.K.; concanavalin A was from Pharmacia Fine Chemicals, S-751 04 Uppsala 1, Sweden.

Antibodies. The following were used. (1) Balb/c mouse anti-(Wistar rat brain) serum, specific for Thy-1.1 alloantigen on AKR mouse thymocytes (Acton *et al.*, 1974). (2) Rabbit anti-(rat brain Thy-1) serum, specific for Thy-1 on rat thymocytes with 70% of antibody against Thy-1 rat-specific xenoantigen, 26% against Thy-1 rat–mouse cross-reacting xenoantigen, and 4% against Thy-1.1 (Barclay *et al.*, 1975); the strength of the antiserum was estimated to be approx. 0.3 mg of anti-(Thy-1) antibody/ml. (3) Rabbit anti-(thymocyte Thy-1) serum, raised against Thy-1 purified by affinity chromatography with lentil lectin as described in the Results section. Two rabbits were immunized three times at 8-day intervals with 30 μg of Thy-1 per injection. The first two doses were in complete Freund's adjuvant and given intramuscularly, the third was in incomplete Freund's adjuvant and given subcutaneously. The animals were bled 8 days after the third injection and analysed as described in the Results section. (4) Purified horse IgG* anti-(rabbit IgG) antibody labelled with ^{125}I (20 μCi/μg) (Morris & Williams, 1975). (5) Purified rabbit $F(ab')_2$ anti-(mouse IgG) antibody labelled with ^{125}I (20 μCi/μg) and prepared as described for anti-(mouse Fab fragment) antibody (Acton *et al.*, 1974). (6) Rabbit anti-(lentil lectin) antibody which was raised by immunization with lentil lectin in the same way as for antiserum (3) above, by using 600 μg of lectin per dose.

* Abbreviations: IgG, immunoglobulin G; Thy-1_{L+}, Thy-1 binding to lentil lectin; Thy-1_{L-}, Thy-1 not binding to lentil lectin; Thy-1_{total}, Thy-1 containing a mixture of Thy-1_{L+} and Thy-1_{L-} purified by antibody affinity chromatography.

Methods

Unless otherwise stated all procedures were carried out at 0–4°C.

Indirect binding assays and units of antigenic activity. Antigen activity was measured by inhibition of indirect radioactive-binding assays; the different types of assay possible are as described by Morris & Williams (1975). To assay antigen in detergent extracts, trace amounts of second antibody were used, whereas in the quantitative analysis of anti-(thymocyte Thy-1) antibody saturating amounts of ^{125}I-labelled horse anti-(rabbit IgG) were added. In all cases the standard assay contained 25 μl of antiserum, 25 μl of inhibitor and 50 μl of target cells. In the second step 100 μl of ^{125}I-labelled anti-(IgG) was added.

In the Thy-1.1 assay (Letarte-Muirhead *et al.*, 1974), Balb/c anti-(Wistar rat brain) serum was added at 1:50 (v/v) (final dilution 1:200) and ^{125}I-labelled anti-(mouse IgG) was used in the second step rather than ^{125}I-labelled anti-(mouse Fab fragment).

In the Thy-1 xenoantigen assay (Morris *et al.*, 1975) an antiserum against purified brain Thy-1 (see under 'Antibodies' above) was used rather than one against whole brain homogenate. The target cells were rat thymocytes, and the antiserum did not need absorption as only 4% of the antibody was against Thy-1.1 antigen. Distinction was not made between rat-specific and rat–mouse cross-reacting xenoantigen, and the serum was added at 1:1500 (v/v) (final dilution 1:6000).

To compare antigenic activities at different stages of the purification a unit of activity was defined as the amount of antigen needed to give 50% inhibition of the appropriate standard binding assay. Thus 1 ml of extract of which 25 μl gave 50% inhibition at a dilution of 1:100 would contain 4000 units of antigenic activity.

Preparation and solubilization of thymocyte membranes. Thymus glands were obtained from 70–100 rats (approx. 10^{11} thymocytes) and membrane was prepared by the Tween-40 method (Morris *et al.*, 1975) with volumes increased in proportion with increased cell numbers. The membrane was resuspended in 25 ml of 0.01 M-Tris–HCl, pH 8.0, plus 0.02% (w/v) NaN_3, to which was added 25 ml of 4% (w/v) deoxycholate in the same buffer. After 60 min at 0°C the extract was centrifuged for 6750000 *g*-min, leaving solubilized antigen in the supernatant.

Gel filtration in deoxycholate. Upward-flowing columns of Sephadex G-200 were eluted with deoxycholate buffer. For gel filtration of solubilized membrane, 50 ml of the supernatant was loaded on to a Pharmacia K-50 (5 cm × 90 cm) column and 20 ml fractions were collected. For final purification of Thy-1 obtained by antibody affinity chromatography, a Pharmacia K-26 (2.6 cm × 90 cm) column was used, and 5 ml fractions were collected.

Affinity chromatography with columns of Sepharose 4B coupled with lentil lectin and concanavalin A. *Lens culinaris* phytohaemagglutinin (referred to as lentil lectin) was prepared from lentils by the method of Howard *et al.* (1971) as modified by Hayman & Crumpton (1972). The lentil lectin was pure as judged by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and was coupled to activated Sepharose 4B (Cuatrecasas, 1970) at a ratio of between 3 and 14mg of lectin to 1ml of packed swollen beads. The Sepharose 4B–lectin was always used in amounts such that saturation did not occur during absorption. In large-scale preparations, as in Table 1, 2.5ml of beads with 14mg of lentil lectin/ml was sufficient.

Before use, the lentil lectin–Sepharose 4B column was washed with deoxycholate buffer, then with 0.5M-methyl α-D-glucopyranoside in deoxycholate buffer, and finally with deoxycholate buffer. The sample was then applied and the column washed with deoxycholate buffer until the E_{280}^{1cm} reading was zero. Bound material was then eluted with 0.5M-methyl α-D-glucopyranoside in deoxycholate buffer and the eluate assayed for protein and antigenic activity.

Affinity chromatography with concanavalin A–Sepharose 4B (10mg/ml of beads) was carried out in the same way.

Affinity chromatography with antibody–Sepharose 4B columns. γ-Globulin [obtained by precipitation with 16% (w/v) Na_2SO_4] from rabbit anti(brain Thy-1) and anti(lentil lectin) antisera was coupled to Sepharose 4B at 20–35mg of protein/ml of beads as described by Cuatrecasas (1970) for anti-insulin antibody. Before use, anti-(Thy-1) columns were pre-eluted with 3vol. of 0.05M-diethylamine–HCl buffer, pH11.5 (Read *et al.*, 1974), and washed with deoxycholate buffer. This eluted a small amount of IgG (detected by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis) and thereafter IgG was not detectable in any fractions.

For affinity chromatography, columns of volume 9–15ml were set up with a 5ml column of normal rabbit IgG–Sepharose 4B preceding the antibody column (to absorb aggregates and decrease non-specific binding). Deoxycholate extract of thymocyte membrane was passed through the column at approx. 0.3ml/min and washed through with deoxycholate buffer until no further E_{280}-absorbing material emerged. Then the upper column was disconnected and the antibody column eluted at 0.7ml/min with 2 column-volumes of 0.05M-diethylamine–HCl, pH11.5, plus 0.5% deoxycholate, followed by deoxycholate buffer again. The eluted fractions were immediately brought to pH8.3 with solid glycine and then dialysed overnight against deoxycholate buffer. Antigenic activities and protein contents were then determined. Columns were used three times without detectable loss of antigenic activity.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. This was carried out as described by Fairbanks *et al.* (1971), by using 5.6% (w/v) acrylamide gels (containing 1% sodium dodecyl sulphate) and 0.2% sodium dodecyl sulphate in the electrophoresis buffer as the standard system. When 5, 7.5, 10 and 12.5% (w/v) gels were used, the different concentrations were achieved by adding more of the acrylamide–bisacrylamide mixture. Samples were concentrated for electrophoresis by precipitation with 20% (w/v) trichloroacetic acid for 60min at 0°C followed by washing twice with acetone (0°C), which removed both deoxycholate and trichloroacetic acid. Some variation in the effectiveness of precipitation of deoxycholate extracts was observed; thus in all cases where purity was being estimated gels were also run with material concentrated by ultrafiltration (see below). Sodium dodecyl sulphate (1% or more to give a sodium dodecyl sulphate/protein ratio of >7:1) and 4M-urea, plus either 2mM-iodoacetamide or 1% dithiothreitol, was added to the precipitates, which were solubilized by heating at 100°C for 3min; reduced samples were then alkylated with iodoacetamide. Gels were stained for protein with Coomassie Blue (Weber & Osborn, 1969) and de-stained electrophoretically with a Canalco de-stainer. For carbohydrate stain the periodic acid–Schiff procedure of Glossmann & Neville (1971) was used except that 25% (v/v) propan-2-ol plus 10% (v/v) acetic acid was used to fix the gels. The relative mobility of the bands was determined by comparison with the mobility of the Bromophenol Blue marker and the apparent mol.wt. estimated by using standard curves constructed with marker proteins (Weber & Osborn, 1969). The marker proteins used (with mol.wts. in parentheses) were: H chain (52000) and L chain (23500) of rabbit IgG; bovine serum albumin (68000); hen ovalbumin (43000); whale myoglobin (17000). Densitometry was carried out on a Gilford 2410-S gel scanner at 550nm for Coomassie Blue, and 560nm for the periodic acid–Schiff stain.

To assay Thy-1.1 and Thy-1 rat xenogeneic antigen activities after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, the concentration of sodium dodecyl sulphate in the gel and buffer was decreased to 0.1% (w/v) and the sample was loaded in 0.1% (w/v) sodium dodecyl sulphate–0.01M-Tris, pH8.0. ^{125}I-labelled hen ovalbumin and ^{125}I-labelled whale myoglobin iodinated as for ^{125}I-labelled antibodies (Jensenius & Williams, 1974) were loaded in trace amounts as internal markers. After electrophoresis the gels to be assayed were cut into 3mm slices which were crushed in 0.5ml of 0.05% (w/v) sodium dodecyl sulphate in 10mM-Tris, pH8.0, counted for radioactivity in a Nuclear

Enterprises γ-radiation counter to determine marker positions, and left overnight at 4°C. The material eluted from every slice after centrifugation was tested for antigenic activity by inhibition of the indirect binding assays. In these assays, the concentration of sodium dodecyl sulphate during absorption was 0.025% (w/v), and this decreased the antigenic activity of pure Thy-1 treated in an identical way, except for the electrophoresis step, to 10% of its activity in deoxycholate buffer. Thus to estimate the recovery of antigen activity from the gel a standard curve of inhibition by Thy-1 in the presence of sodium dodecyl sulphate was used.

Concentration of fractions throughout purification. Fractions were concentrated for analysis or further procedures by ultrafiltration in an Amicon apparatus by using a PM10 filter.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951); bovine serum albumin (concentration determined by using $E_{280}^{1cm} = 6.74$ for a 1% solution) was used as standard. When assays were carried out in the presence of deoxycholate the standards used to construct the curve contained the same amount of deoxycholate as the unknowns.

Results

Procedures used in the purification of the Thy-1 molecule

A summary of the procedures used in purification of the Thy-1 molecule is shown in Fig. 1. In all cases the purification of Thy-1 was assessed by measurement of its antigenic activities (Thy-1.1 and Thy-1 xenoantigens) by inhibition assays (see under 'Methods'). The first step always involved preparation of crude thymocyte membrane followed by solubilization in deoxycholate. After this, a combination of affinity chromatography (using antibody or lectin columns) and gel filtration resulted in purification of the Thy-1 molecule. The steps summarized in Fig. 1 are discussed in detail below.

Estimation of the degree of purification required

In a typical preparation of Thy-1, approx. 1×10^{11} thymocytes were used. Given that there are approx. 6×10^5 antigenic sites per thymocyte for Thy-1.1 (Acton *et al.*, 1974) or Thy-1 xenoantigens (Morris & Williams, 1975) it can be calculated that 1×10^{11} cells have about 100nmol of Thy-1. Taking a mol.wt. of 28000 this amounts to 2.8mg of antigen. This can be compared with a total protein content of 1810mg per 1×10^{11} thymocytes (Table 1). Thus it can be estimated that purification of the order of 650-fold is required to obtain pure antigen on the basis of protein content.

Preparation of crude membrane with Tween-40

Morris *et al.* (1975) showed that if thymocytes are suspended in 2% (w/v) Tween-40 at 0°C most of the Thy-1.1 antigen is released as a membrane fragment which sediments at $4.5 \times 10^6 g$-min but not at $1 \times 10^5 g$-min. The advantage of this method is that it can easily be increased in scale for use with large batches of cells. Table 1 shows that approx. 50% of the Thy-1.1 antigen activity is recovered in the membrane fraction at a ninefold purification. In Plate 1 the gel profiles for membrane material analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and stained with Coomassie Blue for protein and with periodic acid–Schiff reagent for carbohydrate are shown. Plate 1(*a*) shows that a large number of protein bands are present, but that the periodic acid–Schiff-staining profile is simpler (Plate 1*e*). The main bands, which are likely to be glycoproteins, are labelled from 1 to 5 and have apparent mol.wts. of 32000, 60000, 90000, 110000 and 150000 respectively (in 5.6% acrylamide gels). Band 2 is broad, and band 4 appears as a doublet in gels when small amounts of material are electrophoresed. In Plate 1(*e*) there is another band of periodic acid–Schiff-staining material

Table 1. *Purification of Thy-1.1 antigen by affinity chromatography on lentil lectin–Sepharose 4B*

Fraction	$10^{-4} \times$ Total Thy-1.1 activity (units/fraction)	Protein (mg/fraction)	Relative specific activity	Yield of antigenic activity (%)
Cells (1.3×10^{11})	84	2360	1	100
90000*g*-min pellet	15	1428	0.3	18
4500000*g*-min supernatant	1.5	714	0.06	2
4500000*g*-min pellet (=membrane)	42.3	128	9	50
Membrane in deoxycholate	42.3	128	9	50
6800000*g*-min pellet	2.0	18	3	2
6800000*g*-min supernatant (=soluble fraction)	27.6	108	7	33
After Sephadex G-200 chromatography	14.9	14	30	18
Sepharose 4B–lectin unbound fraction	8.3	9	26	10
Sepharose 4B–lectin bound and eluted fraction	8.3	0.9	260	10

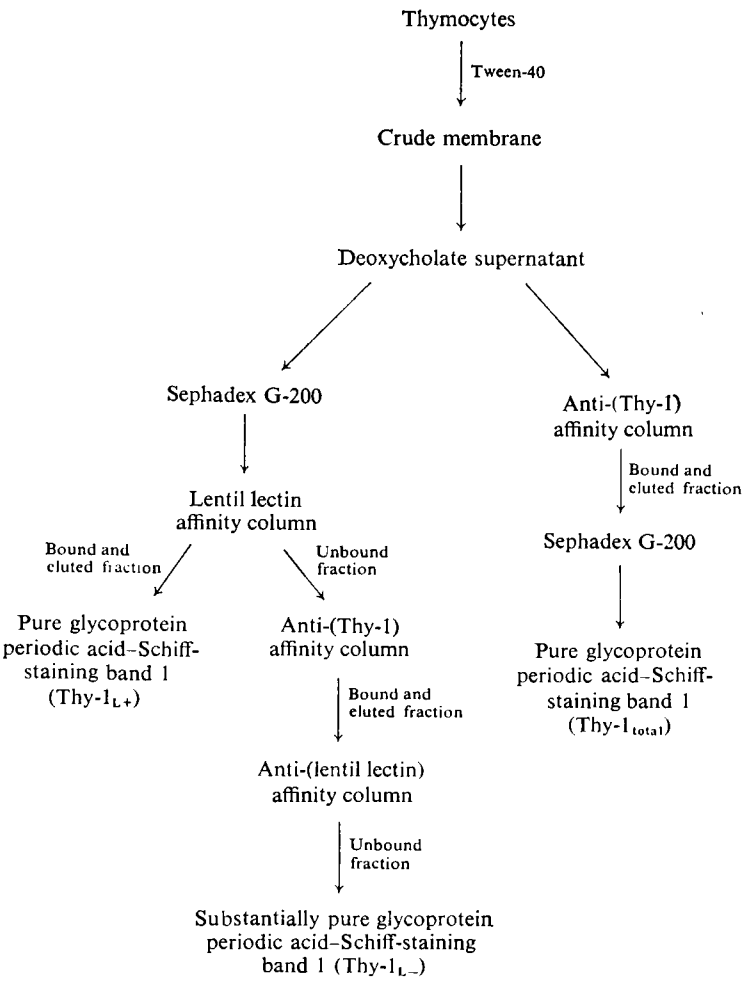


Fig. 1. *Flow sheet for the purification of the Thy-1 molecule*

running slightly ahead of the Bromophenol Blue marker and this is probably glycolipid (Glossman & Neville, 1971).

The periodic acid–Schiff staining pattern is similar to that obtained by Ladoulis *et al.* (1974) for rat thymocyte plasma membrane prepared by conventional methods.

Solubilization in deoxycholate

Thy-1.1 antigen is effectively solubilized by deoxycholate (Letarte-Muirhead *et al.*, 1974), and in the present preparations the same method was used except that the ratio of membrane protein/deoxycholate was increased to 1:8 (w/w), which was the maximum ratio compatible with solubilization. At this ratio, membrane from 1.3×10^{11} cells was solubilized in 50ml of 2% (w/v) deoxycholate, and 65% of the Thy-1.1 activity of the crude membrane was found in the 6.8×10^{6}*g*-min supernatant (Table 1). Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of material from the solubilized extract gave gels which did not differ markedly from those carried out with membrane in either protein (compare Plate 3*a* with Plate 1*a*) or carbohydrate (compare Plate 3*e* with Plate 1*e*) bands. The only difference was a decreased glycolipid band; this material may be lost during trichloroacetic acid precipitation and acetone extraction in the presence of deoxycholate.

Gel filtration

The deoxycholate extract of membrane (50ml) was applied to a large column (1800ml; 5cm×90cm) of Sephadex G-200 and eluted with deoxycholate buffer. The elution profile was as previously described for chromatography on a smaller column (Morris *et al.*, 1975), and most of the Thy-1.1 activity was eluted at the elution volume of ovalbumin. The fractions containing Thy-1.1 activity were pooled and contained 13 and 55% respectively of the protein and antigenic activity applied to the column (Table 1); a 4.3-fold purification of antigen resulted. The pooled fraction contained a number of polypeptides as detected by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Plate 1*b*), but only one periodic acid–Schiff-staining component (Plate 1*f*). As might be expected this was the low-molecular-weight band 1 seen in the whole-membrane profile (Plate 1*e*).

Affinity chromatography with lentil lectin and concanavalin A columns

In preliminary experiments a deoxycholate extract of crude thymocyte membrane was passed through a lentil lectin–Sepharose 4B column which was then washed with buffer and eluted with methyl α-D-glucopyranoside. The fractions were assayed for Thy-1.1 antigen and, as Fig. 2(*a*) shows, a substantial proportion of the antigen was not retarded by the column, whereas the rest bound to the column and was eluted by methyl α-D-glucopyranoside. Re-running of the unretarded (Fig. 2*b*) and eluted fractions (Fig. 2*c*) confirmed that they were distinct and henceforth they are referred to as 'Thy-1.1_{L-}' and 'Thy-1_{L+}'.

In large-scale preparations the pooled Thy-1.1-containing fractions from a Sephadex G-200 column were passed through a lentil lectin column in conditions where saturation was not reached, and the unretarded and eluted fractions were recovered and analysed. The unretarded fraction had a decreased specific activity for Thy-1.1 antigen (per mg of protein), and contained similar protein bands after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis as the fraction applied to the column (Table 1 and Plate 1*c*). This fraction also retained part of periodic acid–Schiff-staining band 1 (Plate 1*g*) seen in the starting G-200 fraction, but this was clearly and reproducibly decreased in amount. The eluted fraction was purified for Thy-1.1 antigen ninefold compared with the Sephadex G-200 fraction, and the overall purification was 260-fold compared with the starting cells (Table 1). This approaches the estimated requirement of a 650-fold purification and suggested that Thy-1_{L+} should be a major component of this fraction. In fact when sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out, only one major band was seen after staining for protein (Plate 1*d*) or carbohydrate (Plate 1*h*). There was also one minor protein band present, but this was almost certainly lentil lectin eluted from the column, since it was obtained in washings of the column before and after application of the post-G-200 material.

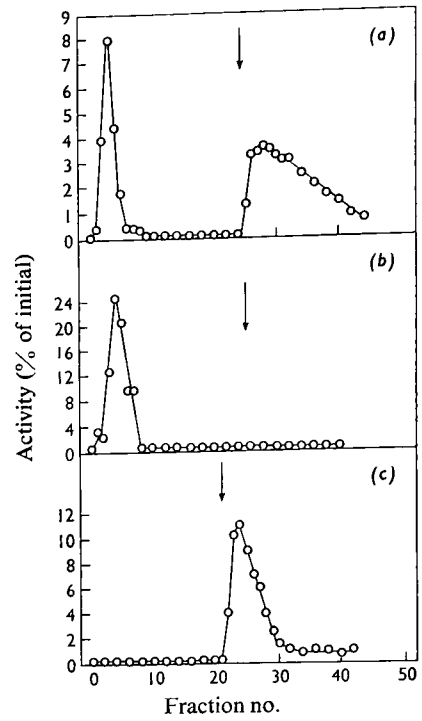
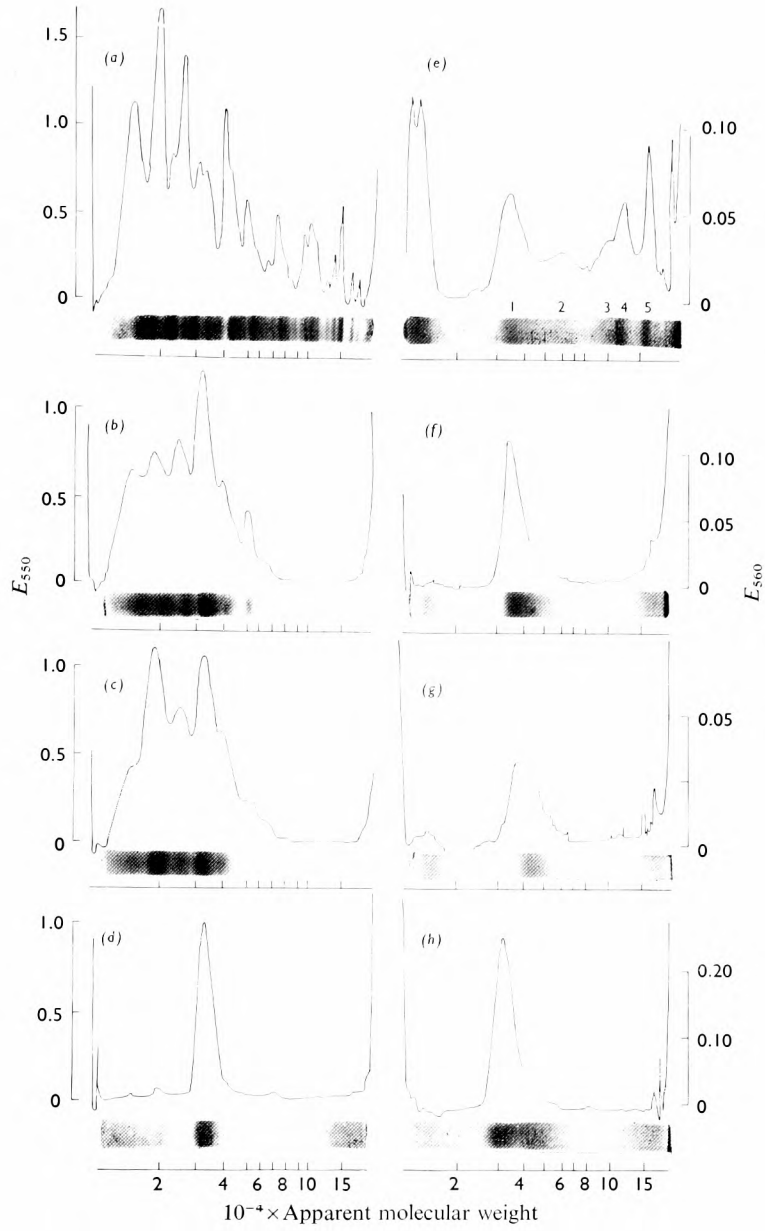


Fig. 2. *Affinity chromatography of Thy-1.1 on Sepharose 4B–lentil lectin*

(*a*) 2ml of a 2% deoxycholate extract from thymocyte membranes prepared from 1.3×10^9 cells was applied to a 2.0ml Sepharose 4B–lentil lectin column (3mg of lectin/ml of beads) which was then washed with deoxycholate buffer (44ml) and eluted with 0.5M-methyl α-D-glucopyranoside in deoxycholate buffer; an arrow marks the beginning of elution. The collected fractions (2ml) were assayed for Thy-1.1 activity, which is expressed as the percentage of the activity applied to the column. (*b*) shows chromatography as in (*a*) except that the applied fraction was the material which did not bind to lentil lectin (fractions 2–5 from *a*). (*c*) shows chromatography as (*a*) except that the applied fraction was the material which had bound to lentil lectin (fractions 26–36 from *a*).

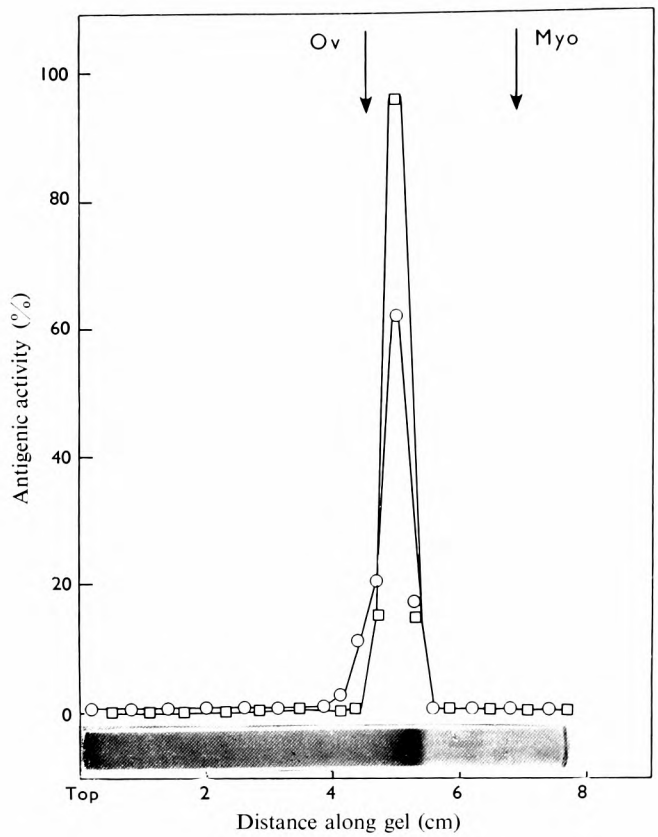
To verify that the Thy-1.1 antigenic activity coincided with the major band in Plate 1(*d*) and 1(*h*), material eluted from gel slices after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was assayed for antigenic activity. Sodium dodecyl



EXPLANATION OF PLATE 1

Purification of Thy-1_{L+} *analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

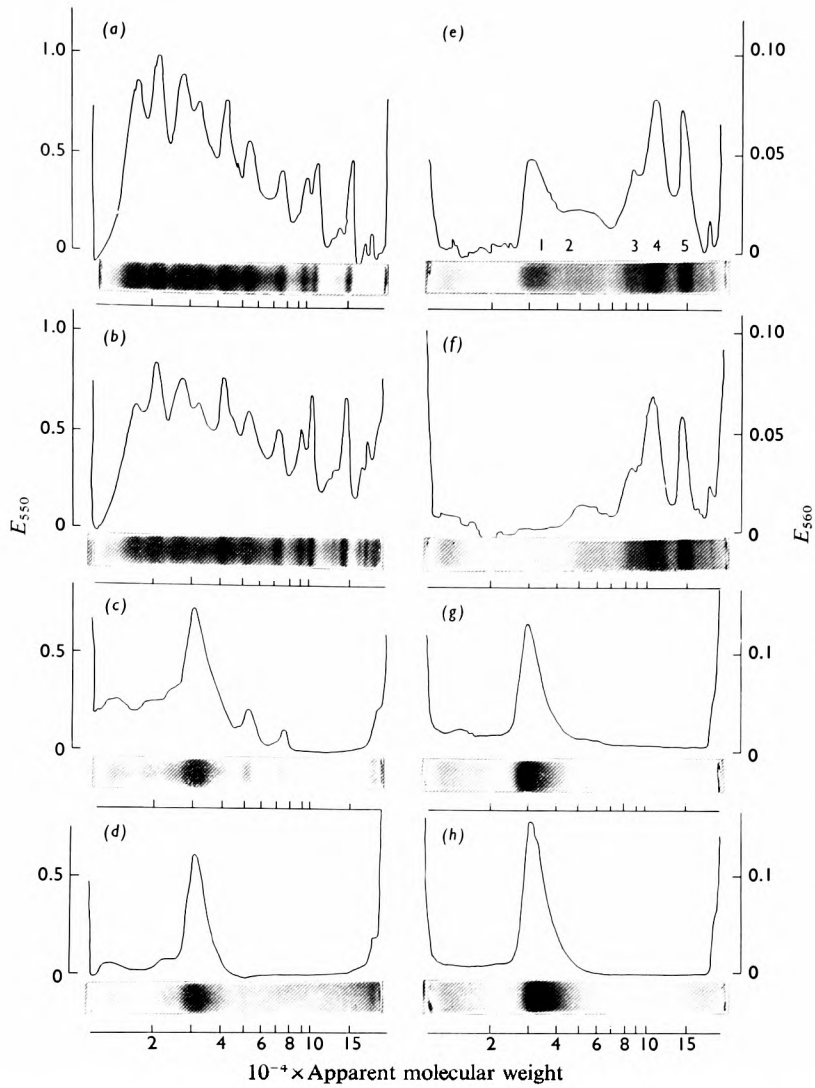
Fractions after reduction and alkylation were electrophoresed on 5.6% gels and stained with Coomassie Blue (*a*, *b*, *c*, *d*) or periodic acid–Schiff reagent (*e*, *f*, *g*, *h*). The following fractions are shown: Tween-40 membrane, 60μg (*a*) and 340μg (*e*); Thy-1.1 active fraction from Sephadex G-200, 50μg (*b*) and 150μg (*f*); lentil lectin unretarded fraction, 50μg (*c*) and 180μg (*g*); lentil lectin absorbed and specifically eluted fraction, 13μg (*d*) and 30μg (*h*).



EXPLANATION OF PLATE 2

Assay of Thy-1.1 *and Thy*-1 *xenoantigenic activity after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

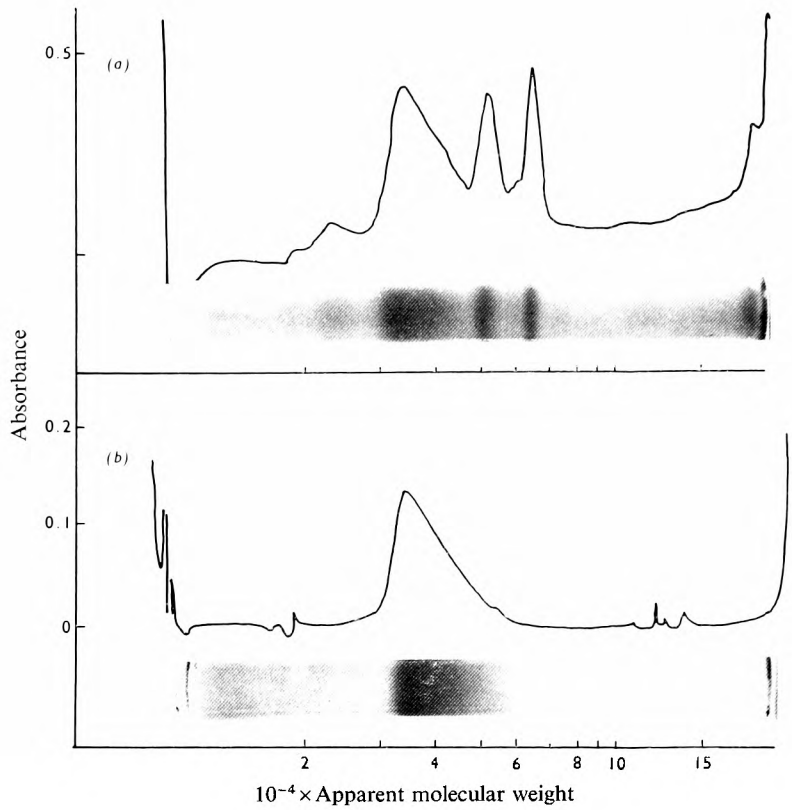
2.5μg of Thy-1 purified by the lentil lectin column was electrophoresed on a 5.6% acrylamide gel containing 0.1% sodium dodecyl sulphate with ^{125}I-labelled whale myoglobin (Myo) and ^{125}I-labelled hen ovalbumin (Ov) as markers. After electrophoresis the gel was sliced and assayed for Thy-1.1 activity ( ) and Thy-1 xenoantigen activity ( ) as described under 'Methods'. Also shown is the photograph of a gel on which 7μg of Thy-1 was electrophoresed under the same conditions and stained with Coomassie Blue.



EXPLANATION OF PLATE 3

Purification of Thy-1_{total} *analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

Fractions, after reduction and alkylation, were electrophoresed on 5.6% gels and stained with Coomassie Blue (*a*, *b*, *c*, *d*) or periodic acid–Schiff reagent (*e*, *f*, *g*, *h*). The following fractions are shown: deoxycholate extract of Tween-40 membrane, 80μg (*a*) and 350μg (*e*); deoxycholate extract depleted of Thy-1 antigens, 80μg (*b*) and 350μg (*f*); fraction eluted from antibody column by pH11.5 buffer, 20μg (*c*) and (*g*); eluted fraction after Sephadex G-200 chromatography, 20μg (*d*) and (*h*).



EXPLANATION OF PLATE 4

Thy-1_L- *analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

23μg of the Thy-1_L- fraction obtained by using the antibody column (Table 3) was electrophoresed after reduction and alkylation on 5.6% gels and stained with Coomassie Blue (*a*) or periodic acid-Schiff reagent (*b*).

sulphate caused a tenfold decrease in the Thy-1.1 antigenic activity compared with that in deoxycholate buffer. However, the inhibition curve was not altered in shape and analysis of the gels was possible. Plate 2 shows that Thy-1.1 antigenic activity was in the same position on the gel as the major protein band visible if a gel run in the same conditions was stained with Coomassie Blue. In addition to assaying Thy-1.1 activity in this experiment, Thy-1 xenoantigen activity was also measured. As shown below, the glycoprotein purified with lentil lectin has strong Thy-1 xenoantigenic activity as well as Thy-1.1 activity, and this activity coincided with Thy-1.1 on the polyacrylamide gels (Plate 2).

Thus it appeared that the glycoprotein bound to, and eluted from, lentil lectin–Sepharose 4B was pure Thy-1_{L+}, and that the residual glycoprotein found in the unretarded fraction (Plate 1*g*) was Thy-1_{L-}. If this were so, it was possible that another agglutinin might bind the Thy-1_{L-}. Concanavalin A was tried for, although it binds to the same sugar residues as lentil lectin, it has a binding constant for methyl α-D-mannoside and methyl α-D-glucopyranoside 50 times higher than that of lentil lectin (Stein *et al.*, 1971). Thus the Sephadex G-200 fraction containing Thy-1.1 activity was passed twice through a lentil lectin column and then chromatographed on a concanavalin A–Sepharose B absorbent. Approx. 50% of the activity was bound and could be eluted with methyl α-D-glucopyranoside. This supported the view that Thy-1_{L-} was also a glycoprotein.

Affinity chromatography with anti-(brain Thy-1) antibody columns

To further substantiate the above results another affinity method for purification was tried. Antibody against purified Thy-1 from rat brain was coupled to Sepharose 4B and used to absorb antigen from a deoxycholate extract of thymocyte membrane. Throughout the purification both Thy-1.1 and Thy-1 xenoantigens were assayed.

Details of the affinity chromatography are described in Fig. 3 and Table 2. When deoxycholate extract was passed through the antibody affinity column (Fig. 3*a*), antigen was depleted from a large volume, and, when the column was saturated, both Thy-1.1 and Thy-1 xenoantigens appeared simultaneously in the eluate.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out on the starting extract and on the antigen-depleted effluent (at 60ml in Fig. 3) and the results are shown in Plate 3(*a*) and 3(*b*) for protein and Plate 3(*e*) and 3(*f*) for carbohydrate stains. No clear removal of any protein band can be associated with absence of antigen, but the removal of periodic acid–Schiff-staining band 1 occurred in the antigen-depleted fractions. After

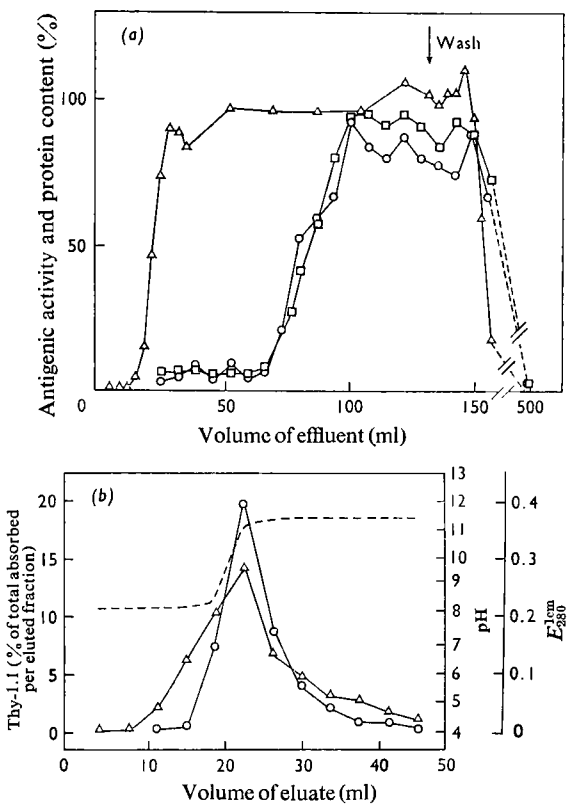


Fig. 3. *Affinity chromatography for Thy-1 with an antibody–Sepharose 4B column*

Membrane from 8×10^{10} thymocytes was solubilized in 160ml of 1% deoxycholate, and 140ml of the extract was passed through a 15ml column of Sepharose 4B to which was coupled rabbit anti-(rat brain Thy-1) antibody. As shown in (*a*), the effluent was assayed for protein (△), Thy-1.1 antigen (○) and Thy-1 xenoantigen activity (□) and the results are plotted as a percentage of the antigenic activity or protein content of the starting extract (100% = 450μg/ml of protein, 1.8×10^3units/ml of Thy-1.1 antigen, and 7.6×10^3units/ml of Thy-1 xenoantigen). After washing, the column was eluted with pH11.5 buffer as in (*b*), where the pH (----), E_{280}^{1cm} (△) and Thy-1.1 antigen activity (○) of the eluted fractions are shown. Antigen activity is plotted as percentage of the total absorbed antigen recovered per eluted fraction, and each point represents one fraction.

the column became saturated with antigen, the periodic acid–Schiff-staining band 1 reappeared (not shown but identical with those in Plate 3*a* and 3*e*).

Elution of the column with 0.05M-diethylamine buffer, pH11.5, is shown in Fig. 3*b*. It can be seen that 44% of the absorbed Thy-1.1 antigenic activity was

Table 2. *Purification of Thy-1_{total} with antibody columns*

Affinity chromatography with antibody columns was as described in Fig. 3 and data shown below are pooled from the experiment in Fig. 3 plus one other.

Fraction	Protein (mg)	Thy-1.1 antigen			Thy-1 xenoantigen		
		$10^{-4}\times$ units	Relative sp. activity	Yield (%)	$10^{-4}\times$ units	Relative sp. activity	Yield (%)
Membrane extract from which antigen was depleted	43	17.3	1	100	74.3	1	100
Fraction eluted by pH11.5 buffer	2.1	8.8	10.5	51	40	11	54
Fraction after Sephadex G-200 column chromatography	0.7	5.3	19	31	25	22	35

recovered in a peak associated with material having absorbance at 280nm. This peak also contained 54% of the absorbed Thy-1 xenoantigen activity (Table 2). Analysis of the eluted material by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed one main protein band with an apparent mol.wt. of 32000, plus a number of less-prominent bands (Plate 3*c*). Only one periodic acid–Schiff-staining band was found and this corresponded to the major protein band (Plate 3*h*). For further purification the material eluted from the affinity column was chromatographed on Sephadex G-200 and the Thy-1.1 antigenic activity was eluted at a Stokes radius of 3.0nm as before. Analysis of this fraction by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed only the major protein band (Plate 3*d*) and periodic acid–Schiff-staining band 1 (Plate 3*h*). (This preparation is henceforth referred to as 'Thy-1_{total}'.)

Parameters of the purification by antibody affinity chromatography are summarized in Table 2 for Thy-1.1 and Thy-1 xenoantigen. It is noteworthy that yields and purification are calculated with respect to deoxycholate extract and thus yield from starting cells would be about half of that shown, and the purification factor about ten times greater (compare with Table 1).

Thus the results of purification by an antibody column were consistent with those obtained by lectin affinity chromatography and supported the view that the part of periodic acid–Schiff-staining band 1 not retarded by lentil lectin was Thy-1_{L-}, since all of this band was removed by the antibody column. To further establish this, the antibody column was used to purify Thy-1_{L-} from a Sephadex G-200 fraction which had been passed twice through a lentil lectin column. Thy-1.1 and Thy-1 xenoantigenic activities were depleted from the passed fraction, as found for whole extract in Fig. 3(*a*), and analysis by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed complete removal of periodic acid–Schiff-staining band 1 found in the starting material shown in Plate 1(*g*).

When the column was eluted with pH11.5 buffer, 41% of the Thy-1.1 antigen was recovered and the eluted fraction was similar after electrophoresis to that eluted from whole extract (Plate 3*c*), with the exception of one extra major polypeptide band. This band had the same mobility as lentil lectin and it was considered likely that it was lectin which had eluted from previous columns and then bound to carbohydrate on the antibody affinity column. This view was confirmed when the band was removed without loss of antigenic activity by an anti-(lentil lectin) affinity column (see under 'Methods').

Details of the recovery of antigenic activity and of the purification are given in Table 3, and the final purification approached that for Thy-1_{L+} (Table 1). In Plates 4(*a*) and 4(*b*) are shown gels stained for protein and carbohydrate after electrophoresis of the eluted fraction after removal of contaminating lectin. The fraction contained one carbohydrate band running in approximately the same position as periodic acid–Schiff-staining band 1, but the band was broader, indicating apparent heterogeneity (see below). The protein pattern showed one major band (50–60% of the stain by densitometry), which was in the same position as the carbohydrate band, and two prominent contaminant proteins of higher molecular weight. Analysis of antigenic activity after electrophoresis as in Plate 2 showed association with the glycoprotein, but not with the contaminant bands.

Antigenicity of the various preparations

To obtain a direct comparison of the specific activities of the various Thy-1 preparations, inhibition assays were done at the same time for Thy-1.1 antigen and for the Thy-1 xenoantigens. The results are shown in Table 4 as the protein (in ng) of each fraction required for 50% inhibition of the respective binding assays. This provided a valid comparison as the inhibition curves for the three preparations were roughly parallel in each assay.

Table 3. *Purification of Thy-1_{L-} by an antibody column*

Fraction	Protein (mg)	Thy-1.1 antigen $10^{-4}\times$ units	Relative sp. activity	Yield (%)	Thy-1 xenoantigen $10^{-5}\times$ units	Relative sp. activity	Yield (%)
Extract after Sephadex G-200 chromatography and passage through a lentil lectin column	10.5	8.0	1	100	2.8	1	100
Fraction eluted by pH11.5 buffer and after passage through an anti-(lentil lectin) column	0.51	2.7	7	34	1.1	8	39

Table 4. *Amount of antigen preparation needed for 50% inhibition of standard binding assays*

Values shown are means of the individual determinations given in parentheses.

Thy-1 fraction	Amount of fraction (ng) needed for 50% inhibition of assay for: Thy-1.1	Thy-1 xenoantigens
Thy-1_{L+}	7.8 (6, 7, 10.3)	1.9 (1.7, 1.8, 2.1)
Thy-1_{total}	12.6 (11, 11.6, 15.2)	2.8 (2.1, 3.2, 3.1)
Thy-1_{L-}	19 (17, 21)	4.5 (4.3, 4.8)

In all cases less protein was needed for the inhibition of the Thy-1 xenoantigen assays than for Thy-1.1. This may be due to the higher affinity of the hyperimmune rabbit antiserum and is in agreement with previous observations (Morris *et al.*, 1975). The three separate preparations of antigen all carried the same antigenic determinants, since all gave total inhibition of each assay. In terms of specific activity all preparations were of the same order, except that Thy-1_{L-} had a lower activity, as would be expected as it is less pure when analysed by sodium dodecyl sulphate – polyacrylamide-gel electrophoresis. It is also possible that there was some loss of antigenic activity in the preparations purified by antibody columns, since Thy-1_{total} appears as pure as Thy-1_{L+}, yet had a lower specific activity.

Further characteristics of the purified preparation determined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Thus far sodium dodecyl sulphate–polyacrylamide-gel electrophoresis has been used as a criterion of purity of reduced and alkylated antigen preparations; in addition to this, electrophoresis was carried out to further characterize the antigens.

The effect of reduction was first examined, and unreduced antigen ran with the same mobility as reduced and alkylated material in 5.6% gels.

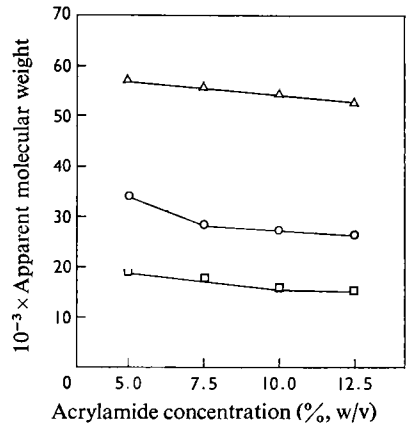


Fig. 4. *Apparent molecular weight of Thy-1_{L+} on gels of different acrylamide concentrations*

The molecular weight of Thy-1_{L+} (○), fetuin (△) and avidin (□) was estimated after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by using gels at four different acrylamide concentrations as shown on the abscissa.

Secondly, the effect on electrophoresis of the concentration of acrylamide in the gel was determined and apparent molecular weights were measured. It is known that reduced and alkylated polypeptides electrophorese in sodium dodecyl sulphate with a mobility proportional to log(mol.wt.) (Weber & Osborn, 1969), whereas many glycoproteins behave anomalously (Glossmann & Neville, 1971; Segrest *et al.*, 1971). Usually the glycoproteins appear larger than predicted (compared with protein markers), particularly when run on gels with a low percentage of acrylamide. In Fig. 4 the apparent molecular weight is plotted against acrylamide concentration and the result compared with that obtained by using two other glycoproteins, fetuin and avidin. The apparent

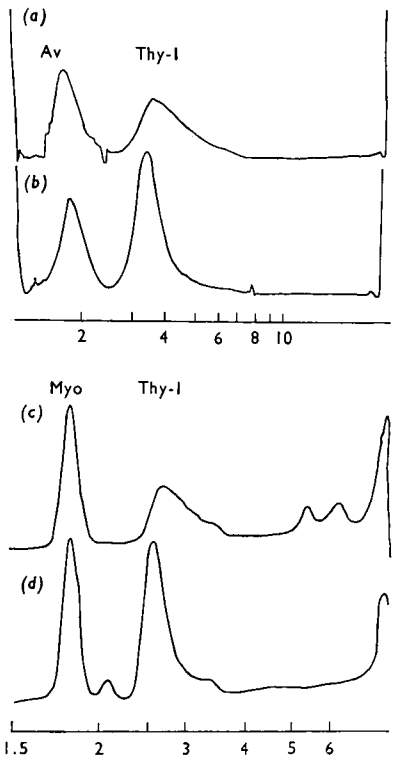


Fig. 5. *Differences between Thy-1 preparations after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

(*a*) Thy-1_{L-} (23μg) and (*b*) Thy-1_{L+} (17μg) were electrophoresed with 50μg of avidin on 5.6% gels and stained with periodic acid–Schiff reagent. (*c*) Thy-1_{L-} (23μg) and (*d*) Thy-1_{L+} (17μg) were co-electrophoresed with 5μg of myoglobin in 12.5% gels and stained with Coomassie Blue. All samples were reduced and alkylated.

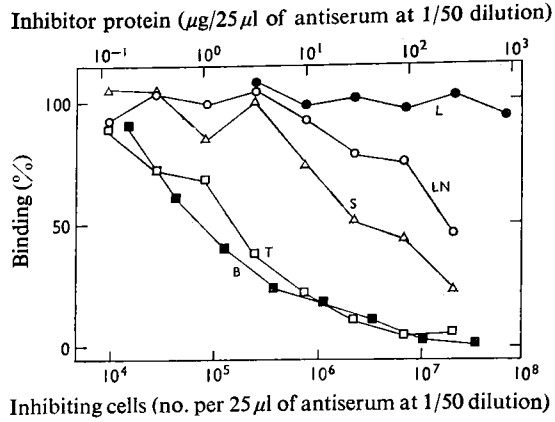


Fig. 6. *Inhibition of the binding of rabbit anti-Thy-1_{L+} antibodies to rat thymocytes*

Antiserum at a dilution of 1/50 was pre-absorbed with the amounts of tissue shown on the abscissa and after centrifugation was assayed for residual binding by using indirect binding assays described by Morris & Williams (1975). Excess of rat thymocyte target cells was used, and the second antibody [^{125}I-labelled horse anti-(rabbit IgG)] was added in saturating amounts (20μg/ml). Inhibition by liver homogenate (●), lymph-node cells (○), spleen cells (△), thymocytes (□) and brain homogenate (■) is shown. One abscissa (defining inhibiting cell numbers) applies to □, △ and ○, and the other (defining protein) applies to ■, ● and □.

mol.wt. of the Thy-1_{L+} fell from 34000 to 26000 in going from 5.0 to 12.5% gels. The more correct value is likely to be that determined from the 12.5% gels since both avidin and fetuin approach their correct mol.wts. of 15600 and 50500 respectively on the 12.5% gel (Glossmann & Neville, 1971). The apparent mol.wt. was determined a number of times on 12.5% gels and a mean±S.E.M. of 25300±240 (four determinations) was obtained for Thy-1_{L+}. In contrast, the apparent mol.wt. of Thy-1_{L-} was 27200±390 (five determinations). This difference was also found on 5.6% gels, where the values were 31600±400 (five determinations) and 34800±380 (seven determinations) for Thy-1_{L+} and Thy-1_{L-} respectively.

Finally, the apparent heterogeneity, particularly of Thy-1_{L-}, was analysed by using sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Thy-1_{L+} and Thy-1_{L-} were electrophoresed along with markers to act as internal standards, and the gels were stained for protein or carbohydrate. Typical results of gel scans are shown in Fig. 5, where (*a*) shows electrophoresis on 5.6% gels of Thy-1_{L-} and (*b*) that for Thy-1_{L+}, both stained with periodic acid–Schiff reagent; the marker is avidin. Figs. 5(*c*) and 5(*d*) also show Thy-1_{L-} and Thy-1_{L+} respectively, but electrophoresis was on 12.5% gels which were stained for protein, and the marker was myoglobin. For both carbohydrate and protein stain, and on 5.6 and 12.5% gels, Thy-1_{L-} was more heterogeneous in the direction of high molecular weight, but not of lower molecular weight as compared with Thy-1_{L+}. In Fig. 5 the small differences in apparent molecular weight between Thy-1_{L-} and Thy-1_{L+} can be seen when gels (*a*) and (*b*), and (*c*) and (*d*), are compared. [As Thy-1_{L-} is heterogeneous towards higher-molecular-weight forms, readings of mobility on gels were taken to the leading peak (Plate 4 and Fig. 5), which is most comparable with

the more symmetrical peak of Thy-1_{L+} (Plate 1*d* and Fig. 5).]

Immunogenicity of purified Thy-1

To further check that the purified glycoprotein was identical with the Thy-1 molecule as defined by its antigenicity, the antisera raised by immunization of two rabbits with Thy-1_{L+} was analysed (see under 'Antibodies' for the immunization schedule). From previous observations (Morris & Williams, 1975) rabbit antisera against Thy-1 would be expected to contain antibodies against a component absent from liver, but found in large amounts on brain and thymocytes and in very much smaller amounts on spleen and lymph-node cells. Both antisera against the pure preparation showed this pattern in absorption analysis which is illustrated for one in Fig. 6.

The antibody could be further analysed for specificity against Thy-1.1 antigen, Thy-1 rat-specific xenoantigen and Thy-1 rat–mouse cross-reacting xenoantigen. This was done as described previously (Morris & Williams, 1975) by comparing absorption of binding by rat brain with that by A/JAX (Thy-1.2) and A/Thy-1.1 mouse brain. The two antisera were 45% and 60% specific for rat xenoantigen respectively, and in both cases the rest of the antibody was against the rat and mouse cross-reacting xenoantigen. The absence of significant antibody against Thy-1.1 was not unusual in that analysis of anti-(rat brain) sera binding to thymocytes showed that the appearance of anti-(Thy-1.1) antibodies was an inconsistent event (Morris & Williams, 1975).

Discussion

Procedures for the purification of Thy-1.1

With the procedures summarized in Fig. 1 the Thy-1 glycoprotein can be obtained in two forms and in large enough amounts for chemical analysis. The method using lentil lectin is simple and effective, the only problem being that the lectin appears to be continuously eluted in small amounts from the columns. Lentil lectin exists as a dimer of non-covalently bound subunits (Howard *et al.*, 1971), and if one monomer was coupled to the Sepharose 4B the other could dissociate and be eluted. Lectin can be simply removed from a preparation with an antibody affinity column (see under 'Methods'), and this is possibly the best method for removing minor contamination because even inactivated lectin would probably retain its antigenicity.

Antibody columns should also be very useful in further studies on the Thy-1 molecule. Thy-1_{L-} can be obtained, and also preparations of Thy-1_{total} can be made. Considerable improvement of the method as reported here may be possible with the use of more concentrated antibody, which would increase the specific binding relative to non-specific absorption. Obviously a one-step affinity-chromatography procedure resulting in pure antigen would be desirable.

Properties of the rat Thy-1 molecule

The purifications described above have established that the rat Thy-1 molecule is a glycoprotein and confirm the previous observations (Morris *et al.*, 1975) that it carries xenoantigenic specificities as well as the Thy-1.1 antigen. Heterogeneity in the carbohydrate portion of the glycoprotein was suggested by the finding that only about half of the antigen would bind to lentil lectin columns. Estimates of molecular size by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis gave apparent mol.wt. values of 25300 and 27200 for the main bands of Thy-1_{L+} and Thy-1_{L-} respectively. Both values are in reasonable agreement with an estimate of 28000 for the mol.wt. of Thy-1.1 antigen in deoxycholate, based on determination of hydrodynamic properties (Letarte-Muirhead *et al.*, 1974). As this latter estimate includes deoxycholate bound to the antigen, and as the technique of sodium dodecyl sulphate–polyacrylamide-gel electrophoresis is not reliable for estimates of the molecular weights of glycoproteins, all these values may be overestimates of the true molecular weight. With the pure preparations of Thy-1 an accurate estimate of molecular weight should be possible by using the methods of Tanford *et al.* (1974).

Further heterogeneity seemed to exist in Thy-1_{L-}, since this preparation did not run as a sharp band on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The heterogeneity was towards apparently higher-molecular-weight forms, and this, together with the larger size of the main band, is consistent with the existence of extra carbohydrate on this form of Thy-1. It is possible that addition of carbohydrate obscures lentil-lectin-binding sites, but this can only be established by chemical analysis.

The rat Thy-1 glycoprotein has a single polypeptide chain as judged by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of reduced and unreduced antigen. Histocompatibility antigens are associated with a shorter polypeptide-chain-$\beta 2$ microglobulin, which occurs in large amounts in the membranes of most tissues (Tanigaki & Pressman, 1974). No evidence for this was obtained in association with Thy-1. Further, the Thy-1 molecule shows no tendency to form polymers, as appears to be the case with the major erythrocyte glycoprotein (Slutzky & Ji, 1974; Tuech & Morrison, 1974).

The above properties of the rat Thy-1 molecule are at variance with suggestions of some workers as to the molecular nature of mouse Thy-1. Atwell *et al.*

(1973) have suggested that Thy-1.2 antigen is a protein of mol.wt. 60000, and other groups of workers (Vitetta *et al.*, 1973; Esselman & Miller, 1974) have suggested that the antigens are on a glycolipid molecule. In contrast with this, Sauser *et al.* (1974) have identified mouse Thy-1.2 as a molecule of size more similar to that of rat Thy-1. Moreover, Trowbridge *et al.* (1975) found that a molecule similar to rat Thy-1 could be precipitated from detergent extracts of iodinated mouse thymocytes with antiserum to rat brain Thy-1, which cross-reacts with mouse Thy-1 antigen (Barclay *et al.*, 1975). Conventional alloantisera would not precipitate the Thy-1 molecule, possibly owing to low affinity, and this may explain why Atwell *et al.* (1973) and Vitetta *et al.* (1973) obtained different results.

In preliminary experiments we have found that Thy-1.1 is obtained in good yield from membrane prepared by the Tween-40 method from AKR mouse thymocytes. After solubilization in deoxycholate the mouse antigen activity was found to have a Stokes radius of 3.0nm by gel filtration on Sephadex G-200 in deoxycholate. Moreover mouse thymocyte membrane gave a similar pattern of bands staining with periodic acid–Schiff reagent after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis to that with rat membrane. In particular, a prominent band, equivalent to rat periodic acid–Schiff-staining band 1, was seen. Thus it seems possible that the mouse Thy-1 molecule will eventually be found to be very similar to its equivalent in the rat.

Quantitative importance of the Thy-1 glycoprotein in thymocyte membranes

The Thy-1 molecule is likely to constitute a significant proportion of the surface area of mouse and rat thymocytes. The number of Thy-1.2 antigenic sites per cell in the mouse was estimated to be >400000 (Hämmerling & Eggers, 1970), and Thy-1.1 in the mouse and rat, and Thy-1 xenoantigens in the rat, were found at about 600000 sites/cell (Acton *et al.*, 1974; Morris & Williams, 1975). Also, from sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of thymocyte membranes, it is clear that Thy-1 is quantitatively a major glycoprotein. The results for membrane purified by the Tween-40 method are supported by those of Ladoulis *et al.* (1974) on membrane prepared by conventional techniques. They identified a major glycoprotein of apparent mol.wt. 27000 on rat thymocyte, but not spleen lymphocyte membrane. This glycoprotein was almost certainly the Thy-1 molecule, and its absence from spleen is consistent with the presence of only very small amounts of Thy-1 antigens in the spleen and lymph-node cells of the rat (Acton *et al.*, 1974; Morris & Williams, 1975).

Given that the Thy-1 glycoprotein can be clearly identified on gels after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by staining for carbohydrate, the presence of a similar molecule might be evident in gels of thymocyte membrane from other species. As stated above, this is so for mouse thymocytes, but the literature suggests that this does not hold for rabbit or calf thymocyte membranes (Schmidt-Ullrich *et al.*, 1974, 1975). Also, in our laboratory, no periodic acid–Schiff-staining band equivalent to rat Thy-1 could be identified in membrane prepared by the Tween-40 method from human thymocytes (Dr. J. Fabre, unpublished work). It would be surprising if a functionally equivalent glycoprotein were not present in other species, and it may be that sodium dodecyl sulphate–polyacrylamide-gel electrophoresis gives misleading results for glycoproteins in comparison between species. In support of this is the finding that for erythrocyte membranes of different species there are marked differences in the major glycoprotein bands, whereas the protein band pattern is relatively constant (Hamaguchi & Cleve, 1972).

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Purification of the Thy-1 Molecule from Rat Brain

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The present paper describes the characterization of the Thy-1 molecule from rat brain. The molecule was recognized by its antigens, which could be solubilized from brain membrane with deoxycholate. In the solubilized form the Thy-1 antigens were associated with a homogeneous component with the following hydrodynamic properties: $s_{20,w} = 2.2$S, $\bar{v} = 0.72$ml/g and Stokes radius = 3.0nm. The mol.wt. of the deoxycholate–antigen complex was estimated to be 27000; these values are not significantly different from those obtained thymocyte Thy-1. Brain Thy-1 was further purified by affinity chromatography with lentil lectin coupled to Sepharose 4B, and more than 80% of the antigen was bound. The material eluted with methyl α-D-glucopyranoside was then filtered on a column of Sephadex G-200, and only one glycoprotein was found in the antigenically active fraction. On sodium dodecyl sulphate–polyacrylamide-gel electrophoresis the glycoprotein was very similar to the Thy-1 from thymocytes that binds to lentil lectin. Its apparent mol.wt. on 12.5% acrylamide gels was 24000, and it electrophoresed as a symmetrical band. Brain Thy-1 was antigenically indistinguishable from thymocyte Thy-1 when analysed with rabbit antisera raised against brain or thymocyte Thy-1.

Antigens of the Thy-1 (θ) molecule are found in large amounts on thymocytes and in the brain of adult mice and rats. In neonatal animals of the same species the antigens are present in low amounts in brain, and they increase in specific activity from birth to reach adult values at about 3 weeks of age (Reif & Allen, 1964, 1966; Douglas, 1972; Acton *et al.*, 1974; Morris & Williams, 1975). This increase correlates with developmental events such as the formation of synaptic junctions, the adoption of behavioural patterns and the onset of bioelectric activity (Aghajanian & Bloom, 1967; Tilney, 1933; Deza & Eidelberg, 1967), and precedes the main period of myelination (Davison & Peters, 1970). Other cell-surface antigens show a similar increase in specific activity (Schachner & Hämmerling, 1974; Schachner, 1974).

The Thy-1 antigen is found in all regions of mouse and rat brain in various amounts (Reif & Allen, 1964; Moore *et al.*, 1971; Grabar *et al.*, 1974), but there may be considerably less on peripheral nerves (R. T. Acton, J. Addis, G. F. Carl & W. F. Bridgers, unpublished work). In cultures of foetal mouse brain, Thy-1 is present on neuronal-like cells and on an indistinct class of cells, but not on glial-like cells (Mirsky & Thompson, 1975). The expression of Thy-1 antigen on neuronal cell lines is variable, with small amounts present on one mouse neuroblastoma of peripheral sympathetic origin (Schachner, 1973), but apparently not on others (Joseph & Oldstone, 1974). Thy-1.1 is found on some rat neuronal cell lines (R. T. Acton, J. Addis, G. F. Carl & W. F. Bridgers, unpublished work). Although the data are confused, a differential localization of Thy-1 within nervous tissue may eventually be found.

The molecular nature of brain Thy-1 has not been established, although Esselman & Miller (1974) suggest that the Thy-1 antigens are on gangliosides in the mouse. In rat thymocytes there is no evidence for Thy-1 antigen on gangliosides, and a glycoprotein of apparent mol.wt. 25000 has been characterized as the Thy-1 molecule (Letarte-Muirhead *et al.*, 1975). It is important to establish whether the antigen is in the same form in thymocytes and brain since antigens can exist on different molecular types, as is clearly established by the existence of ABO blood-group antigen on glycolipid or glycoprotein molecules (Hakomari & Kobata, 1974).

Thus in the present paper we describe the purification of the Thy-1 molecule from rat brain, and its characterization in crude extracts and purified form. The antigen could be solubilized from crude brain membrane with deoxycholate, but not with non-ionic detergents (Letarte-Muirhead *et al.*, 1974), and was purified by affinity chromatography with lentil lectin, followed by gel filtration on Sephadex G-200.

Materials and Methods

Unless otherwise stated all procedures were at 0–4°C and all methods were as described in Letarte-Muirhead *et al.* (1975).

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Antibodies

To prepare rabbit anti-(rat brain Thy-1) sera, two rabbits which had been primed with brain glycoprotein obtained after lentil lectin affinity chromatography were further immunized with pure Thy-1 5 months later. The priming involved two intramuscular injections of 1mg of glycoprotein in complete Freund's adjuvant, followed by subcutaneous injection of 1mg in incomplete Freund's adjuvant. The boosting with pure Thy-1 involved three injections of 40μg of Thy-1 given intravenously at weekly intervals. Animals were bled weekly after this.

Crude membrane preparation from brain and solubilization in deoxycholate

Whole brains were removed from rats of 6–12 weeks of age and frozen in liquid N_2 for storage at −70°C. This procedure had no effect on Thy-1.1 activity compared with fresh material. Brain homogenate was prepared by using a Potter–Elvehjem homogenizer with rat brains suspended in 10vol. of 0.32M-sucrose. The large debris was removed by centrifugation for 16000*g*-min and the pellet washed once. The pooled supernatants were centrifuged for 2000000*g*-min and the membrane pellet obtained was resuspended in 0.05M-NaCl–0.01M-Tris–HCl, pH7.4, to give 12mg of protein/ml. An equal volume of 4% (w/v) Lubrol-PX in the same buffer was added and the mixture stirred on ice for 60min. The extract was centrifuged at 6000000*g*-min and the pellet obtained was resuspended in 0.02% (w/v) NaN_3–0.01M-Tris–HCl, pH8.0, to give 12mg of protein/ml. An equal volume of 4% (w/v) deoxycholate in the same buffer was then added, and the mixture was frozen and stored overnight at −20°C. On thawing it was centrifuged at 6000000*g*-min and the pellet extracted again with 2% (w/v) deoxycholate in the above buffer. The supernatants were pooled to give the solubilized Thy-1 fraction.

Affinity chromatography with lentil lectin coupled to Sepharose 4B

A column (35ml) of lentil lectin–Sepharose 4B (10mg of lectin/ml of swollen beads) was used in a typical preparation of Thy-1 from 63g of rat brain. The column was pre-washed with 1M-methyl α-D-glucopyranoside in 1% (w/v) deoxycholate buffer, then with 0.5% (w/v) deoxycholate buffer. The solubilized brain membrane fraction was then passed through the column at 15ml/h, and the column was washed with 3–4 column-volumes of 0.5% (w/v) deoxycholate buffer. Bound material was then eluted with 1M-methyl α-D-glucopyranoside in 1% (w/v) deoxycholate buffer at 5ml/h. Protein was estimated from the E_{280}^{1cm} readings, and Thy-1.1 antigenic activities were assayed.

Sucrose-gradient centrifugation

This was carried out in H_2O and ^2H_2O as described by Morris *et al.* (1975) except that 1% (w/v) deoxycholate was used in the gradients and ^{125}I-labelled myoglobin was used as an extra marker. Deoxycholate extract was sedimented on the gradients and for analysis in ^2H_2O the sample was dialysed against deoxycholate buffer in ^2H_2O before application.

Gel filtration in deoxycholate

To measure Stokes radius, and in preparative procedures, Pharmacia K-26 and K-50 upward-flowing columns containing Sephadex G-200 were used (Letarte-Muirhead *et al.*, 1974, 1975).

Concentration of fractions

Fractions were concentrated by ultrafiltration with a Bio Fibre 80 beaker (Bio-Rad Laboratories, Bromley, Kent, U.K.) dialysing simultaneously with 0.01M-Tris–HCl, pH8.0, where it was desirable to decrease the deoxycholate concentration (e.g. in concentrating the fractions after Sephadex G-200 chromatography in 0.5% deoxycholate). Further concentration was effected with an Amicon Diaflo ultrafiltration apparatus by using a PM 10 membrane which had been equilibrated with deoxycholate.

Analytical methods

Protein was determined by Hartree's (1972) modification of the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Samples in deoxycholate were estimated either by comparing them with standards in the same concentration of deoxycholate or by trichloroacetic acid precipitation of the sample and the removal of deoxycholate with acetone.

Phospholipid was determined from the phosphorus present in a chloroform–methanol (2:1, v/v) extract. Phosphorus was determined by a modification of the method of Bartlett (1959) and 1μg of phosphorus was taken to be equivalent to 25μg of phospholipid.

Results

Estimation of the amount of Thy-1 in brain and the degree of purification required

It can be estimated that there is approx. 2.8mg of Thy-1 per 1×10^{11} thymocytes (Letarte-Muirhead *et al.*, 1975). If the inhibition of the Thy-1.1 assay by brain homogenate is compared with that produced by thymocytes, an estimate of the amount of Thy-1 in brain can also be made. Assuming the same molecular weight as for thymocyte Thy-1 (see below),

Table 1. *Purification of brain Thy-1*

A unit of activity is defined as the amount of antigen needed to give 50% inhibition of the appropriate binding assay. Specific activity is the number of units of activity per mg of protein and is related to that of brain homogenate.

			Thy-1.1 antigen			Thy-1 xenoantigens		
Fraction	Protein (mg)	Phospholipid (mg)	$10^{-5}\times$activity (units)	Relative sp. activity	Yield (%)	$10^{-5}\times$activity (units)	Relative sp. activity	Yield (%)
Brain homogenate	7300	2900	18.7	1.0	100	81.5	1.0	100
Crude membrane pellet	2880	2000	13.0	1.8	70	65.5	2.0	80
Lubrol-PX pellet	1600	1200	8.0	2.0	43	63.0	3.5	77
Deoxycholate extract	1440	799	13.0	3.5	70	34.0	2.1	42
Eluted from lentil lectin	74	10	4.5	24	24	28.0	34	34
After Sephadex G-200 chromatography	2.0	<0.1	2.2	440	12	16.0	720	20

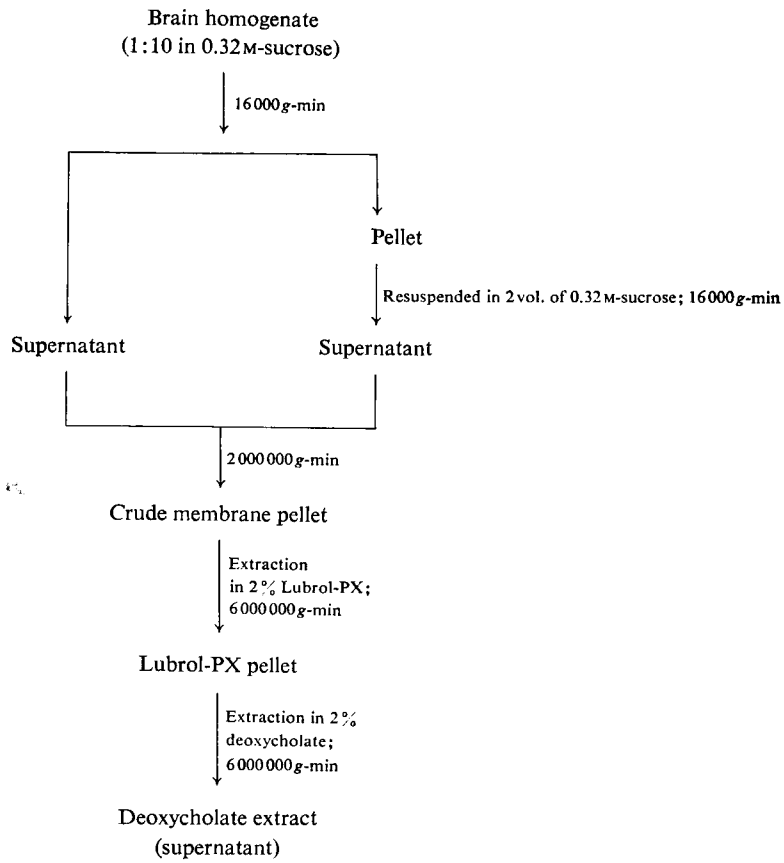


Fig. 1. *Scheme for the solubilization of brain Thy-1*

this amount would be 95μg of Thy-1 antigen per g wet wt. of brain, or 145μg per average rat brain. Thus on a per rat basis there is more Thy-1 in brain than thymus, which contains about 40μg per animal (1.5×10^9 thymocytes).

In a typical preparation of brain Thy-1 (see below) 63g wet wt. of brain was used, and this contained about 6.0mg of Thy-1 compared with 7300mg of protein (Table 1). Thus a purification of about 1200-fold should result in pure Thy-1.

Solubilization of Thy-1 antigens

The scheme for the solubilization of brain Thy-1 is shown in Fig. 1 and details of yields of antigen activity and of protein are listed in Table 1. The first step was the preparation of a crude membrane fraction from which solubilization in detergent was attempted. Non-ionic detergents did not inactivate Thy-1.1 antigen, but nor did they give effective solubilization (Letarte-Muirhead *et al.*, 1974). Deoxycholate did not solubilize crude membrane very well, but, if membrane was first extracted with 2% Lubrol-PX, effective solubilization of Thy-1.1 antigen was possible from the Lubrol-PX pellet. Moreover, solubilization was improved when membrane in deoxycholate was frozen–thawed before centrifugation. With the procedures outlined in the Materials and Methods section and in Fig. 1, about 50% of the Thy-1 antigens (Table 1) from brain homogenate were obtained in a 6×10^6g-min deoxycholate supernatant.

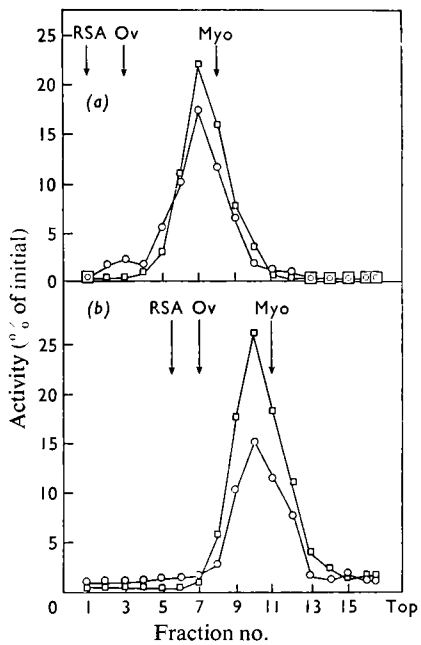


Fig. 2. *Sucrose-gradient centrifugation of solubilized Thy-1 in deoxycholate*

(*a*) shows the distribution of Thy-1.1 (○) and Thy-1 xenoantigenic (□) activities, after centrifugation for 40h at 44000rev./min ($r_{av.}$ 8.35cm), of deoxycholate extract of brain membrane on a 5–20% (w/v) sucrose gradient in 1% (w/v) deoxycholate buffer in H_2O. Activity is expressed as the percentage of total antigenic activity in the original extract. The positions of marker proteins sedimenting on the same gradient are shown: RSA, rat serum albumin; Ov, ovalbumin; Myo, myoglobin. (*b*) shows antigenic activities and marker positions after centrifugation as above but with gradients and extract prepared in ^2H_2O.

Sedimentation of brain Thy-1 on sucrose density gradients

To determine the nature of the solubilized antigen the deoxycholate extract was subjected to zone sedimentation on sucrose gradients in H_2O and ^2H_2O; such an analysis at an early stage in the purification may indicate whether multiple forms of the antigen exist.

The results are shown in Fig. 2 and the position of sedimentation of Thy-1.1 and Thy-1 xenoantigens is plotted in comparison with the positions of marker proteins. There is a high recovery of antigenic activity and both antigens move in an identical way as homogeneous components. The $s_{20,w}$ and $\bar{v}$ values were calculated as described by Meunier *et al.* (1972) and were 2.2S and 0.72ml/g respectively.

The results shown in Fig. 2 suggested that the antigens were solubilized in deoxycholate and gave no evidence for multiple forms of Thy-1 antigens in rat brain.

Affinity chromatography on lentil-lectin columns

To further purify Thy-1 from the deoxycholate extract, affinity chromatography on lentil lectin–Sepharose 4B columns was carried out. The results are shown in Fig. 3, and the major part of the protein,

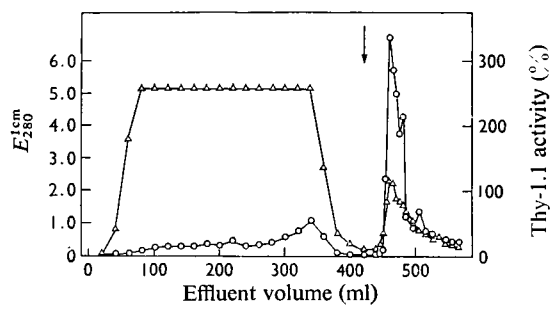
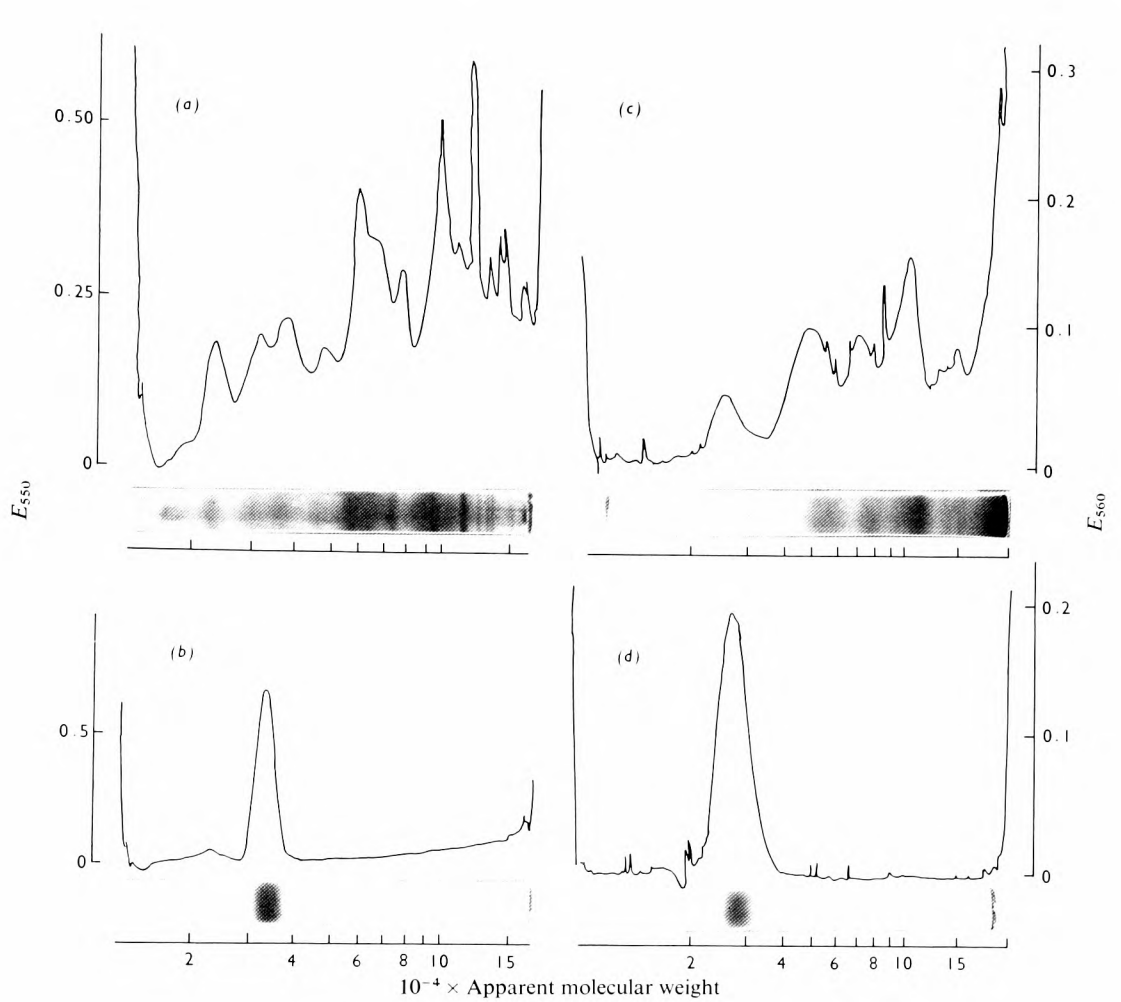


Fig. 3. *Affinity chromatography with lentil lectin–Sepharose 4B*

325ml of Thy-1 extract in deoxycholate was loaded on to a lentil lectin–Sepharose 4B affinity column (volume 35ml) and bound material was eluted with 1M-methyl α-D-glucopyranoside in 1% (w/v) deoxycholate buffer. Elution commenced at the point indicated by the arrow. Thy-1.1 antigenic activity was assayed (○) and expressed as a percentage of that in the starting extract (3600units of Thy-1.1 antigenic activity/ml). Protein is indicated by E_{280}^{1cm} (△); the background E_{280}^{1cm} values for 2% (w/v) deoxycholate and 1M-methyl α-D-glucopyranoside in 1% (w/v) deoxycholate buffer were 0.23 and 0.21 respectively.



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of fractions during purification

The following samples were loaded on to 5.6% acrylamide gels in sodium dodecyl sulphate: 45μg (*a*) and 220μg (*c*) of material eluted from lentil lectin; 9μg (*b*) and 22μg (*d*) of the Thy-1 fraction after rechromatography on Sephadex G-200. Gels were stained for protein (*a* and *b*) or carbohydrate (*c* and *d*) and scanned at 550nm and 560nm respectively.

as indicated by E_{280}^{1cm} values, was not retarded, whereas the Thy-1.1 activity was bound, and was eluted with methyl α-D-glucopyranoside. In this experiment the affinity column was approaching saturation, but under non-saturating conditions (e.g. in the first fractions through the column) more than 80% of the Thy-1.1 activity was bound. This is in contrast with Thy-1 from thymocytes; in that case there is a clear distinction between the Thy-1 which binds to lentil lectin and that which is unretarded, the two forms being present in approximately equal amounts (Letarte-Muirhead *et al.*, 1975).

The yields obtained with the Thy-1 xenoantigen assay indicate that approx. 80% of the Thy-1 loaded on to the affinity column is eluted with methyl α-D-glucopyranoside. In terms of Thy-1.1 antigen activity there appeared to be a loss of Thy-1, but this may be more apparent than real, since the Thy-1.1 assay is less reproducible than the Thy-1 xenoantigen assay, and backgrounds are more affected by deoxycholate. The overall yield after the lentil lectin step was in reasonable agreement for the two assays. The profile in Fig. 3 clearly shows that this a particularly good purification step, since the major part of the Thy-1 activity is eluted with only 5% of the protein and less than 2% of the phospholipids loaded on to the affinity column (Table 1).

Gel filtration on Sephadex G-200 in deoxycholate

To further purify Thy-1, the material eluted from the lentil lectin column was chromatographed on Sephadex G-200. This was done on small columns (2.6cm×90cm) to measure Stokes radius, and on large columns (5.0cm×90cm) in preparative procedures. In Fig. 4 the result of column measurement of Stokes radius is shown, and Thy-1.1 antigen emerges at the same elution volume as ovalbumin and has a Stokes radius of 3.0nm (Tanford *et al.*, 1974). The antigen is separated from the bulk of the protein and the Sephadex G-200 column provides a very good purification step (Table 1). On some occasions gel filtration was repeated to achieve complete purification (see later).

Analysis of the purification by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

From the results given in Table 1, Thy-1 should be a major component in the antigenically active fraction after gel filtration, and may also be detectable in the post-lentil-lectin fraction. The samples from these stages were analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and stained for carbohydrate and protein. The results are shown in Plate 1, and after gel filtration only one major glycoprotein band remains (Plate 1*b* and 1*d*), and this band can be detected in the material eluted from

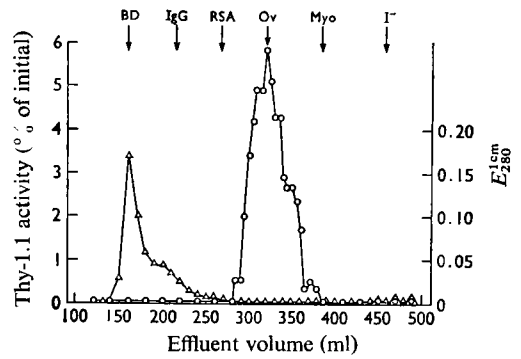


Fig. 4. *Chromatography of eluate from lentil lectin on Sephadex G-200 in deoxycholate*

5ml of eluate from lentil lectin was chromatographed on a column (2.6cm×90cm) of Sephadex G-200 in 0.5% (w/v) deoxycholate buffer. Fractions (5ml) were assayed for Thy-1.1 activity (○), which is expressed as the percentage of the activity of the loaded extract, and the E_{280}^{1cm} (△) measured. The elution position of protein markers run immediately before and after the fractionation are shown: IgG, bovine IgG (immunoglobulin G); RSA, rat serum albumin; Ov, ovalbumin; Myo, myoglobin. The void and total volumes are indicated by Blue Dextran (BD) and $Na^{125}I$ (I^-).

lentil lectin (Plate 1*a* and 1*c*). After one passage of this material through Sephadex G-200, a minor glycoprotein was also detected (not shown), but this could be eliminated by a second step of gel filtration (Plate 1*b* and 1*d*).

To be sure that the antigenicity was associated with the major glycoprotein, a gel was run in 0.1% sodium dodecyl sulphate, and antigenic activities were assayed from the gel in exactly the same way as described for thymocyte Thy-1 (Letarte-Muirhead *et al.*, 1975). Both Thy-1.1 and Thy-1 xenoantigens ran with the glycoprotein band.

Reduction and alkylation of Thy-1 had no effect on its mobility; thus the molecule consists of a single polypeptide chain.

The homogeneity and apparent molecular weight were also established after sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and it can be seen from Plate 1(*b*) and 1(*d*) that brain Thy-1 electrophoreses as a symmetrical band. The apparent molecular weight by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis varied with acrylamide concentration in the same manner as did thymocyte Thy-1, and the mean±S.E.M. was 31300± 1000 on 5.6% acrylamide gels (seven determinations) and 24100±150 (four determinations) on 12.5% gels.

Immunogenicity of brain Thy-1

To show further that the glycoprotein purified by this procedure was identical with the Thy-1

molecule, antisera were raised in two rabbits against purified brain Thy-1 (see the Materials and Methods section) and analysed for binding to thymocytes. From previous studies (Morris & Williams, 1975) such sera should have antibodies against a component absent from liver, but found in large amounts on thymocytes and brain and in much smaller amounts on spleen and lymph-node cells. This pattern was also obtained for rabbit anti-(thymocyte Thy-1) (Letarte-Muirhead *et al.*, 1975), and, when the anti-(brain Thy-1) was analysed in the same way, the same result was obtained for both sera.

The concentration of antibody was estimated as described by Morris & Williams (1975), and the bleeds of highest activity from one rabbit contained 0.3 mg/ml of anti(Thy-1), whereas the other contained 0.1 mg/ml.

The antisera were further analysed for their specificities against Thy-1 by absorption with A/Thy-1.1 and A/Jax congenic mouse brain and Wistar-rat brain. The results are shown for the more active serum in Fig. 5, and it can be seen that the absorption was 26, 30 and 100% for A/Jax and A/Thy-1.1 mouse and Wistar-rat brain respectively. Thus 70% of the antiserum was against the rat-specific Thy-1 xenoantigen, 26% against the rat–mouse cross-reacting xenoantigen and only 4% against Thy-1.1. This serum was used for antibody affinity columns to purify thymocyte Thy-1 (Letarte-Muirhead *et al.*, 1975).

In serum of the second rabbit, the antibody specificities as described above were 45, 37 and 18% respectively.

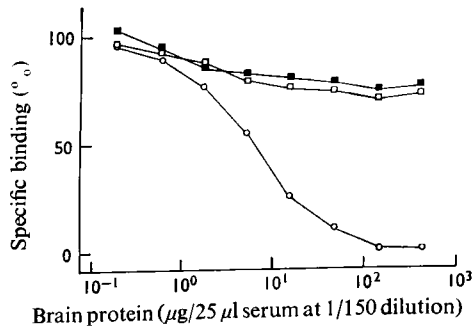


Fig. 5. *Analysis of the specificities of rabbit anti(brain Thy-1) serum*

Antiserum at a dilution of 1/150 was preincubated with brain homogenate from Wistar rat (○), A/Thy-1.1 mouse (□), or A/Jax mouse (■) and after centrifugation was analysed for residual binding by using indirect binding assays as described by Morris & Williams (1975). Excess of rat thymocyte target cells was used, and the second antibody [^{125}I-labelled horse anti-(rabbit IgG)] was added in saturating amounts (20 μg/ml).

Antigenicity of rat brain Thy-1

Antigenicity of brain Thy-1 and thymocyte Thy-1 was compared by studying inhibition of the binding of rabbit anti-(brain Thy-1), or rabbit anti-(thymocyte Thy-1_{L+}) sera (Letarte-Muirhead *et al.*, 1975) to thymocyte target cells. This analysis should reveal if thymocyte Thy-1 displays antigens not present on brain Thy-1. The results are shown in Fig. 6 and it is clear that the brain Thy-1 and thymocyte Thy-1 molecules cannot be distinguished in terms of their antigenicity. The two assay systems differed in their sensitivity to inhibition and this was probably due to differences in affinity rather than to antigenic specificity. The rabbit anti-(thymocyte Thy-1) serum was an early bleed after limited immunization, whereas the anti-(brain Thy-1) serum was obtained after prolonged immunization.

To summarize the similarities between brain Thy-1 and thymocyte Thy-1_{L+}, the amount of antigen required for 50% inhibition of the Thy-1.1 and Thy-1 xenoantigen assays is given in Table 2. The specific activities for the antigens are similar in all cases, and this is consistent with estimates of

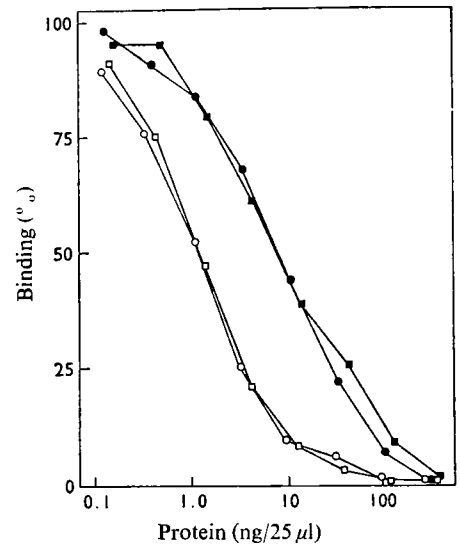


Fig. 6. *Inhibition of rat Thy-1 xenoantigen assays by purified Thy-1 from brain and thymocytes*

Rabbit anti-(brain Thy-1) serum (25 μl) at a dilution of 1/1500 was incubated with 25 μl dilutions of Thy-1 purified by lentil lectin affinity chromatography from brain (○) and thymocytes (□). Similarly rabbit anti-(thymocyte Thy-1) at 1/400 was incubated with Thy-1 from brain (●) and thymocytes (■). The binding of residual antibodies to excess of target cells (1×10^6 rat thymocytes per assay) was revealed with trace amounts of ^{125}I-labelled horse anti-(rabbit IgG) as described by Morris & Williams (1975) and expressed as a percentage of the binding of unabsorbed antiserum.

Table 2. *Amount of antigen preparation needed for 50% inhibition of standard binding assays*

Values are given as the means±S.E.M. with the numbers of determinations given in parentheses. Two assays for Thy-1 xenoantigens are used: (*a*), with rabbit anti-(brain Thy-1), and (*b*), with rabbit anti(thymocyte Thy-1).

	Amount of fraction (ng) needed for 50% inhibition of assays for		
		Thy-1 xenoantigens	
Thy-1 fraction	Thy-1.1	(*a*)	(*b*)
Thy-1 (brain)	8.5±1.1 (4)	1.4±0.1 (5)	5.4±2.0 (3)
Thy-1_{L+} (thymocytes)	10.5±2.3 (3)	1.9±0.3 (4)	8.0±3.3 (3)

purity for thymocyte Thy-1 and brain Thy-1 obtained by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Discussion

Purification of Thy-1 from brain

The large quantities of Thy-1 in rat brain and the availability of the tissue make it a good source for the purification of quantities of antigen sufficient for chemical characterization. With thymocytes preparing membrane is a good initial purification step, but with the brain, already mostly membrane, this gives little purification. However, a relatively simple procedure using two detergents has been successful in purifying Thy-1 to homogeneity.

Hydrodynamic properties of Thy-1 in deoxycholate

Sucrose-gradient centrifugation and gel filtration in deoxycholate were used to determine some hydrodynamic parameters of Thy-1 by methods described in Letarte-Muirhead *et al.* (1974). These apply to antigen plus bound detergent and the molecular size is likely to be an overestimate as parameters are determined by comparison with marker proteins which bind much less deoxycholate than membrane proteins (Helenius & Simons, 1972). The following values were obtained: $s_{20,w}$, 2.2S; $\bar{v}$, 0.72ml/g; Stokes radius, 3.0nm; and from these a frictional coefficient of 1.52 and mol.wt. of 27000 were calculated. These values are not significantly different from those for Thy-1 from thymocytes (Letarte-Muirhead *et al.*, 1974).

Chemical nature of Thy-1

The Thy-1 antigen has been purified from brain and shown to be a single symmetrical glycoprotein band, by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, of apparent mol.wt. 24100. This value is consistent with that of 27000 obtained above from the hydrodynamic properties for the deoxycholate–Thy-1 complex. The glycoprotein appears to constitute the main structure carrying Thy-1 antigens in brain, and no evidence for antigenically active gangliosides as reported by Esselman & Miller (1974) for mouse brain Thy-1 has been obtained.

The apparent mol.wt. of 24100 for brain Thy-1 determined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was consistently slightly lower than for thymocyte Thy-1 that binds to lentil lectin (Thy-1_{L+}, mol.wt. 25300), and considerably lower than the thymocyte Thy-1 that does not bind (Thy-1_{L-}, mol.wt. 27200). Thus the brain Thy-1 is more similar to thymocyte Thy-1_{L+}, and a brain equivalent of Thy-1_{L-} is yet to be found, although it could exist in the 10–20% of brain Thy-1 that did not bind to lentil lectin. In terms of antigenicity and immunogenicity, brain Thy-1 could not be distinguished from thymocyte Thy-1_{L+} or Thy-1_{L-}, and the differences between these three forms of Thy-1 must be established by chemical analysis.

Importance of Thy-1 in rat brain

The Thy-1 glycoprotein may play an important role in brain function, and the increase in the amount of antigen during development is perhaps significant. The clear determination of the localization of Thy-1 in the brain, yet to be achieved, may also suggest possible functions. The studies on distribution of Thy-1 antigens referred to in the introduction indicate that preferential localization may be found, but so far this has been mainly limited to studies by absorption analysis using fractions of brain. Localization by immunological techniques on histological sections has been attempted (Moore *et al.*, 1971; Grabar *et al.*, 1974), but convincing results are yet to be obtained, probably because specific antisera of high concentration have not been available.

As an alternative to examining antigen, the distribution of Thy-1 glycoprotein could be determined. A number of workers have studied glycoprotein of brain synaptosomal membrane, and bands produced on sodium sulphate–polyacrylamide-gel electrophoresis that could indicate Thy-1 were only sometimes found (Banker *et al.*, 1972; Morgan *et al.*, 1973; Zanetta *et al.*, 1975; Waehneldt *et al.*, 1971; Gurd *et al.*, 1974). Given the characterization of Thy-1 glycoprotein in the present paper, more definitive experiments should now be possible.

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