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B LYMPHOCYTE ACTIVITIES IN THE OPOSSUM,  
TRICHOSURUS VULPECULA

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ABSTRACT

The evolution of vertebrate immunity from the level of the protochordates to that of the metatherians is reviewed.

Using standard methods IgG, IgM and IgA were isolated from the serum or intestinal fluid of the Australian brush-tailed opossum, Trichosurus vulpecula. These were characterized in terms of their molecular weights, amino acid and carbohydrate compositions and values for their concentrations in serum were calculated. Two forms of IgG were seen which differed in their abilities to bind to insoluble matrices and also in their molecular weights. No antigenic differences were seen between them on analysis by agar diffusion. The molecular weight of the IgA seen in intestinal fluid and results from its analysis by agar diffusion suggest that the molecule may lack secretory component.

B lymphocytes were identified by their surface immunoglobulin and their complement and Fc receptors. The number of these cells in blood and various lymphoid tissues of T.vulpecula was found to be similar to the values reported for mice and humans. Lymphocyte fractionation on nylon wool columns confirmed that the markers employed were associated with an adherent cell population.

Blood lymphocytes were stimulated in vitro with a range of mitogens and the degree of transformation achieved with each was assessed by the cells' uptake of tritiated thymidine. Insoluble concanavalin A, pokeweed mitogen and lipopolysaccharide, in that order, were the most effective of the mitogens used on unfractionated blood lymphocytes. These three mitogens were further used in studies in which nylon wool fractionation of blood

lymphocytes was used to prepare B cell- and T cell-enriched cultures. Lipopolysaccharide was the only mitogen to stimulate B cells more than T cells. Insoluble concanavalin A consistently stimulated T cells to a greater extent than B cells as did pokeweed mitogen.

The ultrastructure of mitogen-stimulated cells was studied by electron microscopy and it was shown that lipopolysaccharide induced the formation of plasmablasts which resembled those of eutherians.

Mitogen-stimulated cells were also analysed for their production of immunoglobulins, the levels of de novo synthesised materials being measured by their incorporation of isotope-labelled leucine provided in the culture medium. Both secreted and intracellular proteins were measured in this way. Lipopolysaccharide, pokeweed mitogen and insoluble concanavalin A all induced significantly increased levels of 19S and 7S secreted proteins, these proteins being separated by gel filtration. Pokeweed mitogen induced the synthesis of significantly increased levels of both 19S and 7S intracellular proteins, while lipopolysaccharide and insoluble concanavalin A significantly increased the levels of 19S protein only. The presence of IgM and IgG in the 19S and 7S fractions was shown by their precipitation with class-specific antisera.

The immune responses of T.vulpecula to a particulate and a soluble antigen were compared with those of rabbits to the same antigens. Sheep erythrocytes, at two dose levels, were injected intravenously. The responses of opossums to  $5 \times 10^9$  erythrocytes were appreciably more rapid than those of the rabbits. The responses of the two species to  $25 \times 10^9$  erythrocytes were similar in the titres attained and the time taken to do so. The distribution of haemagglutinating activity between IgM and



IgG was studied and found to be essentially the same for both species for both levels of antigen. The responses of opossums to bovine serum albumin injected intramuscularly with Freund's adjuvants were similar to those of rabbits.

It is concluded that the B cell-dependent immune functions of T.vulpecula are as efficient as those of other metatherians and compare favourably with those of eutherians.

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ABBREVIATIONS USED IN TEXT

BGG	bovine gamma globulin
BSA	bovine serum albumin
Con.A	concanavalin A
cpm	counts per minute
CR	complement receptor
DEAE-cellulose	diethyl amino ethyl cellulose
DNP	dinitrophenyl sulfonic acid
DxSO <sub>4</sub>	dextran sulphate
FcR	Fc receptor
FCS	foetal calf serum
HGG	human gamma globulin
IEP	immuno-electrophoresis
Insol.Con.A	insoluble concanavalin A
Insol.Con.A-LP	insoluble concanavalin A, laboratory prepared.
Insol.Con.A-W	insoluble concanavalin A, washed.
KLH	keyhole limpet haemocyanin
Lan.C	lanotoside C
LPS	lipopolysaccharide
2-ME	2-mercaptoethanol
MEM	minimal essential medium (Eagle's)
MLR	mixed lymphocyte reaction
MW	molecular weight
PBA	polyclonal B cell activator
PBS	phosphate buffered saline
PEG	polyethylene glycol
PHA	phytohaemagglutinin
PPD	purified protein derivative
Prot.A	protein A
PWM	pokeweed mitogen
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
SI	stimulation index
SmIg	surface membrane immunoglobulin

SRBC	sheep red blood cells
TCA	trichloroacetic acid
TNP	trinitrophenyl sulfonic acid
ZC	complement coated zymosan particles.



PREFACE

This study of some aspects of the immune capacity of Trichosurus vulpecula, the Australian brush-tailed opossum, can be justified on several grounds. Firstly, marsupials have been little studied from an immunological point of view, their geographical restriction making them unavailable to most investigators. Secondly, there is the relationship that Australian marsupials have to American marsupials, on the one hand, and to placental vertebrates on the other. Marsupials and placentals are believed to have evolved from a common therian stock, differentiation between the two lines beginning about 100 million years ago. The Australian marsupials are thought to be descendants of an American immigrant that dispersed across what is now Antarctica in the late Cretaceous period. Subsequently, Australian marsupials evolved in isolation for some 70 million years. It is therefore of interest to compare the immune capabilities of T.vulpecula, a relatively recent marsupial, with those of its ancient, unchanged didelphoid stock and with those of eutherian mammals. Thirdly, T.vulpecula has acquired a particular relevance to New Zealand. Since its introduction from Australia, it has flourished and conservative estimates put its present population in excess of 60 million. The species is involved in two zoonoses in this country, namely, tuberculosis and leptospirosis.

T.vulpecula seems highly susceptible to Mycobacterium tuberculosis, the disease being rapidly progressive in these animals. Foci occur throughout the country in which infected opossums, grazing bush-pasture fringes, maintain the infection in dairy cattle making eradication of tuberculosis from cattle difficult and presenting a health hazard to man. Furthermore, T.vulpecula shares its bush habitat with deer, animals of recognised economic potential. Tuberculosis is seen in captured wild deer and the

threat that this poses both to the health of the handlers of deer carcasses and to an expanding venison market does not need emphasising.

As regards to leptospirosis, up to 70 per cent of sexually mature opossums from farm land and 30 per cent from bush are infected with Leptospira balcanica. This organism has been reported as causing leptospirosis in man in Europe, but its relevance to the disease in New Zealand remains to be established.

Consequently, any information relating to the immune capabilities of the opossum could conceivably be useful in gaining a wider understanding of the epidemiology of these two diseases.

The present investigation examines some aspects of the immune competence of T.vulpecula. It is limited to B cell functions and considers the behaviour of these cells in vitro and in vivo and examines the immunoglobulins that they produce.

## CHAPTER 1

### THE PHYLOGENY OF VERTEBRATE IMMUNITY

#### 1.1 Introduction

Vertebrate animals have a number of mechanisms which protect them against infection. These are conveniently divided into innate and acquired forms. Innate resistance is a function of certain physical and chemical features of the individual. Acquired immunity is further divided into immunity mediated by cells, predominantly lymphocytes and macrophages, and immunity mediated by antibodies. Phagocytosis can be regarded as a property common to both forms of resistance, being effective in the absence of antibody but greatly enhanced by its presence.

Invertebrates, too, are capable of resisting infection by potentially pathogenic organisms. However, their defence systems are considered to be less sophisticated than those of vertebrates and appear to be restricted to cellular mechanisms. While phagocytosis of foreign material is effectively achieved by a range of cell types, there is, to date, no convincing evidence that invertebrates produce factors akin to vertebrate immunoglobulins, either in functional or structural terms.

The recognition of "non-self" material is a primitive function evident even in Protozoa. The difference between vertebrate and invertebrate immune potential lies in the range of cells available to these animals which are capable of making this distinction. Both groups have phagocytic cells capable of detecting and removing foreign material. However, it is the occurrence in vertebrates of antigen-sensitive lymphocytes that results in the development of antibody-secreting plasma

cells or in the proliferation of effector cells engaged in the many aspects of cell mediated immunity.

It is the purpose of this review to outline the development of immune competence throughout the vertebrate phyla to the metatherian level.

## 1.2 Protochordates

The ascidians, a group of sessile tunicates which belong to the Protochordate group, are considered by many to share an ancestral stock with the vertebrates (Berrill, 1955). Tunicates have well-defined phagocytic cells and amoebocytes (Overton, 1966) and lymphocyte-like cells (Warr et al., 1977). That ascidians are capable of mounting some form of immune response was shown by Cantacuzene (1919) who described an increased activity of phagocytic cells of Ascidia mentula following the injection of these animals with a marine bacterium. Amoebocytic cells from injected animals agglutinated the bacteria at the cells' surfaces; cells from control animals did not show this reaction. Neither the specificity of the reaction nor the role of humoral factors in it were investigated.

Marchalonis and Warr (1978) described a protein in the haemolymph of Pyura stolonifera which is capable of binding to both dinitrophenyl (DNP) and erythrocyte antigens. This protein showed a charge heterogeneity, a molecular weight of 65,000 to 70,000 daltons and, on polyacrylamide gel analysis, it resembled the mammalian u chain. The authors proposed that the tunicates might express a primitive u-like chain ancestral to all subsequent immunoglobulin polypeptide chains.

There is a colony specificity in some compound ascidians that is manifested by fusion between colonies when the adjacent colonies are of the same species, and

non-fusion when the colonies are of different species (Bancroft, 1903). Such allogeneic recognition has been described in several species (Tanaka *et al.*, 1970). Tanaka (1973) has described two types of factor involved in non-fusion. One, found in the test matrix and blood, brings about the disintegration of incompatible test cells and granular amoebocytes. The second factor is released from disintegrated cells and constricts ampullae or blood vessels thereby causing an area of necrosis. These studies indicate that immuno-incompatibility in tunicates includes not only non-self recognition, but also subsequent antagonistic reactions leading to localised cell death. This dual response to foreignness is characteristic of specific immune reactivity.

### 1.3 Fish

Fish were the earliest vertebrate animals to evolve and can be divided into four main classes; these are the extinct Placodermi, the Agnatha, the Chondrichthyes and the Osteichthyes (Table 1.1).

#### a. Agnatha

The most primitive living vertebrates are the hagfish and the lamprey. The Pacific hagfish, Eptatretus stoutii, has natural serum agglutinins for sheep red blood cells (SRBC), the levels of which can be raised by injection of SRBC (Linthicum and Hildemann, 1970). E.stoutii also responds to Keyhole Limpet haemocyanin (KLH) (Thoenes and Hildemann, 1970) and Streptococci (Raison *et al.*, 1978). Anti-streptococcal antibodies consist of  $\mu$ -like heavy chains and light chains, the latter having a molecular weight somewhat greater than those of many advanced vertebrates. This finding is in marked contrast to that of De-Ioannes and Hildemann (1975)

Table 1.1 A classification of fishes

(After Romer, 1955)

Class	Placodermi	: Extinct
Class	Agnatha	
Order	Cyclostomata	
	<u>Eptatretus stoutii</u>	: Pacific hagfish*
	<u>Petromyzon marinus</u>	: Sea lamprey
	<u>Lampetra japonica</u>	: Arctic lamprey
Class	Chondrichthyes	
Subclass	Elasmobranchii	
	<u>Heterodontus francisci</u>	: Horned shark
	<u>Rhinobatos productus</u>	: Guitar fish
	<u>Triakis semifasciata</u>	: Leopard shark
	<u>Ginglymostoma cirratum</u>	: Nurse shark
	<u>Mustelus canis</u>	: Smooth dogfish
	<u>Dasyatis centroura</u>	: Stingray
	<u>Dasyatis americana</u>	: Southern ray
Subclass	Holocephali	
Class	Osteichthyes	
Subclass	Choanichthyes	
Order	Crossopterygii	
Order	Dipnoi	
	<u>Neoceratodus forsteri</u>	: Australian lungfish
	<u>Protopterus aethiopicus</u>	: African lungfish
Subclass	Actinopterygii	
Superorder	Chondrostei	
	<u>Polyodon spathula</u>	: Paddle fish
Superorder	Holostei	
	<u>Amia calva</u>	: Bowfin
Superorder	Teleostei	
	<u>Lutjanus griseus</u>	: Gray snapper
	<u>Carassius auratus</u>	: Gold fish
	<u>Cyprinus carpio</u>	: Carp

## Superorder Teleostei (contd)

<u>Epinephelus itaira</u>	: Giant grouper
<u>Pleuronectes platessa</u>	: Plaice
<u>Osteoglossus bicirrhosum</u>	: Arowana
<u>Prionotus evolans</u>	: Sea robin
<u>Salmo gardneri</u>	: Rainbow trout
<u>Lepomis macrochirus</u>	: Bluegill
<u>Chromis caeruleus</u>	: Damselfish
<u>Dascyllus aruanus</u>	: Damselfish
<u>Anaplocoma fimbria</u>	: Sablefish

\* Species names are mentioned in text.

who, using immunoglobulins from non-immunised E. stoutii, were unable to demonstrate the presence of heavy chains by acrylamide gel electrophoresis.

The Sea lamprey, Petromyzon marinus, produces antibodies to bacteriophage f2 (Marchalonis and Edelman, 1968a), human erythrocytes (Pollara et al., 1970) and Brucella antigens (Litman et al., 1970). Analysis of the anti-phage antibody shows that it consists of non-covalently bonded  $\mu$  chain-like heavy chains and light chains which show an extensive charge heterogeneity, suggesting a wide range of combining specificities. The sedimentation coefficient of the molecule is dependent on the protein concentration, being 6.6S at low protein concentrations and 14S at higher concentrations. Somewhat different results have been described following analysis of lamprey anti-human 'O' erythrocyte antibody (Litman et al., 1970). Migrating as if an alpha globulin, the molecule consists of four non-covalently linked polypeptide chains each with a molecular weight of 90,000 daltons; no evidence of light chains was found. The authors suggested that this molecule was a  $\mu$ -chain polymer. A natural agglutinin for horse erythrocytes has been characterized in the Sea lamprey (Marchalonis and Edelman, 1968b). This large molecule (48S) consists of smaller subunits which are bound together by disulphide bonds. Fijii et al. (1979) showed the production of haemolytic and haemagglutinating antibodies in the Arctic lamprey (Lampetra japonica) to sheep erythrocytes. Following repeated injections, heat stable antibody was produced after 4 weeks. A considerable secondary response was also demonstrated.

Cell mediated immune functions have been demonstrated in cyclostomes. The Pacific hagfish rejects skin allografts and shows a specific, longterm memory in this



respect (Hildemann and Thoenes, 1969). Delayed type hypersensitivity reactions to Freund's complete adjuvant and skin allograft rejection have been shown in P. marinus (Good et al., 1972). Further evidence of T-cell influenced functions comes from the observation of mixed lymphocyte reactions (MLR) of cells from hagfish and lamprey larvae (Cooper, 1971). Furthermore the presence of "T cells" in these primitive animals is indicated by the finding that lymphocytes from P. marinus respond to phytohaemagglutinin (PHA), a recognized T-cell mitogen (Olson, 1967; Cooper, 1971; Du Pasquier, 1976a; Cohen, 1977).

Neither hagfish nor lampreys have been shown to possess plasma cells, the cells producing antibodies in these species being small to large lymphocytes (Jordan, 1938; Good et al., 1966; Hildemann, 1972). Hagfish blood lymphocytes are derived from diffuse haemopoietic foci found in the gut wall; in the genus Myxine the anterior kidney acts as a further haemopoietic area (Manning and Turner, 1966).

The lamprey is a more advanced cyclostome than the hagfish and is the most primitive animal to have both thymic and splenic tissues (Finstad et al., 1964; Good et al., 1966). No histologic evidence of thymic tissue is seen in E. stoutii while in P. marinus, protothymus pharyngeal lymphoid accumulations develop in epithelial foci within the 2nd to 5th pharyngeal pouches. Finstad et al. (1964) demonstrated that the hagfish "spleen" consists of haemopoietic foci throughout the gut mucosa. The lamprey has a more organised haemopoietic mass within an infolding of the anterior gut. A similar tissue is found in the gill region and protovertebral arch, the latter possibly being the phylogenetic precursor of bone marrow (Good et al., 1972). A proliferative response following antigenic stimulation has been described

in the lamprey, notably in the protovertebral arch (Finstad and Fichtelius, 1965).

In producing antibodies and rejecting skin allografts cyclostomes display basic B and T cell functions. Whether or not two such separate cell populations exist at this level is not known. With the further phylogenetic emergence of organised lymphoid tissues these two forms of immune competence become more readily definable.

#### b. Chondrichthyes

The elasmobranchs have thymuses divisible into a cortex, densely populated with small lymphocytes, and a medulla (Finstad et al., 1964). The spleens of elasmobranchs are differentiated into a red pulp with blood sinuses and a lymphocytic white pulp. Haemopoietic areas are also found in the gut wall and kidneys. While plasma cells have been described in advanced elasmobranchs, such as the Leopard shark, Triakis semifasciata, and the Nurse shark, Ginglymostoma cirratum, they are absent in more primitive forms, such as the Horned shark, Heterodontus francisii, and Guitar fish, Rhinobatus productus (Enole et al., 1958).

Differential mitogen responses by lymphocytes of the Nurse shark (Lopez et al., 1974) indicate lymphocyte heterogeneity in the form of T and B cells in these animals. Unfractionated shark lymphocytes were shown to respond to concanavalin A (Con.A) but not to PHA. However, density gradient fractionation gave a population which was responsive to PHA, a finding which the authors took to indicate the presence of suppressor cells in this species.

Sharks responds to a range of antigens including proteins (Marchalonis and Edelman, 1965, 1966a), protein-hapten conjugates (Voss and Sigel, 1971), bacteria

(Schulkind et al., 1972) and viruses (Sigel and Clem, 1963). Two immunoglobulins with sedimentation coefficients of 17S and 7S have been described (Marchalonis and Edelman, 1965, 1966a; Fuller et al., 1978). Amino acid analysis and agar diffusion studies of these immunoglobulins from the Smooth dogfish, Mustelus canis, show them to be antigenically identical (Marchalonis and Edelman, 1965). Analysis of cysteine-containing fragments from the heavy and light forms of immunoglobulin from the Nurse shark further indicate that they belong to the same class (Klapper and Clem, 1977). The Leopard shark has a high molecular weight immunoglobulin which, in addition to light chains and  $\mu$ -like heavy chains, has a polypeptide chain of 20,000 daltons which may be analogous to the J chain of mammalian IgA and IgM (Klaus et al., 1971).

The identification of elasmobranch immunoglobulin as an IgM analogous to that of mammals is based on the electrophoretic characteristics of the molecule (Marchalonis and Edelman, 1965), its high carbohydrate content (Marchalonis and Edelman, 1966a; Clem and Small, 1967), its ultrastructure as determined by electron microscopy (Feinstein and Munn, 1969; Parkhouse et al., 1970) and circular dichroic analysis (Litman et al., 1971c).

IgM showing various degrees of polymerization occurs in different species of elasmobranchs. While the Dogfish has only 7S and 17S forms the stingray, Dasyatis centroura, has an immunoglobulin with a molecular weight of 360,000 daltons, apparently a dimer of the basic 7S unit, and small amounts of pentameric 19S forms (Marchalonis and Schnofeld, 1970). Johnston et al. (1971) have shown that the major immunoglobulin in the Southern ray, Dasyatis americana, is a pentameric form.

A notable difference between the immunoglobulins of lampreys and sharks is that in the latter species the heavy and light chains are bonded with disulphide linkages rather than non-covalent forces (Marchalonis and Edelman, 1965).

### c. Osteichthyes

Bony fishes have both a spleen and a well developed thymus (Good et al., 1966). In these fish, the spleen is not essential for antibody production; splenectomy of the teleost gray snapper, Lutjanus griseus, does not appear to affect its ability to make circulating antibodies (Good et al., 1966).

Distinct gut-associated lymphoid foci are also found in most bony fishes and it is thought that these may be involved in local antibody synthesis (Good et al., 1966; Manning and Turner, 1966). Plasma cells are seen in all groups of Actinopterygii (Manning and Turner, 1976).

When cultured with mitogens teleost lymphocytes show proliferative responses which suggest the presence of T and B cell-like lymphocytes (Etlinger et al., 1976a,b). While Rainbow trout (Salmo gairdneri) blood lymphocytes and splenocytes were stimulated by Con.A, lipopolysaccharide (LPS) or purified protein derivative (PPD), thymocytes were stimulated only by Con.A. Etlinger et al. (1978) further showed that LPS and PPD induced the formation of plasma cells and the polyclonal production of antibody in cultures of trout blood lymphocytes. Further evidence indicating a T cell or T cell-like function in teleosts has been provided by the demonstration of carrier-effects in responses to protein-hapten conjugates (Avtalion et al., 1975; Stolen and Makela, 1975; Yocum et al., 1975).

Organ cultures of thymus cells derived from fish immunized with protein antigens produce specific IgM-type antibody (Ortiz-Muniz and Sigel, 1971; Sailendri, 1973). The thymuses of phylogenetically more advanced vertebrates, lacking B cells, are incapable of this activity (Wright and Cooper, 1976).

Surface immunoglobulin has been demonstrated on the lymphocytes of cartilaginous (Ellis and Parkhouse, 1975) and bony (Emmerich et al., 1975; Warr et al., 1976, 1979; Clem et al., 1977) fishes. Studies in mice had suggested that the u chains of cell surface immunoglobulin may differ from those of secreted immunoglobulins (Melcher et al., 1975; Melcher and Uhr, 1976). Warr and Marchalonis (1977) showed that, in fact, the heavy chain of Goldfish (Carassius auratus) surface membrane immunoglobulins were some 10,000 daltons lighter than the heavy chains of serum IgM. Warr et al. (1979) showed IgM in the lysates from spleen and thymus cells of the trout, S.gardneri.

Bony fishes produce antibodies to protein (Everhart and Shefner, 1966; Hodgins et al., 1967; Avtalion, 1969; Trump, 1970), hapten (Smith and Potter, 1967; Clem and Small, 1970; Ambrosius and Fiebig, 1972), viral (Uhr et al., 1962; Sigel and Clem, 1965) and bacterial (Chiller et al., 1969a,b) antigens. Following immunization with SRBC, antibody secreting cells can be detected in the spleen and pronephros of the teleost Bluegill (Lepomis macrochirus) (Smith et al., 1967) and Rainbow trout (Chiller et al., 1969b). Antibody activity is associated with an IgM-like molecule which has a somewhat lower molecular weight than its mammalian equivalent (Fletcher and Grant, 1969; Acton et al., 1971b; Litman et al., 1971b; Marchalonis, 1971b). Of particular interest is the fact that only one form of the IgM molecule, a pentamere, usually exists in the sera of bony fishes. Two exceptions to this are seen in the

holostean Bowfin, Amia calva, which has 13.6S and 6.6S molecules (Litman et al., 1971b) and the grouper, Epinephulus itaria, which shows 16S and 6.4S forms of IgM (Clem, 1971). The tetrameric conformation of the 13.6S and 16S molecules, suggested by sedimentation coefficient determinations, has been confirmed by electron microscopy (Acton et al., 1971a,b; Shelton and Smith, 1970). There is evidence that antibodies in the secretions of Actinopterygii are similar to those in the serum. For example, antibodies present in plaice (Pleuronectes platessa) serum and mucus are both of a high molecular weight and have similar carbohydrate and amino acid compositions (Manning and Turner, 1976).

The Australian lungfish, Neoceratodus forsteri, and the African lungfish, Protopterus aethiopicus, Crossopterygian fishes of the order Dipnoi, are the most primitive species to show two antigenically distinct immunoglobulin classes (Marchalonis, 1969; Litman et al., 1971a). This observation has added significance when it is remembered that Crossopterygian species were ancestral to land vertebrates. N. forsteri has a high molecular weight protein (19S) which forms a dimer (23S). The 19S form is a pentameric structure and this has the characteristics of mammalian IgM. The second immunoglobulin has a sedimentation coefficient of 5.9S and a molecular weight of 120,000 daltons, somewhat less than that of mammalian IgG. Whether or not this low molecular weight immunoglobulin is a forerunner of IgG in higher species is not known. The 19S and 5.9S forms have common light chains but antigenically distinct heavy chains.

Antibody responses in fish are generally suppressed by low ambient temperatures. The Warm Water carp, Cyprinus carpio, produces antibodies at 20°C to 25°C, but not if maintained at 12°C (Pliszka, 1938a,b).

However, the teleost sablefish, Anaploma fimbria, from the cold waters of the North Pacific, can produce antibodies at 5 to 8°C (Ridgeway, 1962), indicating a degree of adaptation in the immune system.

First set allografts are rejected in the Arowana, Osteoglossum bicirrhosum, with a median survival time of 18 days. By comparison, higher teleosts (Carassius, Cyprinus) show allograft median survival times ranging from 3 to 8 days (Borysenko and Hildemann, 1969). Advanced bony fishes (Chromis caeruleus, Dascyllus aruanus) show rapid rejection of first set integumentary allografts and accelerated rejection of second set grafts with a vigorous cellular immunity at least equal to that of laboratory mammals (Hildemann, 1972). Allograft immunity appears more highly developed in teleosts than in elasmobranchs. Associated with this increased cell-mediated capability is the demonstration that MLRs occur in the Rainbow trout (Etlinger et al., 1975), which might indicate the emergence of significant histocompatibility antigens.

In general terms, fish display a broad immunological competence in their ability to respond to a wide variety of antigens and to reject allografted skin, bony fishes generally being more efficient in these respects than cartilagenous fishes. Lymphoid tissue development is impressive in these species with the exception of the primitive elasmobranchs which appear to lack plasma cells. The emergence of T/B cell dichotomy is seen at this phylogenetic level. Perhaps the most significant development within this group is the production by lungfish of two antigenically distinct immunoglobulin classes, an early indication of the complexity of immunoglobulins in advanced species.

## 1.4 Amphibians

The amphibians diverged from the crossopterygian fishes in the Devonian period, approximately 350 million year ago, and formed the stock from which all land vertebrates derived (Romer, 1955). The living amphibians are divided into three orders, the Apoda, the Urodela and the Anura (Table 1.2). The Apoda and Urodela are in some respects more primitive than the Anura. The Anurans undergo a more radical metamorphosis and their immune system is considerably more advanced than those of other amphibians.

### a. Apoda

The Apoda is an aberrant group of legless amphibians adapted for burrowing. Thymus development in this group is in the form of epithelial buds associated with each pair of pharyngeal pouches giving four pairs of thymic nodules (Manning and Turner, 1976). Adult caecilians (Nectrocaecilia cooperi, an apodan) also have spleens, livers and kidneys, which play key roles in immune functions (Cooper, 1976). Caecilians reject first-set allografts after 8 to 11 days (Cooper and Garcia, 1968).

### b. Urodela

The order Urodela includes mud-puppies, newts, salamanders and axolotls. Thymuses and spleens are present in these species, but there are neither lymph nodes nor well-organised gut-associated lymphoid tissues (Cowden and Dyer, 1971; Hightower and St.Pierre, 1971). The bone marrow is not lymphopoietic, instead the inter-tubular tissues of the kidney and the subcapsular layer of the liver take part in lymphocyte formation (Cowden and Dyer, 1971). The thymus develops as a three-lobed organ in the connective tissue behind the mandible.



Table 1.2 A classification of Amphibians  
(After Romer, 1955)

Class Amphibia

Subclass Lepospondyli

Order Apoda

Nectrocaecilia cooperi : Caecilian\*

Order Urodela

Necturus maculosus : Mud-puppies

Pleurodeles waltlii : Newt

Triturus alpestris : Newt

Triturus viridescens : Newt

Ambystoma tigrinum : Tiger salamander

Ambystoma mexicanum : Axolotl

Siredon mexicanum : Axolotl

Order Anura

Bufo marinus : Marine toad

Alytes obstetricans : Midwife toad

Xenopus laevis : South African  
clawed toad

Rana pipiens : Leopard frog

Rana catesbiana : Bullfrog

\* Species named are mentioned in text.

In the newts, Pleurodeles waltlii and Triturus alpestris and the Tiger salamander, Ambystoma tigrinum, thymectomy abolishes or delays the rejection of skin allografts (Tam et al., 1976).

In most urodeles the spleen and intertubular haemopoietic tissue of the kidney are the principal sites of lymphopoiesis, while in some the lamina propria of the intestinal tract is also involved in this function (Cowden and Dyer, 1971). Good et al. (1966) have identified plasma cells in the lamina propria of the mud-puppy, Necturus maculosus. Hendry and Charlemagne (1977) reported the presence of plasmablasts, proplasma cells and plasma cells in P.waltlii which, in their morphological features, resembled their mammalian counterparts.

Urodele splenic lymphocytes are comparable to those of anurans with respect to their ability to undergo blast transformation and mitosis when cultured with mitogens or allogeneic cells (Du Pasquier, 1973; Donnelly et al., 1976; Goldstine et al., 1976). Although urodele and anuran spleen lymphocytes react strongly to the B cell mitogen, LPS, the responses of urodele cells in MLR or in response to Con.A and PHA are characterized by significantly lower stimulation indices than those shown by anuran cells (Collins and Cohen, 1976).

Non-adherent splenocytes of the axolotl, Ambystoma mexicanum, separated by means of nylon wool column fractionation, have a mitogen response profile characteristic of T cells (Collins and Cohen, 1976). The adherent cell population, in addition to its response to LPS, also shows significant Con.A responsiveness. This might indicate either that the elution of T cells from the nylon wool was incomplete or that a sub-set of T cells adhered to the columns. Alternatively, the

adherent cells might have both LPS and Con.A response capabilities (Du Pasquier, 1976a). The in vitro behaviour of urodele lymphocytes might suggest that the T and B lymphocyte dichotomy of higher vertebrates may not be complete at this phylogenetic level.

Cell surface immunoglobulins have been demonstrated on thymic and splenic lymphocytes from P.waltlii (Charlemagne and Tournefier, 1975). Evidence for T and B cell co-operation in the newt, Triturus viridescens, was provided by Ruben et al. (1973) who described a carrier effect in animals injected with trinitrophenyl (TNP)-chicken RBCs and TNP-toad RBCs.

The presence of suppressor of T cells in A.mexicanum was suggested by the finding that the response to horse erythrocytes was greater in thymectomised animals than in controls, a finding which also indicates that horse red blood cells act as a thymus-independent antigen in this species (Charlemagne, 1979).

Studies on the antibody producing capabilities of urodeles are limited; available information suggests that these animals produce predominantly, if not entirely, IgM-type immunoglobulins (Ching and Wedgewood, 1967; Fougereau and Houdayer, 1968; Ambrosius et al., 1970).

Antibody dependent cellular cytotoxicity (ADCC) has been demonstrated in A.mexicanum (MacLennan, 1972; Jurd and Doritis, 1977). Thus, chicken erythrocytes sensitized with rabbit anti-chicken erythrocyte anti-serum were lysed by non-immune splenocytes in the absence of complement: the nature of the cell responsible for this activity was not defined. Autografts survive indefinitely in urodeles while the median survival time for skin allografts is between 30 and 50 days (Cohen,

1968; Cooper and Garcia, 1968). Delayed hypersensitivity has been demonstrated to dinitro-fluorobenzene and heat-killed Mycobacterium tuberculosis in A. mexicanum (Tahan and Jurd, 1979). It was demonstrated that this sensitization can be transferred to unsensitized axolotls by injections of lymphocytes from sensitized animals, but not by serum.

### c. Anura

The anurans, which include frogs and toads, appeared during the Triassic period, about 200 million years ago (Goin and Goin, 1971). They show a considerable immunological advance over the urodeles in possessing a haemopoietic bone marrow and well organised nodules of lymphoid tissue in the gastro-intestinal tract. In advanced members of this group, namely, the Ranidae and Bufonidae, rudimentary lymph nodes make their phylogenetic appearance (Cooper, 1967; Cooper and Schaefer, 1970; Riviere and Cooper, 1972).

The thymus is the first lymphoid organ to develop in anurans (Cooper and Hildemann, 1965) and thymectomy affects their immune capabilities. Larval thymectomy in the midwife toad, Alytes obstetricans, impairs the response to SRBCs (Tochinai, 1975) and affects transplantation reactions by either decreasing or abolishing the allograft rejection capacity (Du Pasquier, 1976b). The kidney, spleen and lymph nodes of anurans are important secondary lymphoid organs and contain lymphocytes of thymic origin (Turpen et al., 1973; Tam et al., 1976). The anuran spleen, described by Cooper and Wright (1976), is involved in antigen trapping and antibody synthesis. Antigen localisation patterns have been described for Salmonella adelaide flagella in Bufo marinus (Diener and Nossal, 1966) and for human gammaglobulin (HGG) in Xenopus (Collie, 1974).

The splenic responses of adult Xenopus to HGG and of larval Rana catesbiana to SRBC have been described (Manning and Turner, 1972; Moticka et al., 1973). Plaque forming cells have been demonstrated in the spleen of the toad, Xenopus laevis (Auerbach and Ruben, 1970) and in the spleen, lymph gland and thymus of Bullfrog larvae (Moticka et al., 1973) following immunization with SRBC. Splenectomy reduces the amounts of antibody produced in these species (Collie and Turner, 1975; Brown and Cooper, 1976). In vitro antibody production by spleen cell cultures from Xenopus and Bufo has been described. Auerbach and Ruben (1970) showed that spleen explants from adult Xenopus can produce specific agglutinins and haemolysins when exposed to sheep or mouse red blood cells in vitro, while cell suspensions of B.marinus spleen produce a primary immune response to S.adelaide flagellin (Azzolina, 1975).

In addition to a thymus and spleen, anurans have a variety of lymphoid structures which have been described by Baculi et al. (1970). Clusters of lymphocytes occur within lymphatic vessels giving rise to jugular bodies (Kent et al., 1964). Blood-filtering lymph node-like structures consisting of encapsulated aggregates of lymphocytes are seen in the ventral region of the neck of Bufoniidae and Ranidae (Cowden and Dryer, 1971). Unlike the cyclostomes, elasmobranchs and teleosts, adult anurans have a bone marrow which, as well as being erythropoietic and myelopoietic, is also lymphopoietic (Baculi et al., 1970). Gut associated lymphoid tissues have also been demonstrated in anurans (Goldstine et al., 1975).

On the basis of their responses to soluble Con.A, PHA and PPD, lymphocytes analogous to the T and B cells of birds and mammals have been described in X.laevis (Manning et al., 1976; Du Pasquier and Horton, 1976).

Donnelly et al. (1976), studying the in vitro proliferative responses of lymphocytes from X.laevis, found that the responses to Con.A and PHA were thymus-dependent, while those to PPD were thymus-independent. Xenopus spleen has been demonstrated to be the primary reservoir of peripheralised T and B cells. The mitogen responses of these cells to Con.A, PHA, LPS and PPD resembled those of mice (Green and Cohen, 1979). Wright and Cooper (1978, 1979) studied the response of spleen lymphocytes of the Leopard frog, Rana pipiens, to PHA and Con.A and found that the dose and time response kinetics were similar to those reported for fish (Etlinger et al., 1975, 1976b; Cuchens et al., 1976), reptiles (Cuchens et al., 1976), birds (Sallstrom and Alm, 1973, 1974), metatherian mammals (Fox et al., 1976; Ashman and Keast, 1976; Ashman et al., 1977) and many eutherian mammals (Harvey et al., 1974; Mumford et al., 1975). Ultrastructural studies of R.pipiens lymphocytes following in vitro stimulation with PHA showed a transformation sequence from small lymphocytes to lymphoblasts (Wright et al., 1979). T and B cell co-operation occurs in amphibians. Thus carrier specificity has been shown in T.viridescens (Ruben, 1975) and has been implicated in the response of R.pipiens (Edwards et al., 1975).

As in fish, surface membrane immunoglobulin can be detected on both thymic and splenic lymphocytes of anurans and can be shown to be synthesised by these cells (Du Pasquier et al., 1973; Jurd and Stivenson, 1976; Hadji-Azimi, 1977; Nagata and Katagiri, 1978). Thymic lymphocytes are the first to show surface membrane immunoglobulin (Cooper, 1976).

While urodeles appear restricted to the production of IgM, anurans can produce both heavy and light immunoglobulins. Uhr et al. (1962) showed the production of 19S and 7S antibodies in Bullfrog (R.catesbiana) in

response to immunization with bacteriophage ØX174. Marchalonis and Edelman (1966b) using bacteriophage f2 confirmed these findings. Early antibody activity was limited to the 19S molecules, the low molecular weight antibodies appearing 35 days after immunization. By 58 days, over 90 per cent of the antibody activity was localised in the 7S protein. The 19S and 7S immunoglobulins of R.catesbiana resembled the IgM and IgG classes of mammals in polypeptide structure (Marchalonis, 1971a). The two immunoglobulin classes were antigenically related through the presence of shared light chains and distinguished by the possession of distinct  $\mu$  and gamma type heavy chains. B.marinus was found to produce only 19S antibodies to S.adelaide flagella (Diener and Marchalonis, 1970; Marchalonis and Germain, 1971), in contrast to its ability to produce both 19S and 7S classes of immunoglobulin to bovine serum albumin (BSA) (Acton et al., 1969). These results and related findings (Maung, 1963; Marchalonis et al., 1969) initially suggested that anuran amphibians can form only 19S antibodies in response to S.adelaide flagellar antigen. Flagellin, the main component of Salmonella flagella is now recognised as being a thymus independent antigen (Armstrong et al., 1969; Diener et al., 1971) and as such stimulates principally, if not entirely, an IgM response (Basten and Howard, 1973; Mitchell, 1974).

The intensity of the immune response of anurans, in terms of antibody production, is comparable to that of mammals, most of the antigens used giving rise to both 19S and 7S antibodies (Marchalonis et al., 1970).

A major histocompatibility complex homologue has been described in X.laevis, which controls graft rejection, MLR activity and certain red blood cell antigens

(Du Pasquier et al., 1975). ADCC using chicken erythrocytes sensitized with rabbit anti-erythrocyte anti-serum has been shown to occur with spleen cells from X.laevis (Jurd and Doritis, 1977).

Prompt and vigorous allograft reactions in frogs, even during larval development, shows that cell mediated immunity at the anuran level is comparable to that seen in birds and mammals (Hildemann and Haas, 1961). Thymic lymphocytes are implicated as the effector cells since early thymectomy impairs allograft rejection (Cooper and Hildemann, 1965; Curtis and Volpe, 1971; Horton and Manning, 1972). However, once the secondary lymphoid organs have been developed in late larval life, thymectomy no longer affects immune capacity (Tam et al., 1976).

Anurans reject skin allografts faster than do urodeles; the more advanced anurans, such as the Ranidae, being more efficient in this respect than the more primitive forms exemplified by Xenopus (Baculi and Cooper, 1970). Mixed lymphocyte reactions in the toad, X.laevis have been demonstrated (Du Pasquier and Miggi-ano, 1973). Du Pasquier et al. (1975) proposed the presence of a major histocompatibility complex following the observation that the control of MLR and graft rejection were functions of the same region of the chromosome.

The immune response in amphibians is temperature dependent. Upon challenge with S.adelaide flagellin B.marinus produced antibody-forming cells and antibodies more promptly at 37°C than at 22°C (Diener and Marchalonis, 1970).

Within the Amphibia, certain trends in the evolution of immune capacity become evident; notable is the increasing sophistication of the lymphoid system, parti-



cularly as seen in certain anuran species. A lymphopoietic bone marrow contributes to the immune response while there is the phylogenetic appearance of lymphoid organs which, at least structurally, begin to resemble the nodes of mammals.

Analysis of mitogen-induced lymphocyte activities and the occurrence of carrier-effects indicate the presence of two functionally distinct populations of lymphocytes.

While the limited information available suggests that urodeles might be restricted to producing high molecular weight antibody only, the anurans consistently produce both 19S and 7S immunoglobulins. As in Dipnoid lungfish these are antigenically distinct. However, whereas in lungfish the lower molecular weight antibody has a sedimentation coefficient of 5.9S the anuran low molecular weight immunoglobulin sediments at 7S and in physical and chemical properties is analogous to the IgG of mammals.

### 1.5 Reptiles

To-day's reptiles are the remnants of a once flourishing and dominant group of animals. Diverging from primitive amphibians during the Carboniferous period, 200 million years ago, the reptiles expanded into diverse forms to become the dominant species in the late Cretaceous. The reptiles represent a branching point in evolution since two of their forms, the Thecodonts and the Therapsids, were ancestral to birds and mammals respectively. The successful modern forms include the Squamata (lizards and snakes), the Crocodilia (crocodiles, alligators and caymans), the Chelonia (tortoises and turtles) and the primitive Rhynchocephalia, represented by the tuatara (Table 1.3).

Table 1.3 A classification of Reptiles

(After Romer, 1955)

Class Reptilia

Subclass Anapsida

Order Chelonia

<u>Agrionemys horsfieldii</u>	: Tortoise*
<u>Testudo hermanni</u>	: Tortoise
<u>Chrysemys picta</u>	: Painted turtle
<u>Pseudemys scripta</u>	: Turtle
<u>Chelydra serpentina</u>	: Snapping turtle

Subclass Parapsida : Extinct

Subclass Diapsida

Superorder Lepidosauria

Order Rhynchocephalia

<u>Sphenodon punctatum</u>	: Tuatara
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Order Squamata

Suborder Latertilia

<u>Calotes versicolor</u>	: Lizard
<u>Ophisaurus apodus</u>	: Lizard
<u>Basiliscus basiliscus</u>	: Lizard
<u>Tiliqua rugosa</u>	: Sleepy lizard
<u>Iguana iguana</u>	: Iguana
<u>Gekko gekko</u>	: Gekko
<u>Chalcides ocellatus</u>	: Lizard
<u>Mabuya quinquetaeniata</u>	: Lizard
<u>Uromastyx aegyptia</u>	: Lizard
<u>Lacerta viridis</u>	: Lizard

Suborder Ophidia

<u>Spalerosophis diadema</u>	: Snake
<u>Elaphae quadrivirgata</u>	: Snake
<u>Thamnophis ordinoides</u>	: Garter snake

Table 1.3 (contd)

Superorder Archosauria	
Order Crocodylia	
<u>Alligator mississippiensis</u>	: Alligator
Subclass Synapsida	: Extinct

\* Species named are mentioned in text.

Lymphoid tissues in reptiles include the thymus, spleen and gut-associated lymphoid tissues (Pitchappan and Muthukkaruppan, 1977). The thymus disseminates lymphocytes to other organs of the lymphomyeloid complex such as the spleen and lymph nodes (Auerbach, 1961; Pitchappan and Muthukkaruppan, 1977). It has been suggested (Pitchappan and Muthukkaruppan, 1977) that the thymic lymphocyte progenitors in the lizard, Calotes versicolor, originate from blood-borne stem cells, a situation similar to that seen in chickens and mice (Moore and Owen, 1967b; Owen and Ritter, 1969). Thymectomy in adult lizards has been found to reduce the number of plaque forming cells seen in response to SRBC (Muthukkaruppan et al., 1976). No thymus has been found in adult tuatara (Marchalonis et al., 1969). Gut-associated lymphoid tissues and structures resembling Peyer's patches have been demonstrated in the snake, Spalerosophis diadema (Hussein et al., 1979).

The spleen of some reptiles e.g., tuataras, lizards, snakes and turtles, show a distinct separation into white and red pulps (Wetherall and Turner, 1972; Manning and Turner, 1976; Hussein et al., 1979), although Kanakambika and Muthukkaruppan (1972) failed to see this demarcation in C.versicolor.

Small groups of lymphocytes have been found in the kidney and portal tracts of the liver in the lizard, Tiliqua rugosa (Wetherall and Turner, 1972). Multiple aggregates of lymphoid tissues in the cloacal region of the turtle, Chelydra serpentina resemble anatomically the avian bursa of Fabricius (Sidky and Auerbach, 1968). Unlike the avian bursa, however, which assumes its major role early in development, the cloacal lymphoid tissue of turtles is more active in adults than in juveniles and does not function as a primary lymphoid

organ. The oesophagus and small intestine of the snake, Elaphae quadrivirgata, have discrete lymphoid nodules in the mucosa (Manning and Turner, 1976). Accumulations of lymphocytes resembling Peyer's patches have been described in the lizards, Iguana iguana, Gekko gekko and Basiliscus basiliscus (Fichtelius et al., 1968).

Surface membrane immunoglobulin has been demonstrated on reptile lymphocytes. In the tortoise, Agrionemps horsefieldii, Fiebig and Ambrosius (1976) showed that blood and spleen lymphocytes carry both monomeric IgM and what the authors described as IgY. IgY is a 7S immunoglobulin which physico-chemically is more closely related to the chicken immunoglobulin designated IgY by Leslie and Clem (1969) than it is to mammalian IgG (Hadge and Fiebig, 1975). Fiebig and Ambrosius proposed that the IgY of reptiles might be an early phylogenetic counterpart of human IgD since it shows certain physical similarities to that immunoglobulin (Leslie and Clem, 1972). Furthermore, the authors suggested that both the IgM and the IgY might act as antigen receptors, a situation comparable to the combined roles of IgM and IgD in man (Sigel and Klinman, 1978). Whereas thymocytes from fish and amphibians have readily detectable surface membrane immunoglobulin, this could not be demonstrated on tortoise thymocytes.

Polyacrylamide gel analysis of radiolabelled surface membrane immunoglobulin from blood lymphocytes of the alligator, Alligator mississippiensis, revealed two different heavy chain types, indicating that two antigenically different immunoglobulins occur on these cells (Cuchens et al., 1976).

Evidence has been provided for the existence of T-like and B-like cells in A. mississippiensis following glass-wool column fractionation, surface marker studies

and mitogen responsiveness to PHA, Con.A and LPS (Cuchens and Clem, 1979b).

Turtles and tortoises are the reptilian groups whose immune responses have been most studied. The immune responses of turtles against erythrocytes (Noguchi, 1902), Salmonella (Evans et al., 1965), serum proteins (Lykakis, 1968; Coe, 1972a) and protein-hapten conjugates (Leslie and Clem, 1972) have been studied. Tortoises have been shown to respond to SRBC (Rothe and Ambrosius, 1968), bacterial antigens (Maung, 1963; Chartrand et al., 1971) and protein antigens (Frair, 1963; Ambrosius and Frenzel, 1972; Benedict and Pollard, 1972). Immunological memory in the form of a secondary response has been demonstrated in the tortoise, Testudo hermanni (Ambrosius, 1976).

Turtles (Pseudemus scripta and Chrysemys picta) have two antigenically distinct immunoglobulin classes with sedimentation coefficients of 18S and 7.5S and a third class having a sedimentation coefficient of 5.7S. This latter type is antigenically deficient with respect to the 7.5S class and may lack a portion of the Fc region (Lykakis, 1968; Chartrand et al., 1971; Acton et al., 1972; Leslie and Clem, 1972). Only the 5.7S class was found in extracts of turtle egg yolk suggesting that this class may have a selective advantage in its ability to cross certain membranes. As in other species, the earliest antibodies produced in turtles are of high molecular weight (Grey, 1963). Coe (1972) found that antibodies associated with four types of immunoglobulins were produced in C.picta in response to immunization with a range of soluble proteins. These immunoglobulins were designated Ig1 (19S), Ig2 (slightly heavier than 7S) and Ig3 and Ig4 (about 7S). Antibody activity was first found in Ig1 and Ig2 and later

in Ig3 and finally in Ig4. It was not resolved whether Ig2, Ig3 or Ig4 were degradation products of Ig1 or were separate subclasses.

The immune responses of lizards have been studied using Salmonella (Evans et al., 1965), BSA and rat erythrocytes (Wetherall and Turner, 1972), serum proteins (Ambrosius et al., 1969a; Hemmerling, 1971) and SRBC (Kanakambika and Muthukkaruppan, 1972) as antigens. Following immunization with SRBC, C.versicolor produces serum haemolysins and agglutinins as well as blood and spleen plaque-forming cells. Haemolysins appear after a latent period of two weeks and reach a peak during the third to fourth week. Following a second injection of SRBC the development of serum antibody is more rapid but does not increase in titre. A similar finding has been reported for turtles (C.picta) (Grey, 1966) and tortoises (A.horsfieldii) (Rothe and Ambrosius, 1968). In contrast, a rapid and elevated antibody response after a second injection of antigen has been reported in the tortoise, T.hermannii (Ambrosius and Lehmann, 1965) and the lizards, Ophissaurus apodus (Ambrosius et al., 1969b) and T.rugosa (Wetherall and Turner, 1972).

Lizards produce three types of immunoglobulins, a high molecular weight pentamer, similar to IgM (Ambrosius et al., 1969a), a 7.3S immunoglobulin with beta-2 mobility which corresponds to the IgY of birds (Leslie and Clem, 1969) and a 6.8S immunoglobulin which migrates as a beta-1 protein. The 6.8S form appears to be an isotype unique to this reptilian group (Ambrosius, 1976).

The tuatara, Sphenodon punctatum, has immunoglobulins with sedimentation coefficients of 18S and 7S, the 18S molecule, resembling human IgM in its electrophoretic mobility in polyacrylamide gel (Marchalonis et al., 1969). When immunized with S.adelaide flagella

for a period of eight months, tuataras produced antibodies restricted to the 18S class. Diener (1970) noted maximum antibody production in this species two to three months after immunization with S. adelaide flagellin, antibody activity being found only in a high molecular weight (18S) fraction even after six months, a finding consistent with this antigen being thymus independent (Basten and Howard, 1973).

Antibodies are produced in snakes following their immunization with bacterial antigens (Dimow and Slawtschew, 1967), protein antigens (Coe et al., 1976) and parasitic nematodes (Timourian et al., 1961). Coe et al. (1976) immunized the garter snake, Thamnophis ordinoides, with ovalbumin, HGG or KLH in Freund's complete adjuvant. Three immunoglobulin types were seen; an "Ig-M (20S)", an "Ig-1 (9S)" and an "Ig-2 (8.5S)". These workers also demonstrated a secretory immunoglobulin system in T. ordinoides. Ig-M was the only immunoglobulin in bile and was the predominant immunoglobulin in succus entericus, which also had small amounts of Ig-1 and Ig-2. Preliminary evidence indicated that a similar secretory system exists in the turtle, C. picta, (Portis and Coe, 1975). These observations indicate that IgM may have served the function of a secretory immunoglobulin before the evolution of alpha heavy chain genes. Kawaguchi et al. (1978) showed the production of antibody forming cells following injection of sheep erythrocytes and rabbit erythrocytes in the snake, Elapha quadrivirgata. Similar plaque forming cells were also demonstrated in the liver of C. versicolor (Kanakambika and Muthukkaruppan, 1972).

Jurd and Doritis (1977) showed that spleen cells of the lizard, Lacerta viridis could mediate ADCC of chicken red cells coated with rabbit anti-chicken red blood cell serum.



While there is considerable information on the humoral immune functions of reptiles, little is known about their cell mediated immune capacity. Members of the Chelonia, Squamata and Crocodylia all reject first set allografts (Borysenko, 1970; Tercbey, 1970). Rejection at physiologically normal temperatures is prolonged with median survival times of 40 to 90 days. Such rejection could reflect either a primitive response capacity or might indicate the lack of a major histocompatibility complex (Cohen, 1971).

Newborn snapping turtles, C. serpentina, injected with adult allogeneic spleen cells succumbed to acute graft-versus-host reactions when housed at 30°C. Chronic graft-versus-host reactivity with a lower mortality was seen at the more normal temperature of 20°C (Borysenko and Tulipan, 1973). In contrast, mature turtles were not affected by inoculation with spleen cells from adult donors. Blood lymphocytes from A. mississippiensis have been shown to participate in two-way MLR (Cuchens Clem, 1979a).

Antibody production and allograft rejection in reptiles are reduced by low ambient temperature (Evans and Cowles, 1959; Borysenko, 1970; Wetheral and Turner, 1972). An interesting observation by Hussein et al. (1978a,b) was the seasonal variation in lymphoid tissue in the lizards, Chalcides ocellatus, Mabuya quinquetaeniata and Uromastyx aegyptia. In winter the thymuses of these species were highly involuted whereas in other seasons they showed a rich lymphoepithelial organization; splenic white pulp and gut-associated lymphoid nodules were also severely depleted in winter.

The immune system of reptiles resembles that of amphibians. Lymphoid tissue development is comparable in the two groups and antibody production is restricted

to two immunoglobulin classes. The expression of immune competence in both groups is sensitive to ambient temperatures. In this respect the seasonal variation of lymphoid tissue structure represented in reptiles may be significant. Surface immunoglobulin, readily demonstrated on thymocytes from fish and amphibians, was not detected on tortoise thymus cells, a finding consistent with some attempts to demonstrate surface immunoglobulin on human T cells.

### 1.6 Birds

Birds evolved from archosaurian reptiles during the Jurassic period some 165 million years ago (Coilbert, 1967).

The immune system of birds lies anatomically between that of amphibians and reptiles on the one hand, and that of mammals on the other (Good and Finstad, 1967). Many primitive features are retained, notably the presence of collections of lymphoid tissue scattered throughout the soma and the absence of mammalian-type lymph nodes. Along with monotremes, marsupials and mammals, birds develop germinal centres in their peripheral lymphoid tissue, a feature not seen in either fish, amphibians or reptiles (Payne, 1971).

Analysis of the immune functions of avian primary lymphoid tissues have contributed significantly to the concept that cell mediated and antibody mediated immunities are functions of different lymphocyte populations. Birds have two primary lymphoid structures, the thymus and the bursa of Fabricius. Unique to birds, the bursa has long been considered a "cloacal thymus" (Jolly, 1911). Removal of either of these organs profoundly affects the development of immune competence. Glick

et al. (1956) fortuitously showed that bursectomy impairs the antibody forming potential of chickens, an observation that was confirmed and extended by others. On the other hand, graft survival times, unaffected by bursectomy (Warner et al., 1962), were shown to be prolonged following neonatal thymectomy (Warner and Szenberg, 1964) a manipulation which had little effect on antibody production. Studies on the effect of thymectomy in mice (Miller and Osoba, 1967), rats (Jankovic et al., 1962) and rabbits (Archer and Pierce, 1961; Archer et al., 1962) supported these observations and led to the concept that the two forms of immunity are mediated by cells dependent on either a thymic or a bursal influence. The studies of Cooper et al. (1965, 1966) conclusively showed that the thymus and bursa exercise entirely different functions, each controlling the differentiation and development of separate lines of immune cells. Other studies provided proof of a mesenchymal origin of stem cells for both T and B lymphocytes and other blood cells (Moore and Owen, 1965, 1966, 1967a). Consequently, the now accepted two component system of immunological development was proposed (Cooper et al., 1968).

The thymus, arising from the third and fourth pharyngeal pouches, is the first lymphoid organ to develop in the embryo chick and histologically resembles that of mammals (Schrier and Hamilton, 1952; Venzke, 1952). In addition to prolonging the survival time of skin allografts (Warner and Szenberg, 1962; Aspinall et al., 1963) neonatal thymectomy also reduces the delayed hypersensitivity reaction to spinal cord lipid, tuberculin and diphtheria toxoid (Jankovic and Isvaneski, 1963; Cooper et al., 1966) and reduces the severity of graft-versus-host reactions (Simensen, 1962; Cooper et al., 1966).

The bursa of Fabricius arises as a sac-like evagination on the dorsal wall of the cloaca on the fifth day of incubation and is populated with haemopoietic stem cells from the yolk sac seven to eight days later (Katz, 1977). IgM synthesis by bursal lymphocytes begins one to two days later while IgG synthesis is delayed until hatching (Kincade and Cooper, 1971). Most of the IgM produced is retained on the cells' surfaces to act as antigen receptors (Cooper et al., 1972). Following intra-bursal development the mature lymphocytes migrate to peripheral lymphoid organs (Durkin et al., 1971).

The mucosal lining of the bursa is arranged in a number of longitudinal primary folds, each of which is subdivided into secondary folds (Ackerman and Knouff, 1959). This tissue forms a network of lymphoid follicles. The organ is fully developed in the immature bird and undergoes regression at the onset of sexual maturity (Riddle, 1928). Antibody production within the bursa has been reported (Jankovic et al., 1972; Waltenbaugh and Van Alten, 1974).

The bursa plays an important role in the ontogeny of B cell development (Weber, 1975; Weissman, 1975). Bursectomy, whether by hormonal (Glick, 1964), surgical (Mueller et al., 1962; Warner and Szenberg, 1964; Stiffel et al., 1968) or chemical (Glick, 1969) means results in a marked diminution of B cell maturation, an absence of germinal centres and plasma cells from peripheral lymphoid tissues and a reduced ability to produce antibody. Antibody levels comparable to those of intact controls have been reported for the secondary responses in bursctomised birds (Jankovic and Isakovic, 1966, 1967; Rose and Orlans, 1968). However, these antibodies, in contrast to those of control birds, remain sensitive to 2-mercaptoethanol (2-ME) and hence, presumably, are

IgM (Claffin et al., 1966; Jankovic and Isakovic, 1967). Surgically bursectomised birds show an IgG deficiency which is frequently associated with an increased IgM level (Ortega and Der, 1964; Cooper et al., 1966); the development of natural haemagglutinins is depressed in these birds (Graetzer et al., 1963; Jankovic and Isakovic, 1967). Other investigators, however, have reported only slight deficiencies in antibody production in bursectomised birds and postulate the existence of a bursa-independent control mechanism of B cell development (Lerner et al., 1971).

Bursectomy does not affect transplantation immunity (Warner et al., 1962; Aspinall et al., 1963), delayed hypersensitivity reactions (Jankovic et al., 1963) or graft-versus-host reactions (Mueller et al., 1964).

Avian secondary lymphoid tissues include the spleen and lymphoid tissues in the alimentary tract and paracocular and paranasal organs. Lymphoid tissue, with diffuse masses of germinal centres, occurs with an irregular distribution in the lamina propria of the alimentary tract from the pharynx to the cloaca. Prominent lymphoid structures are the 'caecal tonsils' which appear as enlargements of the caudal ends of caeca near the ileo-caeco-colic junction. These organs are important sources of antibody (Jankovic and Mitrovic, 1967), both IgG and IgM having been detected in them (Warner et al., 1969).

Bang and Bang (1968) described various lymphoid tissue accumulations in lacrimal ducts, Harderian glands and ducts and the lateral nasal gland duct; these authors suggested that these tissues provide local immune responses.

The chicken thymus and bursa are populated by T and B lymphocytes respectively. Surface marker studies have shown that lymphoid cells in the bursa are exclusively B cells, while in the thymus, although the majority of cells are T cells, there is a small number of B cells (Kincade et al., 1971; Rabellino and Grey, 1971; Hudson and Roitt, 1973; Albini et al., 1974). While secondary lymphoid organs normally contain a mixture of T and B cells, an exception to this is seen in the paracortical Harderian glands which contain B lymphocytes only (Albini et al., 1974). The bone marrow in chickens is a secondary lymphoid organ (Ivanyi et al., 1972), whereas in mammals it acts as a primary lymphoid organ (Osmond and Nossal, 1974; Ryser and Vassalli, 1974).

Bursal lymphocytes from 14 day-old embryos have IgM surface immunoglobulin (Kincade and Cooper, 1971; Tao-Weidmann et al., 1975). The percentage of IgM-positive bursal lymphocytes increases to reach about 85 per cent at the time of hatching. Bursal lymphocytes expressing surface IgG are detectable by the sixteenth day of incubation (Albini and Wick, 1975) and reach a maximum of about 50 per cent nine days after hatching (Albini and Wick, 1974). IgA has been demonstrated on bursal cells by the 20th day of incubation (Cooper et al., 1976).

Chickens produce antibodies to a range of antigens including serum proteins (Wolfe, 1942; Wolfe and Dilks, 1946; Grey, 1967a; Aitken and Parry, 1974), polypeptides (Gunther et al., 1974), protein-hapten conjugates (Orlans et al., 1968; Yamaha and Benedict, 1975), pneumococcal polysaccharide (Medlin et al., 1973) and heterologous erythrocytes (Aitken and Parry, 1974; Benton and Morgan, 1978). There are species variations in response to any given antigen. Thus, while chickens

respond promptly to BSA (Wolfe, 1942), turkeys (Wolfe and Dilks, 1949), pigeons (Wolfe and Dilks, 1949; Guttman et al., 1971), Guinea fowl (Wolfe and Dilks, 1949), Japanese quail (Leslie and Benedict, 1969) and penguins (Allison and Feeny, 1968) respond poorly to this antigen. White and Nielsen (1975) studied the responses of chickens to both thymus-independent and thymus-dependent antigens. Heat-killed S. adelaide induced a response which was restricted largely to 19S immunoglobulin while SRBC, induced both 19S and 7S antibodies. Chickens show typical carrier-effects in response to immunization with protein-hapten conjugates (Sarvas et al., 1974).

Birds produce antibodies of three distinct immunoglobulin classes which are analogous to mammalian IgM, IgG and IgA (Benedict et al., 1962). Since the heavy chain of chicken 7S antibody is some 10,000 daltons heavier than its mammalian equivalent, Leslie and Clem (1969) designated this immunoglobulin as IgY, although other investigators refer to it as IgG.

A major development within the birds is the phylogenetic emergence of IgA. An avian immunoglobulin corresponding to mammalian IgA was first reported by Lebacq-Verheyden et al. (1972) and Orleans and Rose (1972). IgA is the major immunoglobulin in bile and is found in high concentration in saliva, intestinal and bronchial secretions and oviduct washings, whereas it represents a minor immunoglobulin in serum (Lebacq-Verheyden et al., 1972; Bienenstock et al., 1973; Leslie and Martin, 1973). It is capable of combining with human secretory component (Bienenstock et al., 1972, 1973). By immunodiffusion tests Parry and Aitken (1975) demonstrated the presence of IgA in the saliva and bile of pheasants, Japanese quail, Guinea fowl, turkeys and pigeons.

A possible IgA equivalent has been described in pigeon droppings, lacrimal fluid and saline washings of oviducts (Edwards et al., 1969, 1970).

Watanabe and Kobayashi (1974) identified two types of IgA in chicken external secretions, namely biliary IgA, which existed as a pentamer or tetramer and lacked the secretory component, and intestinal IgA, mainly in a dimeric form complete with secretory component. Further studies by Watanabe et al. (1975) supported these findings. The elution characteristics of chicken secretory component from diethylaminoethyl cellulose and its molecular size closely resemble those of human secretory component (Newcomb et al., 1968; Brandtzaeg, 1971).

Chicken lymphocytes respond in vitro to PHA (Greaves and Roitt, 1968b) or Con.A (Toivanen and Toivanen, 1973); anti-immunoglobulin antiserum also induces proliferative response (Weber, 1973).

Birds show vigorous transplantation immunity indicating the presence of both a strong histocompatibility system and a well developed capacity for cell mediated immunity. The histocompatibility system is referred to as the B system (McDermid, 1964; Hala and Hasek, 1971). Incompatibility to B antigens leads to acute graft rejection (Schierman and Nordskog, 1961) and graft-versus-host reaction (Jaffe and McDermid, 1962). Gunther et al. (1974) studied the antibody response of inbred chickens to a synthetic polypeptide and suggested that the genes controlling the response to this material are linked to those of the major histocompatibility complex. The MLR in birds is controlled by a gene or genes located in the B gene region (Gunther et al., 1974). The similarity of the B system of chickens to the H-2 system of mice and the HLA system of



man reflects the common evolutionary origin of these species.

Studies on cell mediated immunity in turkeys (Healey et al., 1962), ducks (Hasek et al., 1966) and chickens (Gilmour, 1963; Hasek et al., 1966) have been reported. Acute rejection of allografted tissue with rapid first set responses and a second set memory are seen. Allograft immunity in chickens is thymus-dependent (Aspinall et al., 1963); thus irradiated, bursectomised chickens show typical acute allograft rejection (Perey and Dupuy, 1970). Similarly, Warner et al. (1971) showed delayed type hypersensitivity reactions in normal and bursectomised chickens. Several other studies have shown that delayed hypersensitivity responses can be elicited in bursectomised chickens (Jankovic et al., 1963; Palladino et al., 1978).

Birds represent a significant stage in immune phylogeny. The avian bursa of Fabricius is seen as a primary lymphoid organ essential for the maturation of antigen-sensitive B cells. Extirpation of this lymphoid mass in conjunction with thymectomy experiments delineated the basis of T and B cell divisions. A third antigenically unique immunoglobulin class, IgA, emerges at this level. Complete with secretory component, avian IgA is the forerunner of secretion-associated immunity of mammals.

### 1.7 Mammals (Prototheria and Metatheria)

The ancestors of the mammals diverged from therap-  
sid reptiles about 200 million years ago (Romer, 1955).  
Three subclasses of mammals survive namely, the  
Prototheria, the Metatheria and the Eutheria (Table 1.4).  
Prototherian mammals, or monotremes, retain a number of

Table 1.4 A classification of Mammals

(After Morris, 1965)

Class	Mammalia	
Subclass	Prototheria	
Order	Monotremata	
Family	Tachyglossidae	
	<u>Tachyglossus aculeatus</u>	: Echidna*
	<u>Ornithorhynchus paradoxus</u>	: Platypus
Subclass	Theria	: Extinct
Subclass	Aliootheria	: Extinct
Subclass	Metatheria	
Order	Marsupialia	
Superfamily	Didelphoidea	
	<u>Didelphis virginiana</u>	: American opossum
Superfamily	Dasyuroidea	
Family	Dasyuroidae	
Family	Notoryctidae	
Superfamily	Perameloidea	
Superfamily	Caenolestoidea	
Superfamily	Phalangeroidea	
Family	Phalangeridae	
	<u>Trichosurus vulpecula</u>	: Brush-tailed phalangers
Family	Vombatidae	
Family	Macropodidae	
	<u>Setonix brachyurus</u>	: Quokka
	<u>Macropus eugenii</u>	: Tammar
Subclass	Eutheria (=Placentalia)	

\* Species named are mentioned in text.

reptilian features. These egg-laying mammals are represented by two extant species, the Spiny ant-eaters or echidna and the Duck-billed platypus, both being restricted to Australia. The Metatheria or marsupials, are pouched viviparous mammals in which the young, after being born at an embryologically immature stage, migrate to, and continue their development within, the maternal pouch, or marsupium. The Eutheria are mammals having a fully developed placenta, young being born at a relatively advanced physiological stage of development.

a. Prototheria

Both the echidna, Tachyglossus aculeatus, and the platypus, Ornithorhynchus paradoxus, have thymuses and spleens histologically comparable to those of eutherians (Diener and Ealey, 1965; Diener, 1970). A notable feature of the echidna is the presence of lymphoid nodules which are intermediate in structure between the jugular bodies of amphibians and the lymph nodes of eutherians (Diener and Ealey, 1965). They are found within lymphatic vessels and occur in sites where lymph nodes would be expected in placental mammals. Whereas the lymph nodes of eutherians show follicles within a diffuse cortex and a medulla with sinuses and plasma cells, the lymphoid nodules each act as individual follicles. Schofield and Cahill (1969) have described lymphoid elements within the gastrointestinal tract of the echidna.

The cellular responses in echidna lymphoid nodules was studied by Diener et al. (1967a) following the injection of isotope-labelled flagellar antigen of S. adelaide. Active germinal centres were seen within nodules within three hours of antigen injection. A

significant uptake of antigen was also seen in the appendix, Peyer's patches and Hassall's corpuscles of the thymus.

Two types of antibodies were produced by the echidna (Diener et al., 1967b). The initial response was characterized by 2-ME-sensitive antibody while 2-ME-resistant antibody was dominant late in the primary response: these appeared analogous to the IgM and IgG of eutherians. Although a secondary response could be demonstrated it was of a much smaller magnitude than that of placental mammals.

Atwell et al. (1973) determined that the IgM of the echidna has a pentameric structure and a molecular weight of  $950,000 \pm 57,000$  daltons, while the IgG has a molecular weight of  $150,000 \pm 9,000$  daltons. Evidence has been provided to show homology between the gamma chains of the echidna and eutherians by partial amino acid sequence studies (Atwell and Marchalonis, 1977) and by their ability to bind to Staphylococcus aureus protein A (Marchalonis et al., 1978).

#### b. Metatheria

North American opossums (Didelphis virginiana) are born after a gestation period of 12.5 days at which time they have neither circulating lymphocytes nor organised lymphoid tissues. A lymphoid system similar to that of eutherian mammals (Rowlands, 1976) develops soon after birth (Block, 1964).

A single thoracic thymus is present (Johnstone, 1898; Yadav, 1973a) and is the first organ to contain lymphocytes which are seen on the second day in the pouch. The cortex of the adult thymus shows closely packed small and medium lymphocytes while the medulla

consists of a mixture of epithelial cells and lymphocytes. The lymph nodes resemble those of eutherian mammals having abundant lymphocytes and germinal centres in the cortex and plasma cells and sinusoids in the medulla (Rowlands, 1976). Lymphocytes may be found in a cervical node on the third day and are regularly found in other lymph nodes by the sixth day (Zimmermann, 1940; Rowlands et al., 1964). The spleen is triangular or triradiate in form, the internal structure being typically mammalian although it lacks ellipsoids and sinusoids (Perrorr, 1966). Splenic lymphocytes appear between the seventeenth and twentieth pouch days while plasma cells and secondary nodules are seen around day 60: tonsils and Peyer's patches appear later.

Newborn opossums lack serum immunoglobulins. However, 12 hr after attachment to the maternal teat both IgG- and IgA- like proteins, apparently derived from the dam, appear in the circulation (Hindes and Mizell, 1976). While IgM has been found in some animals after 14 days, many animals of this age lack this immunoglobulin.

No measurable antibody production was observed when embryos younger than eight days were immunized with S. typhosa flagellar antigen (Rowlands et al., 1974). There was regular production of antibody after eight days and after 20 days the level of serum antibodies increased.

Rowlands and Dudley (1969) investigated the immune response of opossum embryos to bacteriophage f2 and to BSA coupled with DNP. Embryos aged five days or more at the time of immunization showed an immune response to bacteriophage f2 while embryos 15 days or older responded to the hapten. IgM antibodies were most prominent in the responses but IgG antibodies could also be detected.

Rowlands et al. (1972) observed that there was a notable increase in the antibody activity of serum obtained from opossum embryos which were more than 40 days old when immunized with bacteriophage f2 compared with the response of younger animals. They suggested that improved recognition of antigen, increased numbers of antigen-responsive lymphoid cells or development of new classes of immunoglobulins could be the reason for this increased responsiveness. Antibodies were detected in serum seven days after immunization and peak antibody responses were reached 14 days or more after immunization; a second injection of antigen increased the level of antibodies only slightly. The earliest antibodies detected by sucrose density gradient analyses were 13S "embryonic" antibodies which disappeared when IgM antibodies were produced. IgG antibodies were never prominent and were last to appear in measurable amounts. The authors suggested that the capacity for immunological memory is not well established at this age.

Several antigens have been used to study the immune response of adult North American opossums. These include particulate antigens such as bacteria (Taylor and Burrell, 1968), bacteriophage (Rowlands and Dudley, 1969; Rowlands, 1970; Rowlands et al., 1972) and SRBC (Marx et al., 1971) and soluble antigens such as BSA, ovalbumin and KLH both with and without hapten conjugation (Rowlands and Dudley, 1969; Taylor and Burrell, 1968; Marx et al., 1971). The level of the immune responses attained appeared related to the type of antigen used. Thus, bacterial antigen induced higher antibody levels than did influenza virus which, in turn, was more immunogenic than KLH; BSA induced only feeble responses.

The responses were seen to follow the eutherian pattern in that a 19S, 2-ME-sensitive antibody was

formed first and was replaced in time by a 7S, 2-ME-resistant immunoglobulin. Genco and Liebert (1970) using DNP-BGG as antigen identified 19S and 7S antibodies and also a subclass of the smaller immunoglobulin. These authors also showed that the hexose contents of the 19S and 7S immunoglobulins were comparable to those of higher vertebrates. They further detected "gamma G" and "gamma 1" immunoglobulins in normal opossum milk and in the saline extracts of opossum intestines. Bell (1977) demonstrated the presence of IgG<sub>2</sub> in the serum of D.virginiana.

In contrast to IgG from 62 other mammalian species, only opossum IgG showed an apparent inability of its Fc region to react with Staphylococcal protein A in gel diffusion (Kronvall et al., 1970a).

Hindes and Mizell (1976) noted an immunoglobulin in the serum and milk of D.virginiana, which, in its electrophoretic mobility in a polyacrylamide gel, resembled eutherian IgA.

Limited studies of cell mediated immunity have been made in Didelphis. Taylor and Burrell (1968) showed that skin reactions associated with either tuberculin sensitivity, experimental allergic encephalomyelitis or contact sensitivity to dinitrochlorobenzene were minimal compared to the responses given by conventional laboratory animals. The passive transfer of delayed hypersensitivity was achieved with peritoneal exudate cells (Taylor, 1968).

Adult Didelphis show first and second set rejection phenomena to skin allografts. The rejection of primary grafts occurred at about 14 days, while second set skin graft rejection was accelerated and occurred at about 6 days, the competence to reject skin allografts deve-

loping later than the capacity to produce antibody (La Plante et al., 1969). Attempts to show MLR in Didelphis have been unsuccessful (Fox et al., 1976; Rowlands, 1976).

In the absence of eutherian predators, Australian marsupials evolved into a wide range of forms occupying vastly different ecological niches. The family Macropodidae embraces 56 species and includes the kangaroos, wallabies, quokkas and tammars (Kirsch and Calaby, 1977).

The quokka, Setonix brachyurus, in common with all marsupials of the family Phalangeridae, has two thymus glands, a subcutaneous cervical thymus and a mediastinal or thoracic thymus (Yadav, 1973a). Small lymphocytes appear in the cervical thymus three days after birth and in the thoracic thymus five days after birth (Yadav and Papadimitriou, 1969).

Thymectomy of S.brachyurus younger than 20 days of age does not result in the typical wasting syndrome seen in thymectomised eutherian mammals (Keast, 1968) although the life spans of these animals are markedly reduced (Ashman et al., 1973) and a severe lymphopenia develops (Yadav et al., 1972). The spleen and para-cortical areas of the lymph nodes are depleted in thymectomised animals (Ashman and Papadimitriou, 1975) while antibody production to SRBC cannot be demonstrated during pouch life (Stanley et al., 1972). Neonatal thymectomy also delays skin graft rejection in pouch young (Yadav et al., 1974) and causes a depression of leucocyte responses to PHA and Con.A (Ashman et al., 1977).

Ashman et al. (1972) showed that quokka leucocyte cultures have a dose response curve to PHA similar to those of man and mice (Festenstein, 1968; Fitzgerald,



1971). The dose response curves of the quokka and the tammar, Macropus eugenii, to Con.A (Ashman et al., 1976) were similar to those recorded for hamster lymphocytes (Singh and Tevethia, 1972), while the response to PWM resembled that obtained for mouse lymphocytes (Janossy and Greaves, 1971).

The immune responses of the quokka to SRBC (Stanley et al., 1966; Yadav, 1973b), bacteriophage ØX174 (Yadav, 1971; Stanley et al., 1972; Yadav, 1973b), S. adelaide flagella (Thomas et al., 1972; Yadav, 1973b) and BSA and DNP-BSA (Thomas et al., 1972; Turner et al., 1972) have been studied. Two classes of antibodies analogous to eutherian IgM and IgG are generally produced. The response to bacteriophage ØX174 also induced an antibody with a molecular weight of 80,000 daltons; this low molecular weight immunoglobulin was not a breakdown product of the IgM or IgG classes (Thomas et al., 1972).

A high antibody titre and a distinct anamnestic response was elicited by bacteriophage ØX174 and S. adelaide flagella, but not with SRBC (Yadav, 1973b). The transition from 19S to 7S occurred rapidly in the secondary response with the phage and the flagellar antigens but with SRBC a large proportion of the antibody in both the primary and secondary responses was in the 19S fraction.

As in North American opossums (Rowlands and Dudley, 1968; Rowlands, 1970) and eutherians (Adler, 1956; Munoz, 1967; Wagner and Freeman, 1970), the IgG antibodies of quokkas (and also tammars and T. vulpecula) were found to be only partially resistant to 2-ME reduction while a portion of the IgM antibodies were resistant to 2-ME reduction (Yadav, 1973b).

Three classes of immunoglobulins have been described from S.brachyurus. These include IgM (Bell et al., 1974a) and IgG immunoglobulins, the latter existing as two subclasses, IgG<sub>1</sub> and IgG<sub>2</sub> (Bell et al., 1974a; Lynch et al., 1975). The IgG subclasses of the quokka are comparable to eutherian IgG<sub>1</sub> and IgG<sub>2</sub> subclasses in their electrophoretic mobilities, carbohydrate content and extinction coefficients: they have antigenically distinct heavy chains and differ in their molecular weights (IgG<sub>1</sub>, 178,000 daltons; IgG<sub>2</sub>, 136,000 daltons) (Lynch et al., 1975). Bell et al. (1974a) described two subclasses of IgG<sub>2</sub>, namely, IgG<sub>2a</sub> and IgG<sub>2b</sub>.

The IgG<sub>1</sub> isotype shows both passive haemagglutinating and homocytotropic antibody activities (Lynch and Turner, 1974b). Lynch and Turner (1974) further showed that this homocytotropic antibody was antigenically related to human IgE.

The IgG<sub>2</sub> of the quokka has a counterpart in D.virginiana (Bell, 1977) and it has been suggested that these immunoglobulins may be descendents of the IgG present when the American and Australian marsupials diverged.

Bell et al. (1974b) identified an immunoglobulin in quokka milk which, in its biological and physico-chemical features, resembled eutherian IgA. In milk the molecular weight of this molecule was in excess of 200,000 daltons; in serum two forms were seen, one with a molecular weight of 150,000 daltons, the other with a molecular weight exceeding 200,000 daltons. This (secretory) IgA was also isolated from urine and gut contents.

Nursing Setonix immunized with either S.adelaide flagella, phage ØX174 or SRBC transmitted antibodies to their young by way of milk (Yadav, 1971). Antibody

activity in the milk and in the serum of the young animal was associated with a 7S molecule. The capacity to absorb antibody through the intestine was lost at an age between 170 and 200 days.

The ability of quokkas to mount cell mediated immune reactions has been studied using skin sensitizing chemicals and skin allografts. Turner et al. (1972) demonstrated delayed hypersensitivity development in animals skin-painted with 2,4-dinitrofluorobenzene. First set skin allografts survived for 8 to 14 days on adult animals, the survival time on animals aged 50 days to one year was 14 to 21 days (Yadav et al., 1974). Juvenile animals, either cervically or totally thymectomised neonatally, rejected allografts as rapidly as did control animals. An explanation put forward for the failure of neonatal thymectomy to induce immunological defects, in contrast to that observed in murine experimental systems (Miller and Osoba, 1967), was that in the absence of the thymus, other lymphoid tissues may replace its function or that a milk-borne substance might maintain the development of lymphoid tissue (Yadav et al., 1974).

Little information is available on the immune capabilities of the Australian brush-tailed opossum, Trichosurus vulpecula. Trichosurus belongs to the family Phalangeridae (Stonehouse and Gilmore, 1977) and is phylogenetically more advanced than the North American opossum (Young, 1962; Walker, 1964).

In contrast to Didelphis, Trichosurus has both a thoracic thymus, with two or four lobes and a pair of superficial cervical thymus lobes (Johnstone, 1898; Yadav, 1973a). In addition, it has lymph nodes, spleen and gastrointestinal lymphoid tissues (Rowlands, 1976);

histologically the lymphoid tissues of Australian marsupials resembles those of North American species (Yadav, 1973b).

The immune responses of T.vulpecula to SRBC, S.adelaide flagella and bacteriophage ØX174 has been studied (Yadav, 1973b). All three antigens induced the production of IgM and IgG antibodies. An additional low molecular weight antibody of 80,000 daltons was seen in the animals immunized with bacteriophage ØX174. The sensitivities of the IgM and IgG antibodies to 2-ME were variable, a different degree of lability being reported for each of the antigens. Serum titres to all the antigens used were higher in Trichosurus than in either quokkas or tammars.

The transmission of antibodies across the gut of suckling opossums has been studied by introducing by gavage antisera to SRBC, bacteriophage ØX174 or S.adelaide flagella (Yadav, 1971). A marked variation was seen in the ages at which young opossums lost their ability to absorb such transferred antibody: a range of ages from 98 to 145 days was recorded, a period of development coinciding with the time the young leave the pouch.

Information on the structure of Trichosurus immunoglobulins is limited. In a comparative study, Atwell and Marchalonis (1975) showed that in terms of their molecular weights, the gamma chains of T.vulpecula were similar to those of other mammals.

### 1.8 Conclusion

The potential for the development of a specific immune defense system is evident in the most primitive living vertebrates. The full exploitation of this potential, as seen perhaps in man, has been dependent on the

parallel evolution of a sophisticated lymphoid system which, in turn, has been supported by a number of accessory effector cells.

While the burgeoning of immune competence is seen most dramatically in the phylogenetic expansion of immunoglobulin classes and subclasses, it is also apparent in the development of cell mediated mechanisms. Cellular mechanisms, in the total absence of soluble effector molecules, are probably the most ancient of all body defenses. Such mechanisms show throughout vertebrate evolution a progression from the "self-non self" recognition of ascidians to the complex surveillance and histocompatibility systems of higher species.

The outline of the phylogeny of vertebrate immunity presented here has attempted to capture some of the highlights of this evolutionary sequence.

## CHAPTER 2

### ISOLATION AND PARTIAL CHARACTERIZATION OF IMMUNOGLOBULINS

#### 2.1 Introduction

T.vulpecula responds to immunization with SRBC or S.adelaide flagella by producing antibodies analogous to eutherian IgM and IgG; when bacteriophage ØX174 is used as antigen an additional low molecular weight antibody is produced (Yadav, 1973b).

This chapter describes the isolation of IgG, IgM and secretory IgA from non-immunized opossums and presents, for the first time, some physical properties of these immunoglobulins.

#### 2.2 Materials and Methods

##### a. Animals

Opossums were trapped from the wild. They were housed individually in wire-mesh cages and fed a diet of soaked seed peas, supplemented with fruit and vegetables and water ad libitum. Adult opossums with a mean body weight of 2.45 kg were used in the experiments.

##### b. Collection of blood and intestinal fluid

Blood was collected from anaesthetised animals. The animal to be anaesthetised was placed in a stainless steel container fitted with a clear Perspex lid. The container was gassed with a mixture of halothane (Fluothane, ICI<sup>\*</sup>) and oxygen. When the opossum became recumbent it was removed from the container and the anaesthesia was maintained using a nose cone.

\* A list of suppliers is given in Appendix I.

Blood was collected by cardiac puncture into Vacutainers (Becton-Dickinson) and allowed to clot at room temperature for 1 hr before being held overnight at 4°C to allow retraction of the clot. The serum was then collected and centrifuged at 750 g for 15 min and stored at -20°C. Serum pools were established, each pool contained equal volumes of at least 5 sera.

Intestinal fluids were collected from animals that had been starved for 24 hr, anaesthetised and killed by exsanguination. The small intestine was divided into two or three segments each of which was flushed with cold Tris-HCl-NaCl buffer, pH 8.0, 0.1M (Appendix II). The washings were pooled, centrifuged, concentrated by dialysis against polyethylene glycol (PEG, molecular weight, 20,000 daltons, Baker) and stored at -20°C.

### c. Isolation of IgG

#### i) Rivanol precipitation techniques

Two methods, based on the precipitation of human serum proteins by Rivanol (2,ethoxy-6,9 (7,10) diamino acridine lactate, Calbiochem), were used to isolate IgG from opossum serum.

As described by Rejnek et al. (1973), four volumes of 0.4 per cent Rivanol in water were mixed with one volume of pooled sera. The pH of the mixture was adjusted to between 7.6 and 7.8 with 1N HCl and the precipitate that formed was removed by centrifugation. The supernatant fluid was freed of Rivanol by passage through a Sephadex G-25 (Pharmacia) column (15cm x 2cm). Sodium sulphate was then added to a final concentration of 20 per cent. The resulting precipitate was deposited by centrifugation, dissolved in a minimal amount of phosphate buffer, pH 8.0, 0.03M (Appendix II) and dialysed over-

night against this buffer. Aliquots were then chromatographed on a diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman) column (15cm x 2cm) equilibrated with the phosphate buffer. A step-wise elution using pH 8.0 phosphate buffers of 0.03, 0.04, 0.05 and 0.1M was used at a flow rate of approximately 40 ml per hour. Seven to 10 ml fractions were collected, the optical densities of each being monitored at 280 nm. The fractions were pooled around obvious protein peaks, concentrated by dialysis against PEG and stored at  $-20^{\circ}\text{C}$ . The pooled fractions were analysed by immunoelectrophoresis (IEP) using rabbit antisera against opossum serum (see below).

In the alternative technique (Heide and Schwick, 1978) 10 ml of pooled sera were diluted with 6.9 ml of distilled water and mixed with 6.5 ml of 3 per cent Rivanol in water, the pH being adjusted to 7.5 with 1N HCl. The precipitate that formed was removed by centrifugation and the Rivanol removed from the supernatant by precipitation with sodium chloride added to give a final concentration of 5 per cent. Following centrifugation the IgG in the supernatant was precipitated with 50 per cent saturated ammonium sulphate. This precipitate was deposited by centrifugation, dissolved in distilled water and the ammonium sulphate precipitation repeated. The second precipitate was dissolved in 2 ml of distilled water and dialysed, firstly against 0.15M sodium chloride overnight and secondly, against 0.03M phosphate buffer, pH 8.0 overnight. This material was chromatographed on DEAE-cellulose using phosphate buffers of 0.03M and 0.05M, each of pH 8.0 for elution. The fractions were collected and analysed by IEP.

#### ii) Protein A-Sepharose CL-4B fractionation

The method used was that described by Ey et al. (1978). 1.5 mg of Protein A-Sepharose CL-4B (Protein A-Sepharose, Pharmacia) were rehydrated in distilled water.



The swollen gel was then equilibrated with PBS, pH 7.2, 0.01M, in a 10 ml syringe barrel. Five ml amounts of pooled sera were always used. Following the elution of unbound proteins with phosphate buffer, pH 8.0, 0.15M, a step-wise elution was carried out using 0.1M sodium citrate-citric acid buffers with pH values of 6.0 and 3.5 (Appendix II). The fractions were collected and analysed by IEP as previously described.

d. Analysis of IgG isolates for subclasses

Results will be presented which show that two forms of IgG were isolated from opossum serum. These forms, designated IgG.SA and IgG.LA, were recognised by differences in their elution characteristics from both DEAE-cellulose and Protein A-Sepharose columns, and consequently, in their electrophoretic mobilities. These two IgGs were examined for antigenic differences. An anti-serum to each was prepared in rabbits (as described below) and cross-absorbed with the heterologous IgG type. The absorbed antisera were then used in agar diffusion against the two isolates. The sources of the two IgGs examined are detailed below as are the methods of antisera absorption and analysis.

e. Isolation of IgM

Five, 5 ml amounts of pooled sera were fractionated on 90cm x 5cm columns of Sephadex G-200 using Tris-HCl-NaCl buffer, pH 8.0, 0.1M, for elution. The optical densities of the fractions were monitored at 280 nm. The leading halves of the first peaks were pooled, concentrated by dialysis against PEG and dialysed overnight against veronal buffer, pH 8.6, 0.05M (Appendix II). This material was then subjected to starch block electrophoresis (Osterland, 1968). In this technique 300 gm of

starch (Sigma) that had been washed with distilled water were equilibrated with barbital buffer, pH 8.6, 0.05M, and poured into a 40cm x 15cm x 0.5cm Perspex mould. The ends of the mould were closed with layers of filter paper strips which also served to remove excess fluid from the starch block. The mould was covered with polyethylene film to control drying and equilibrated at 4°C overnight.

Four to 6 ml amounts of the Sephadex G-200-derived material were applied to a trough in the block, the trough being cut 10 cm in from the cathodal end. Bromophenol blue was used as the tracking dye. The inner electrode vessels contained phosphate buffer, pH 7.5, 0.2M, the outer vessels contained 0.05M barbital buffer, pH 8.6. Electrophoresis was performed at 300 volts for 40 hr at 4°C.

After electrophoresis, 1 cm strips of starch were cut from the blocks and each strip was placed in 4 ml of 0.15M sodium chloride. The protein-containing supernatants were collected following centrifugation and the optical density of each measured at 280 nm.

#### f. Isolation of secretory IgA (sIgA)

Intestinal fluids, collected from 5 animals, were individually concentrated by dialysis against PEG and dialysed overnight against Tris-HCl-NaCl buffer, pH 8.0, 0.1M. These materials were then chromatographed on Sephadex G-200. Following concentration against PEG, fractions containing sIgA were identified by IEP using an antiserum specific for sIgA (see below).

#### g. Lyophilisation of immunoglobulins

Immunoglobulins in buffer solutions were dialysed against 0.05M ammonium bicarbonate for 48 hr. One ml

amounts of either the dialysed materials or the ammonium bicarbonate solution were placed in 2 ml. freeze drying ampoules, frozen at  $-80^{\circ}\text{C}$  and freeze-dried under vacuum for 48 hr.

The ampoules were weighted when empty and after freeze-drying, the difference in weight being a measure of the weight of the immunoglobulin. Lyophilised IgG (IgG.LA, defined below) was reconstituted with water and the optimal densities of samples of known protein concentration measured at 280 nm: these values were used to construct a standard curve from which the concentrations of other IgG solutions could be calculated.

#### h. Production of immunoglobulin class specific antisera

Antisera to IgG and IgM were prepared in rabbits by injecting 5 mg of antigens intramuscularly on three occasions at two week intervals. For primary injections the antigen, in saline, was incorporated in Freund's complete adjuvant (Difco) and for subsequent injections in Freund's incomplete adjuvant (Difco). The isolation of the IgG and IgM used for immunization is described above. Antisera to IgG were prepared against:

IgG.SA: This was obtained by Protein A-Sepharose chromatography using sodium citrate-citric acid buffer, pH 6.0, 0.1M. This material is seen as Fraction 2 in Fig. 2.4b.

IgG.LA: This was obtained following the Sephadex G-200 filtration of Fraction 3 of Fig. 2.4a and is seen in well 2, Fig. 2.5.

These antisera were absorbed with lyophilised IgM and sIgA-rich fractions of intestinal fluid until monospecific as judged by IEP against a rabbit anti-opossum serum antiserum.

Antisera to IgM were prepared using as antigen Fraction 1 obtained by starch block electrophoresis of Sephadex G-200-derived material (Fig.2.8a). Anti-IgM antisera were absorbed with lyophilised IgG and sIgA - rich fractions until monospecific as judged by agar diffusion and IEP analysis against a rabbit anti-opossum serum reagent.

Rabbit antisera to sIgA were prepared by the method of Coe (1972b). sIgA in concentrated intestinal washings was precipitated by the addition of non-absorbed rabbit anti-opossum IgG (i.e., anti-IgG, -Fab, -Fc). The precipitated material was washed three times with cold PBS, pH 7.2, 0.01M (Appendix II), resuspended in 0.15M NaCl and emulsified with equal volumes of Freund's adjuvants and used to immunize rabbits by the schedule described above. The resulting antisera were absorbed with lyophilised IgG until monospecific.

All absorptions were carried out by adding the appropriate antigen to an aliquot of antiserum and incubating the mixture at 37°C for 1 hr. Any precipitate that formed was removed by centrifugation and the absorbed antiserum re-tested for specificity by IEP.

Antisera to whole opossum serum were prepared in rabbits by injecting 100 µl amounts of opossum serum intramuscularly in Freund's adjuvants using the immunization schedule described above.

#### i. Immuno-electrophoresis (IEP)

IEP was performed by the method of Scheidegger (1955) using 1.2 per cent Ion-agar (Difco) in veronal buffer, pH 8.6, 0.05M (Appendix II). Electrophoresis was carried out at 100 volts at room temperature for 4 hr. Forty eight hr after the addition of antiserum the

plates were washed for 48 hr in frequent changes of 1.5 per cent sodium chloride solution, dried in contact with filter paper at 37°C and stained with Amido Black (Williams and Chase, 1971), (Appendix II).

j. Agar diffusion

Agar diffusions were performed in 1.2 per cent Ion-agar in 0.15M sodium chloride containing 0.01 per cent sodium azide. Agar diffusion plates were washed, dried and stained as described for IEP.

k. Measurement of serum levels of IgG and IgM

The serum levels of IgG and IgM were measured by the method of Fahey and McKelvey (1965). Gels consisting of 1.5 per cent Ion-agar (Difco) in PBS were prepared. The gels contained the appropriate anti-immunoglobulin antiserum at a concentration shown to be optimal by preliminary experiments. Wells were cut in the agar and charged with 5 µl of either immunoglobulin standards of known concentration or serum samples to be tested. Standards were prepared from lyophilised material to give concentrations ranging from 2.5 to 20 mg per ml for IgG and from 0.25 to 2 mg per ml for IgM. The plates were then left for 24 hr at room temperature in a humid atmosphere. After this time the diameter of the ring of precipitation could be measured directly with a X5 lens. Alternatively, the agar gels were washed, dried and stained as described for IEP plates and the diameter of the stained precipitate measured. A standard curve was prepared for each of the two immunoglobulin classes in which the ring diameter (in mm) was plotted against the standard immunoglobulin concentrations (in mg per ml).

## 1. Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Isolated immunoglobulins were checked for homogeneity by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970; Laemmli and Faure, 1973). The separation gel consisted of 8 per cent acrylamide (BDH) in 0.75M Tris-HCl buffer, pH 8.8 (Appendix II) containing 0.1 per cent SDS; the stacking gel consisted of 3 per cent acrylamide in 0.125M Tris-HCl, pH 6.8 (Appendix II) and also contained 0.1 per cent SDS. The gels were formed in 8.5cm x 0.7cm glass tubes.

Electrophoresis was carried out at 4mA per tube, the running buffer being Tris-Glycine (0.025M Tris, 0.192M Glycine), pH 8.4, containing 1 per cent SDS (Appendix II). Bromophenol Blue, at a concentration of 0.005 per cent, was used as a tracking dye. After electrophoresis the gels were stained overnight with Coomassie Brilliant Blue (Appendix II) and destained with repeated changes of methanol-acetic acid-water (5:1:5) until the gel background was clear.

### m. Molecular weight estimations

SDS-PAGE was also used to estimate the molecular weights of the isolated immunoglobulins and of their heavy and light chains by the combined methods of Weber and Osborn (1969), Davies and Stark (1970) and Laemmli (1970). Sample buffer, containing Bromophenol Blue, and gel buffer were prepared (Appendix II). For proteins with expected molecular weights of 10,000 to 70,000 daltons, 10 per cent gel was used, whereas 5 per cent gel was used for proteins with expected molecular weights of 70,000 to 280,000 daltons. Bovine serum albumin (m.w. 66,000), ovalbumin (m.w. 45,000), pepsin (m.w. 34,000),

trypsinogen (m.w. 14,300) and haemocyanin polymers (with m.w.s of 70,000, 140,000, 210,000 and 280,000) were used as molecular weight markers. These were obtained as kits (MW-SDS-70 Kit and MW-SDS-280 Kit, Sigma).

IgG.LA and IgM were reduced with 2-mercaptoethanol (2-ME) to light and heavy chains by the method of Weber and Osborn (1969) and analysed by SDS-PAGE. Immediately prior to PAGE, the immunoglobulins, at a concentration of 1 mg per ml, were incubated at 37°C for 2 hr in 0.01M sodium phosphate buffer, pH 7.0 (Appendix II), containing 1 per cent SDS and 1 per cent 2-ME.

Electrophoresis was carried out at 8 mA per tube for 5 to 6 hr. The gels were stained with Coomassie Blue as described above. The relative mobilities of the marker proteins were calculated by dividing the distance of protein migration by the distance of tracking dye migration. The migration distance of a broad or diffuse band was taken as the mean of the migration distances of the leading and trailing edges of the band. The relative mobilities were plotted against the known molecular weights of the markers on semi-logarithmic paper; the molecular weights of the immunoglobulins and their component chains were determined from this graph.

#### n. Carbohydrate analyses

The hexose, hexosamine, fucose and sialic acid contents of IgG.LA and IgM were estimated by colourimetric methods. Gravimetrically determined amounts of lyophilised immunoglobulins were dissolved in distilled water to give known protein concentrations of between 1 and 2 mg per ml.

## Hexose

Hexose was estimated by the combined methods of Lustig and Langer (1931) and Weimer and Moshin (1952). Two hundred  $\mu$ l of immunoglobulin solution were mixed with 1 ml of 0.1N NaOH. A blank containing 1 ml of water and a standard containing 1 ml of an equal mixture of galactose and mannose solutions, each at a concentration of 0.1 mg per ml, were prepared. 8.5 ml of orcinol- $\text{H}_2\text{SO}_4$  reagent (Appendix I.I) was added to each tube. The tubes were closed with glass marbles and placed in an 80°C water bath for 15 min. They were then cooled in water and the optical densities of the solutions measured at 540 nm. The hexose contents of the immunoglobulins were estimated from the optical density of the standard, the results being expressed as mg of hexose per 100 mg protein.

## Hexosamine

The method of Rimington (1940) was used. Two hundred  $\mu$ l volumes of immunoglobulin solution were mixed with 2 ml of 3N HCl and hydrolysed in a boiling water bath for 4 hr, the tubes being fitted with air condensers. The hydrolysed samples were neutralised with 3N NaOH and diluted to 10 ml with water. One ml volumes of either the test solution, water or glucosamine (glucosamine HCl, 0.06 mg per ml) were placed in separate test tubes and mixed with 1 ml amounts of acetylacetone reagent (Appendix II). The tubes were capped with marbles and held in a boiling water bath for 15 min. They were then cooled in water and 5 ml of 95 per cent ethanol were added to each. One ml of Ehrlich's reagent (Appendix II) was added to each tube and the contents diluted to 10 ml with 95 per cent ethanol. After 30 min the optical densities were measured at 530 nm. The glucosamine contents of the



immunoglobulins were estimated from the reading of the standard and expressed as mg per 100 mg of protein.

#### Fucose

The fucose contents of the immunoglobulins were measured by the method of Dische and Shettles (1948). In duplicate test tubes 200 ul amounts of the immunoglobulin solutions were mixed with 1 ml amounts of 0.1N NaOH. One ml of distilled water and 1 ml of methyl pentose standard (fucose, 20 ug per ml) were placed in separate test tubes. 4.5 ml of ice-cold  $H_2SO_4-H_2O$  mixture (Appendix II) were added to each of the four tubes which were then held for 3 min in a boiling water bath. After cooling the tubes, 100 ul of cysteine reagent (Appendix II) was added to one of the sample tubes, 100 ul of water being added to the other. After 60 min at room temperature the optical densities were read at 396 and 430 nm with the distilled water blank being used to establish zero. The fucose content, expressed as mg per 100 mg of protein, was calculated from the difference in the readings obtained at 396 and 430 nm and subtracting the values recorded for samples lacking cysteine.

#### Sialic acid

Sialic acid was measured by the method given by Ayala et al. (1951). 4.8 ml of 5 per cent trichloroacetic acid in water were added to 200 ul amounts of immunoglobulin solutions and a sialic acid standard (0.2 mg sialic acid per ml). The tubes were capped with marbles and held in a boiling water bath for 15 min. The tubes were cooled and their contents filtered. Two ml amounts of the immunoglobulin filtrate were placed in separate tubes. One of these had 4 ml of diphenylamine reagent (Appendix II) added to it, the other had 4 ml

of a glacial acetic acid-H<sub>2</sub>SO<sub>4</sub> mixture (Appendix II) added. The filtrate of the standard solution was treated in the same way. A reagent blank consisting of 2 ml of 5 per cent trichloroacetic acid and 4 ml of diphenylamine reagent was prepared. The tubes were capped with marbles and held in a boiling water bath for a further 30 min. After cooling the tubes in water the optical densities of their contents were measured at 530 nm. The sialic acid content, expressed as mg per 100 mg of protein, was calculated from the difference in optical densities in the presence and absence of diphenylamine in order to correct for non-specific development of colour.

#### o. Amino acid analyses

The amino acid compositions of IgG.LA and IgM were determined by the method of Spackman *et al.* (1958) using an automatic amino acid analyser (Beckman, Model, 120C). The immunoglobulins were hydrolysed with 6N HCl for 24, 48 and 72 hr at  $110 \pm 1^{\circ}\text{C}$  under vacuum before being analysed. Analyses were conducted using a single column programme. The concentrations given (see Results) have been obtained by extrapolation to zero time hydrolysis. The amino acid contents are expressed for each amino acid as a percentage of the total recovered (Lisowski *et al.*, 1975).

### 2.3 Results

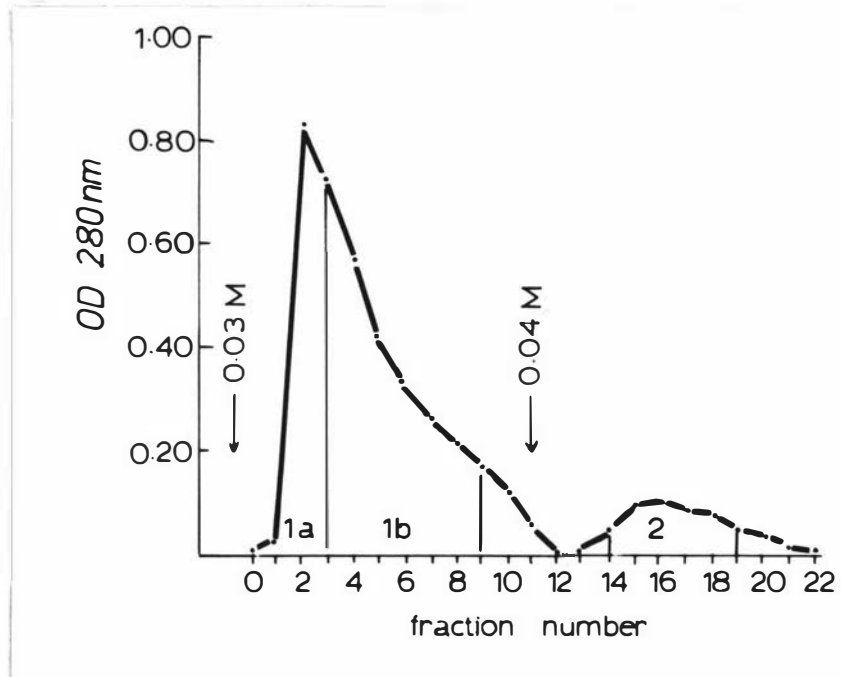
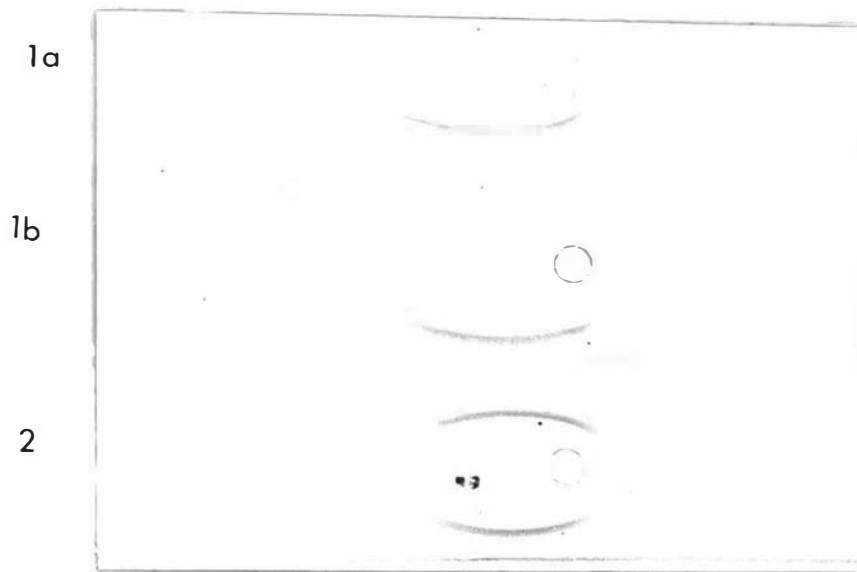
#### a) Isolation of IgG

##### i) Rivanol precipitation techniques

A DEAE-cellulose chromatography elution profile typical of those given by Rivanol-treated (Rejnek *et al.*, 1973) pooled sera is shown in Fig. 2.1a. Stepwise elution

FIGURE 2.1a. Isolation of IgG.LA. Elution profile of supernatant obtained by treating opossum serum with Rivanol (Rejnek et al., 1973). Supernatant fractionated on DEAE-cellulose. Step-wise elution using phosphate buffers, pH 8.0, 0.03M and 0.04M.

FIGURE 2.1b. IEP analysis of fractions obtained by DEAE-cellulose chromatography of Rivanol-treated serum. Wells contain fractions shown in Fig. 2.1a. Troughs contain rabbit anti-opossum serum. Cathode to left.

**2.1a****2.1b**

with pH 8.0 phosphate buffers of 0.03M and 0.04M gave two protein peaks. The IEP analysis of these peaks is shown in Fig. 2.1b, the antiserum used being rabbit anti-opossum serum. While the ascending portion (Fraction 1a) of the first peak gave a single precipitation arc in IEP, the descending portion (Fraction 1b) of this peak showed two arcs. Fraction 2, obtained by elution with 0.04M buffer also gave a single arc in IEP. The single arcs seen in Fractions 1a and 2 and the dominant arc in Fraction 1b resembled IgG in their electrophoretic mobilities. A consistent finding was the slight difference in the mobilities of Fractions 1a and 1b compared with that of Fraction 2. Fraction 1b material was chromatographed further on Sephadex G-200 using Tris-HCl-NaCl buffer, pH 8.0, 0.1M, for elution. Figs. 2.2a and 2.2b show the elution pattern obtained and the IEP analysis of the three fractions examined from the gel filtration. Fractions 1a and 1b each showed single arcs on IEP, their mobilities being characteristic of IgG. Fraction 1c showed, in addition to IgG, the arc noted in Fraction 1b (Fig. 2.1b).

Precipitation of opossum serum proteins by the Rivanol method of Heide and Schwick (1978) gave results similar to those just described. DEAE-cellulose chromatography at pH 8.0, using a stepwise elution (0.03M and 0.05M) gave two protein peaks; a representative elution profile is shown in Fig. 2.3a. IEP analysis of these peaks (Fig. 2.3b) showed that while Fraction 1a from the ascending portion of the first peak (obtained following 0.03M elution) and Fractions 2a and 2b from the second peak (obtained by elution with 0.05M buffer) each gave **double** precipitation arcs, the material in Fraction 1b gave two arcs. The dominant arc in Fraction 1b and the arcs in Fractions 1a, 2a and 2b again resembled IgG in their positions. The second arc in Fraction 1b may have been due to one of the iron-binding proteins,

FIGURE 2.2a. Isolation of IgG.LA. Sephadex G-200 chromatography of Fractions 1b (Figs. 2.1a and 2.1b). Tris-HCl-NaCl, pH 8.0, 0.1M used for elution.

FIGURE 2.2b. IEP analysis of fractions obtained by Sephadex G-200 filtration of Fraction 1b of Fig. 2.1a. Wells contain opossum serum or fractions shown in Fig. 2.2a. Troughs contain rabbit anti-opossum serum. Cathode to left.

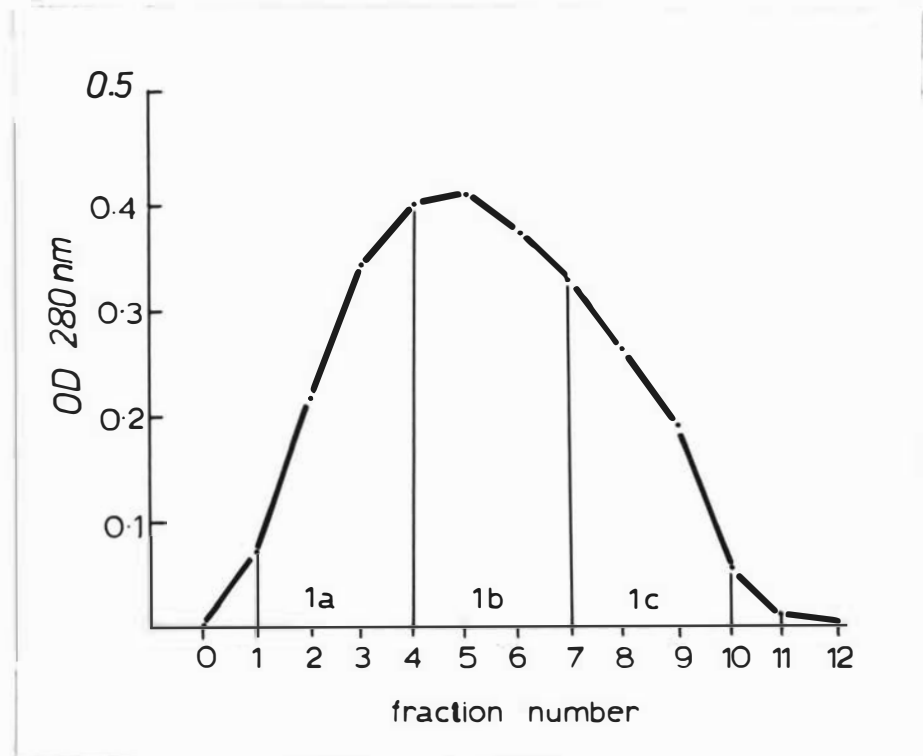
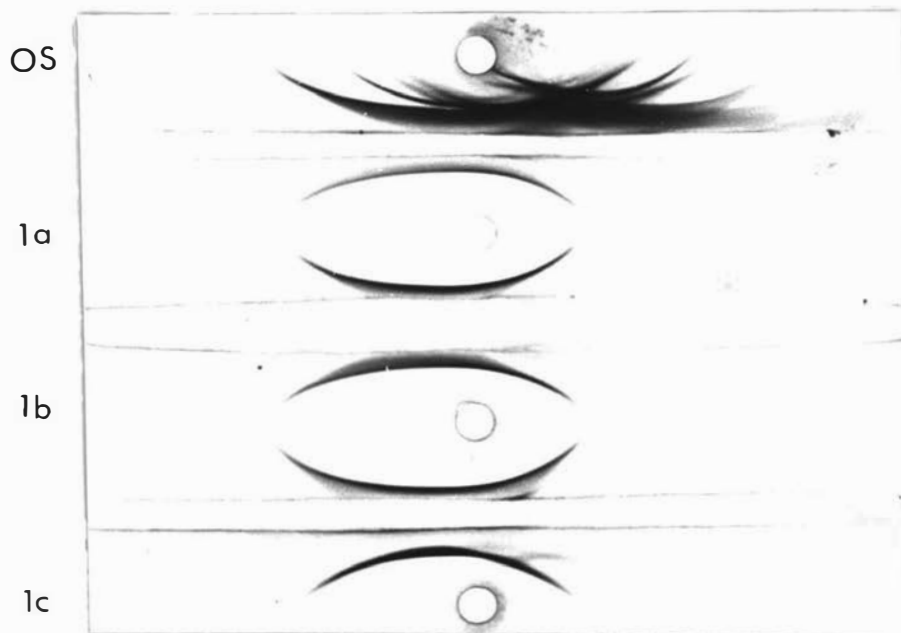
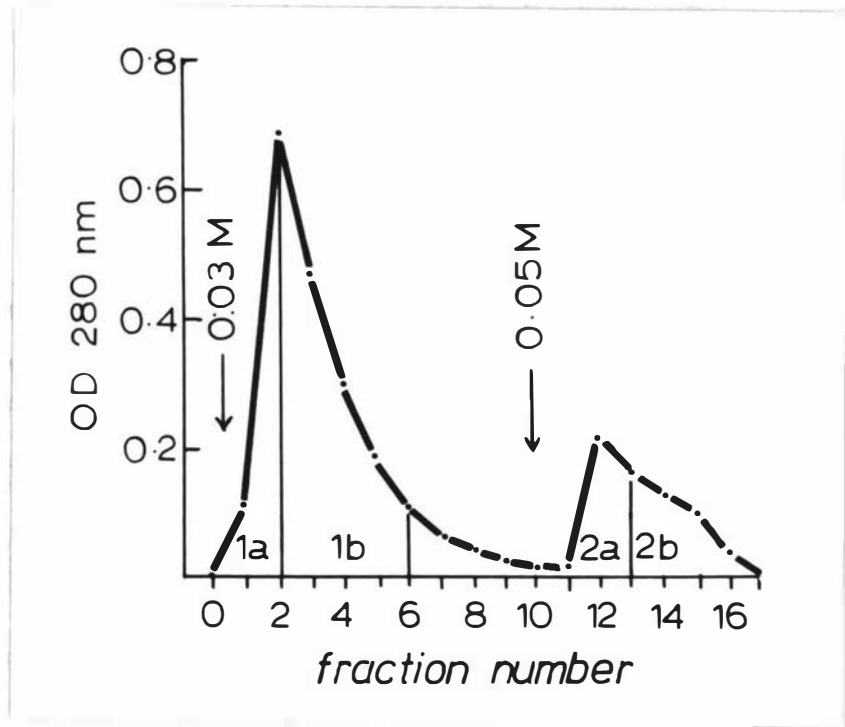
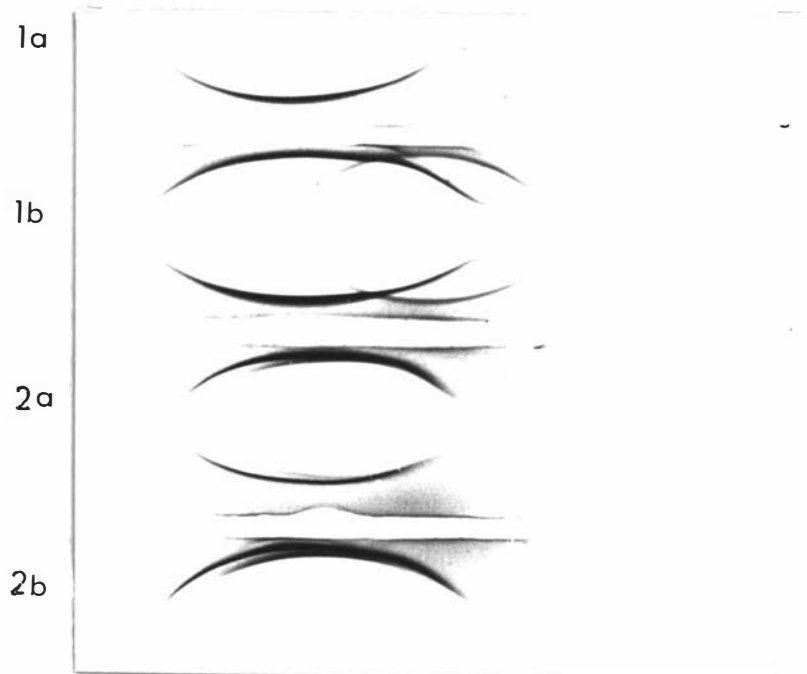
**2.2a****2.2b**

FIGURE 2.3a. Isolation of IgG.LA. Elution profile of supernatant obtained by treating opossum serum with Rivanol (Heide and Schwick, 1978). Supernatant fractionated on DEAE-cellulose. Step-wise elution using phosphate buffers, pH 8.0, 0.03M and 0.05M.

FIGURE 2.3b. IEP analysis of fractions obtained by DEAE-cellulose chromatography by Rivanol-treated serum. Wells contain fractions shown in Fig. 2.3a. Troughs contain rabbit anti-opossum serum. Cathode to left.



**2.3a****2.3b**

transferrin or haemopexin. IEP analysis of Fractions 2a and 2b showed a cathodal "split end" appearance of the precipitation arc, a common feature seen in such analyses. Fraction 1a was compared in IEP with the other immunoglobulin classes isolated. Fig.2.10a shows the homogeneity of this material and illustrates its characteristic electrophoretic mobility. SDS-PAGE analysis of Fraction 1a confirmed the purity of this isolate (Fig. 2.11).

ii) Protein A-Sepharose CL-4B fractionation

The three fractions obtained following the stepwise elution of serum proteins from Protein A-Sepharose columns with sodium phosphate and sodium citrate buffers are shown in Fig. 2.4a. The serum proteins, including some IgG, which did not bind to the column were eluted with the phosphate buffer in the first peak (Fraction 1 of Fig. 2.4b). The IgG which did not bind to the column resembled the IgG eluted in the third peak (Fraction 3) in its electrophoretic mobility on IEP (Fig. 2.4b). While the second peak (Fraction 2) appeared to contain only IgG, the third peak (Fraction 3) contained IgG, IgM and IgA. This was shown by the reaction of Fraction 2 and Fraction 3 materials in IEP against anti-opossum serum and antisera specific for opossum IgG, IgM and sIgA (Fig.2.4c). Thus Fraction 2 reacted with the anti-opossum serum and with an anti-IgG specific antisera but did not react with either anti-IgM or anti-IgA specific antisera. Fraction 3 reacted to varying intensities against all three anti-immunoglobulin antisera. The precipitation lines that formed when Fraction 2 and Fraction 3 were reacted against an anti-IgG reagent differed in their shapes and positions.

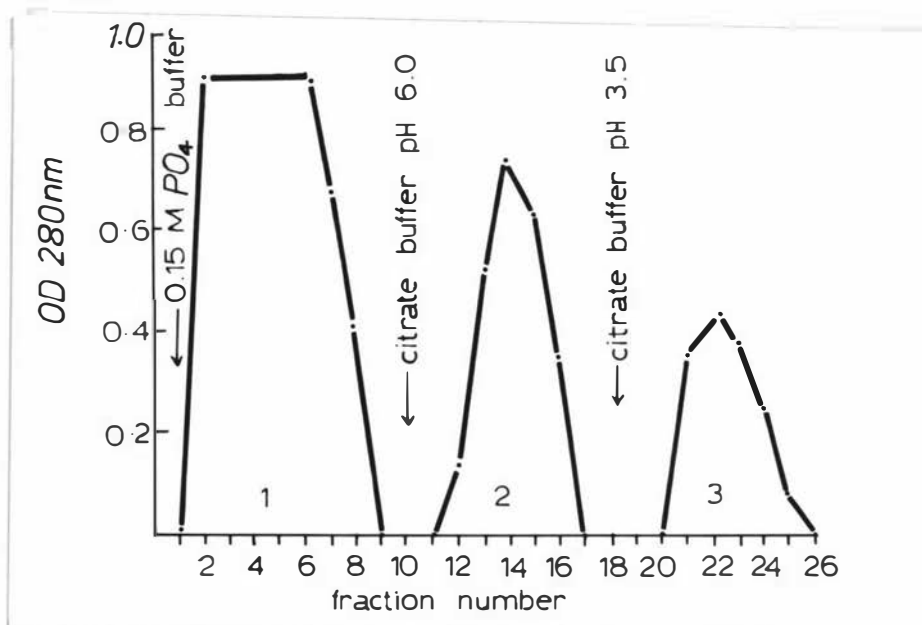
FIGURE 2.4a. Isolation of IgG.SA. Fractionation of serum on Protein A-Sepharose CL-4B columns. Unbound proteins removed with phosphate buffer, pH 8.0, 0.15M; column bound proteins removed with citrate buffers, 0.1M, pH 6.0 and pH 3.5.

FIGURE 2.4b. IEP analysis of fractions obtained by Protein A-Sepharose CL-4B fractionation of serum. Wells contain fractions shown in Fig. 2.4a. Troughs contain rabbit anti-opossum serum. Cathode to left.

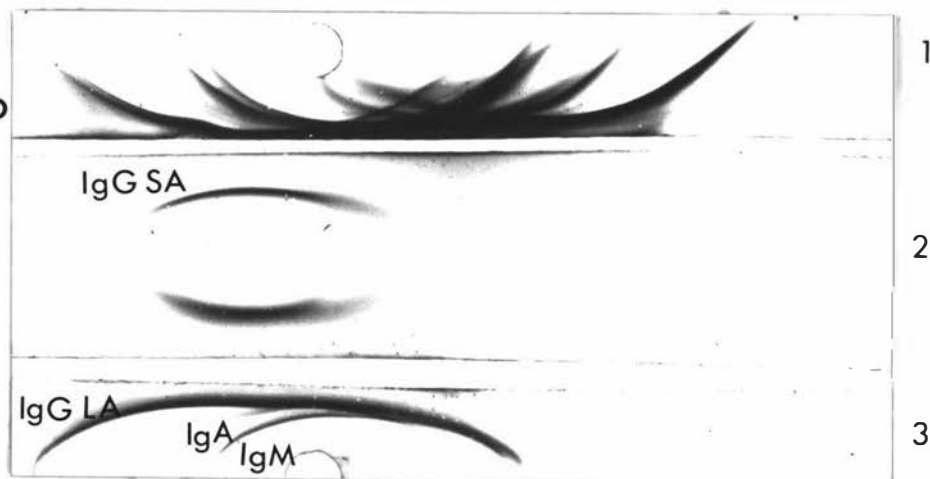
FIGURE 2.4c. IEP analysis of Fractions 2 and 3 (of Figs. 2.4a and 2.4b) obtained by the Protein A-Sepharose CL-4B fractionation of opossum serum. Wells contain fractions shown in Fig. 2.4a. Troughs contain rabbit antisera to opossum IgG.LA (A), opossum serum (B), opossum secretory IgA (C) and opossum IgM (D). Cathode to left.

(Note: Anti-IgA was made monospecific by absorption with opossum IgG.LA. Residual absorbing antigen in this antiserum has precipitated with its corresponding antibody in the anti-opossum serum reagent and formed the line of precipitation between the relevant troughs).

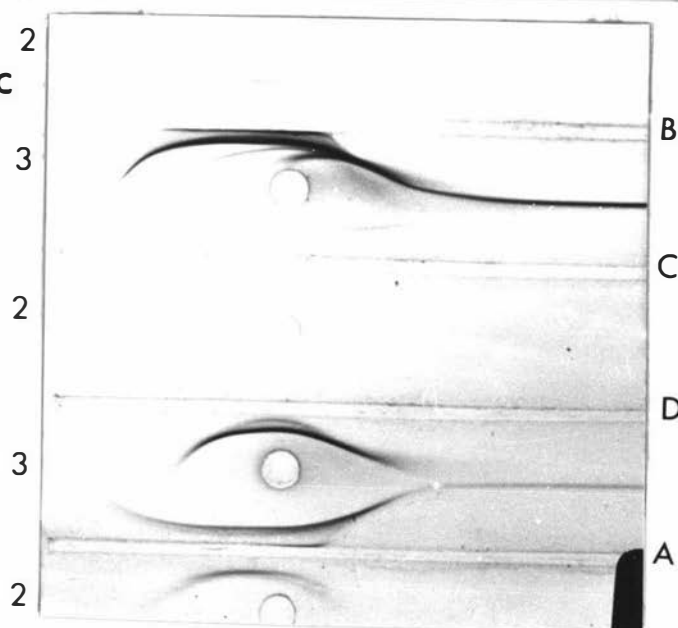
## 2.4a



## 2.4b



## 2.4c



b. Analysis of IgG isolates for subclasses

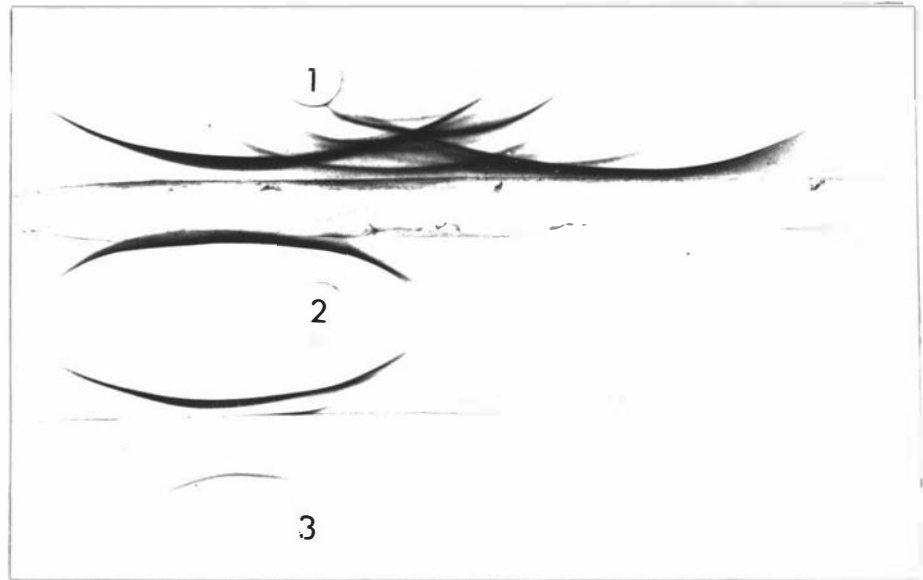
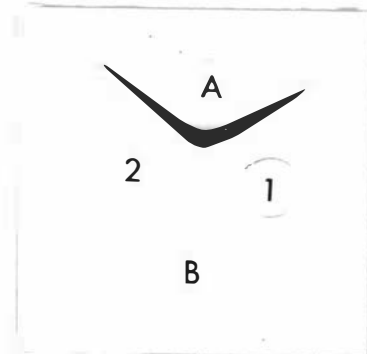
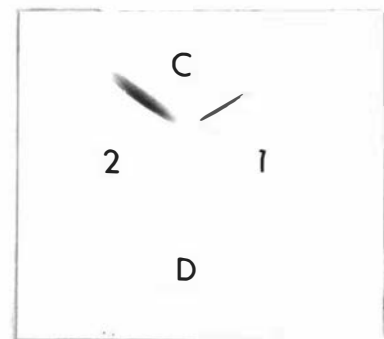
The IgG isolated either by the Rivanol/DEAE-cellulose method or by the Protein A-Sepharose fractionation procedure appeared to be of two forms which differed in their elution characteristics. This difference in binding capacity was reflected in the IgGs' electrophoretic mobilities. The two forms were designated IgG.SA (short arc) and IgG.LA (long arc). Examples of IgG.SA are seen in Figs. 2.1b (Fraction 2) and 2.4b (Fraction 2) while IgG.LA is seen in Figs. 2.1b (Fraction 1a) and 2.4b (the IgG prominent in Fraction 3). Antisera were prepared against IgG.LA and IgG.SA. The IgG.LA used as antigen was obtained by the Sephadex G-200 filtration of Fraction 3 material of Figs. 2.4a and 2.4b; the IgG.SA used was Fraction 2 of Fig. 2.4b. The electrophoretic behaviour of these materials is shown in Fig. 2.5. The IgG nature of IgG.SA had been demonstrated by its reaction with an IgG.LA-specific antiserum and its failure to react with either IgM or sIgA specific antisera (Fig. 2.4c).

Agar diffusion analyses (Fig. 2.6) showed that anti-IgG.SA reacted with both IgG.SA and IgG.LA. The reaction was one of identity, no crossing of precipitation lines or spur formation being seen. Similarly, anti-IgG.LA reacted with IgG.LA and with IgG.SA; again the pattern was one of identity. An aliquot of each antiserum was then absorbed with the heterologous lyophilised antigen (e.g. anti-IgG.SA was absorbed with IgG.LA) until agar diffusion showed a lack of any precipitating activity between the two reactants. The reactions of these absorbed antisera against both their heterologous (absorbing) antigen and their homologous antigens are seen in Fig. 2.6. Absorbed antisera lost their ability to react with their inducing antigen, a result which indicated that IgG.SA and IgG.LA were antigenically identical.

FIGURE 2.5. IEP analysis of IgG.LA and IgG.SA. Wells contain opossum serum (1), IgG.LA (2) and IgG.SA (3). Troughs contain rabbit anti-opossum serum. Cathode to left.

FIGURE 2.6 Agar diffusion analysis of non-absorbed and absorbed anti-IgG.LA and anti-IgG.SA antisera.

- a) Non-absorbed anti-IgG.LA (A) and anti-IgG.LA absorbed with IgG.SA (B) reacted against IgG.LA (1) and IgG.SA (2). Reaction of identity seen between IgG.LA and IgG.SA; lack of precipitation following absorption.
- b) Non-absorbed anti-IgG.SA (C) and anti-IgG.SA absorbed with IgG.LA (D) reacted against IgG.LA (1) and IgG.SA (2). Reaction of identity seen between IgG.LA and IgG.SA; lack of precipitation following absorption.

**2.5****2.6****a****b**

### c. Analysis of anti-IgG antisera

Antisera to IgG were prepared in rabbits as described previously and absorbed with IgM and sIgA until monospecific in IEP. Fig. 2.12a shows the reaction of such an antiserum against whole opossum serum: a single precipitation arc is seen, its position being characteristic of IgG. Agar diffusion analysis confirmed the specificity of such antisera (Fig. 2.12b).

### d. Isolation of IgM

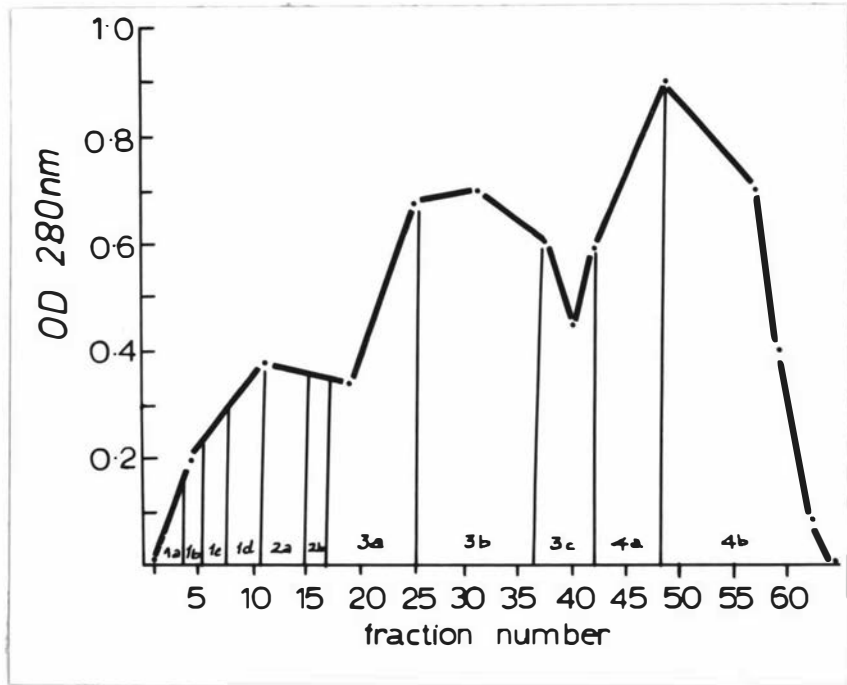
An elution profile representative of those seen following the Sephadex G-200 filtration of opossum serum is shown in Fig. 2.7a. IEP analysis of the fractions showed a distribution of serum proteins characteristic of such separations (Fig. 2.7b). Fractions from the leading edge of the first peak (i.e., Fractions 1a, 1b and 1c) contained putative IgM and a second component which resembled alpha-2-macroglobulin in its electrophoretic mobility. Such fractions from about 5 runs were pooled, concentrated against PEG and subjected to starch block electrophoresis, the result being shown in Fig. 2.8a. IEP analysis of the three fractions selected showed that Fractions 1 and 2 contained a single component, presumably IgM, whereas alpha-2-macroglobulin and traces of other proteins were confined to Fraction 3 (Fig. 2.8b). The electrophoretic behaviour of Fraction 1 material was compared in IEP with those of other immunoglobulin isolates: Fig. 2.10a shows the characteristic IgM-like position of the resulting precipitation arc. SDS-PAGE analysis (Fig. 2.11) confirmed the homogeneity of Fraction 1. The lack of penetration of this protein into the gel was a further indication of its high molecular weight.



FIGURE 2.7a. Isolation of IgM. Elution profile of opossum serum fractionated on Sephadex G-200. Tris-HCl-NaCl buffer, pH 8.0, 0.1M.

FIGURE 2.7b. IEP analysis of fractions obtained by Sephadex G-200 fractionation of opossum serum. Wells contain fractions shown in Fig. 2.7a. Troughs contain rabbit anti-opossum serum.

2.7a



2.7b

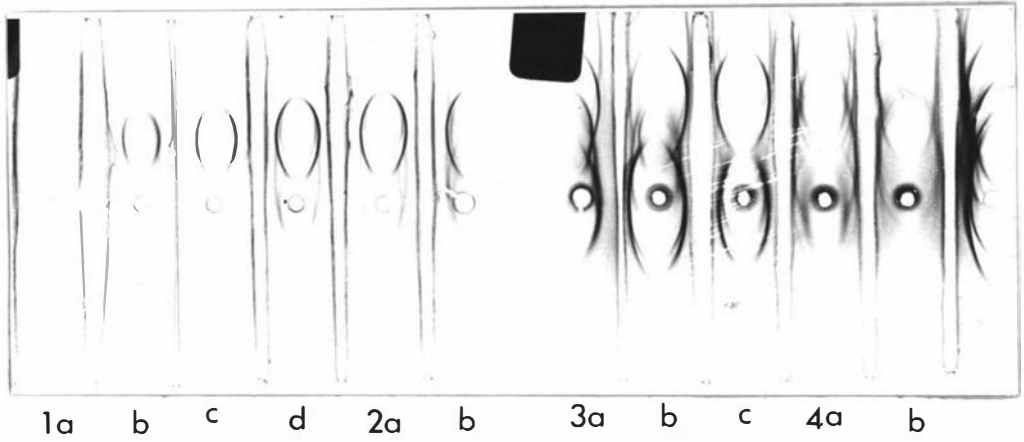
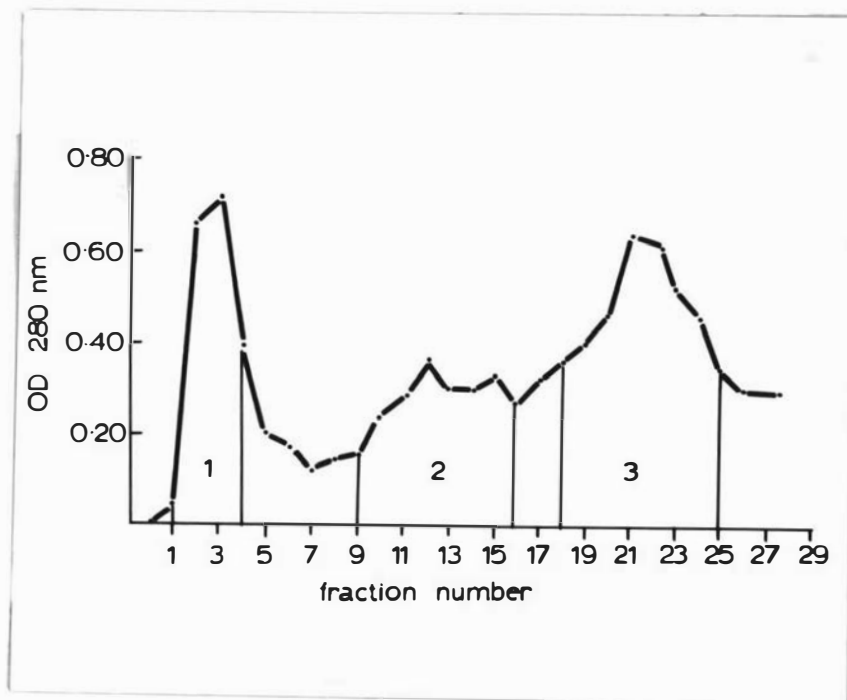


FIGURE 2.8a. Isolation of IgM. Starch block electrophoresis of IgM-enriched serum fractions obtained by Sephadex G-200 filtration.

FIGURE 2.8b. IEP analysis of starch block electrophoresis fractions. Wells contain fractions shown in Fig. 2.8a. Troughs contain rabbit anti-opossum serum. Cathode to left.

**2 - 8a****2..8b**

The IgM isolate and IgG.LA were antigenically related. This was demonstrated by their cross-reaction in agar diffusion when reacted against a rabbit anti-opossum serum (Fig. 2.10b).

e. Analysis of anti-IgM antisera

Antisera were made against starch block Fraction 1 material and absorbed with Rivanol-derived IgG.LA (Fraction 1a, Fig. 2.2b). An analysis of such an IgM-specific antiserum is shown in Fig. 2.12a in which it is reacted against whole opossum serum. A single arc in the IgM position is seen. Fig. 2.12b further shows the specificity of such antiserum in agar diffusion.

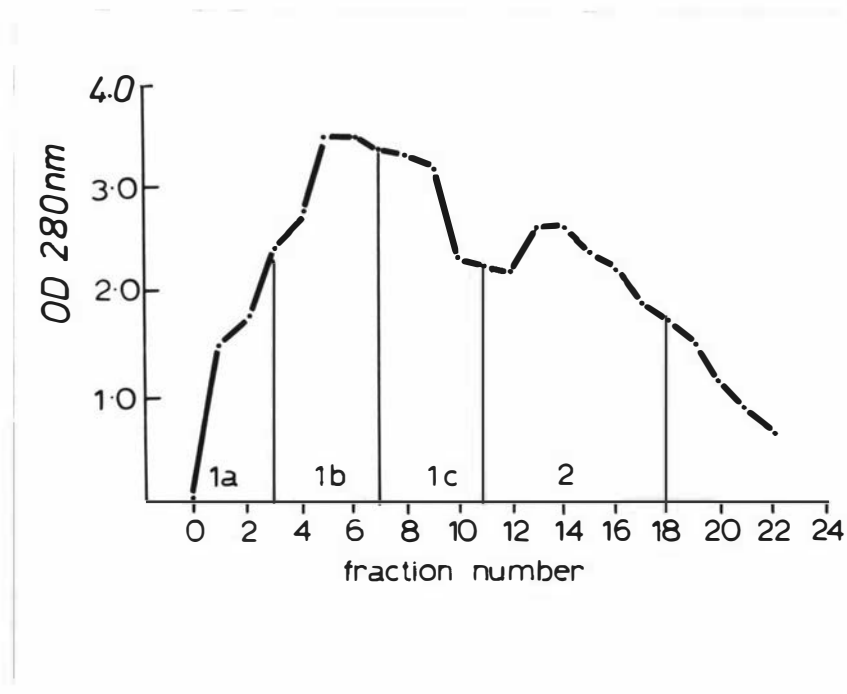
f. Isolation of secretory IgA (sIgA)

When dialysed and concentrated intestinal washings were applied to Sephadex G-200 columns and eluted with Tris-HCl-NaCl buffer, pH 8.0, 0.1M, two ill-defined peaks were seen; Fig. 2.9a is typical of the elution patterns seen. IEP analysis against rabbit anti-opossum serum showed that Fraction 1b and 1c generally gave a single precipitation arc (Fig. 2.9b). Occasionally these fractions showed traces of low molecular weight contaminants on PAGE analysis (Fig. 2.11) which were not evident in IEP. The electrophoretic mobility of the single component in Fractions 1b and 1c was different in IEP to those of the IgG and IgM isolates and resembled that of IgA (Fig. 2.10a). The immunoglobulin nature of this material was shown by its ability to cross-react with IgG and IgM in an agar diffusion (Fig. 2.10b). That this was sIgA was confirmed by its reaction with specific antisera (Fig. 2.12b). SDS-PAGE analysis of the sIgA-containing fractions showed a single intensely staining

FIGURE 2.9a. Isolation of secretory IgA (sIgA).  
Sephadex G-200 fractionation of intestinal washings. Tris-HCl-NaCl, pH 8.0, 0.1M, used for elution.

FIGURE 2.9b. IEP analysis of fractions obtained by Sephadex G-200 fractionation of intestinal washings. Wells contain fractions shown in Fig. 2.9a. Troughs contain rabbit anti-opossum serum. Cathode to left.

## 2.9a



## 2.9b

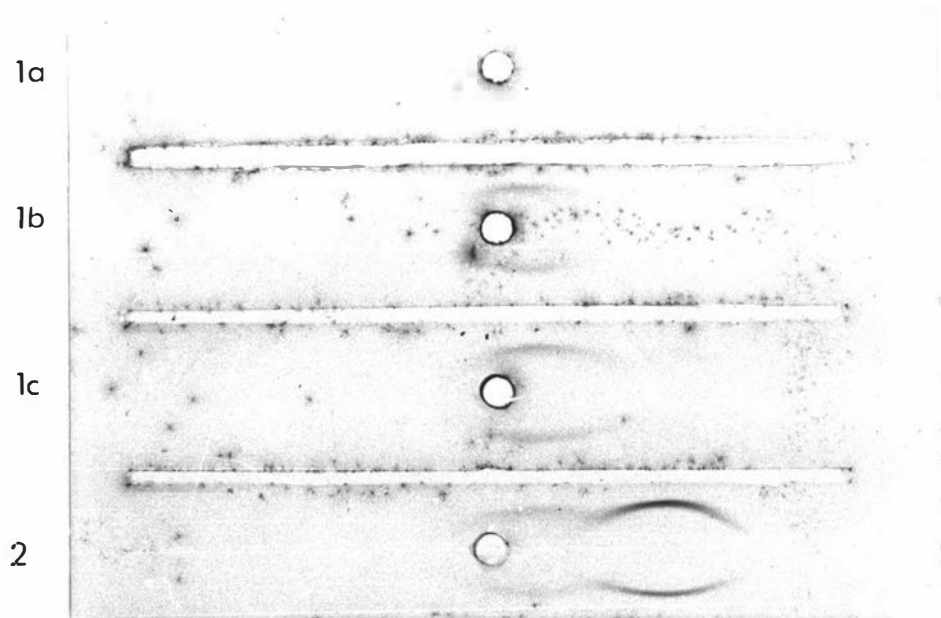


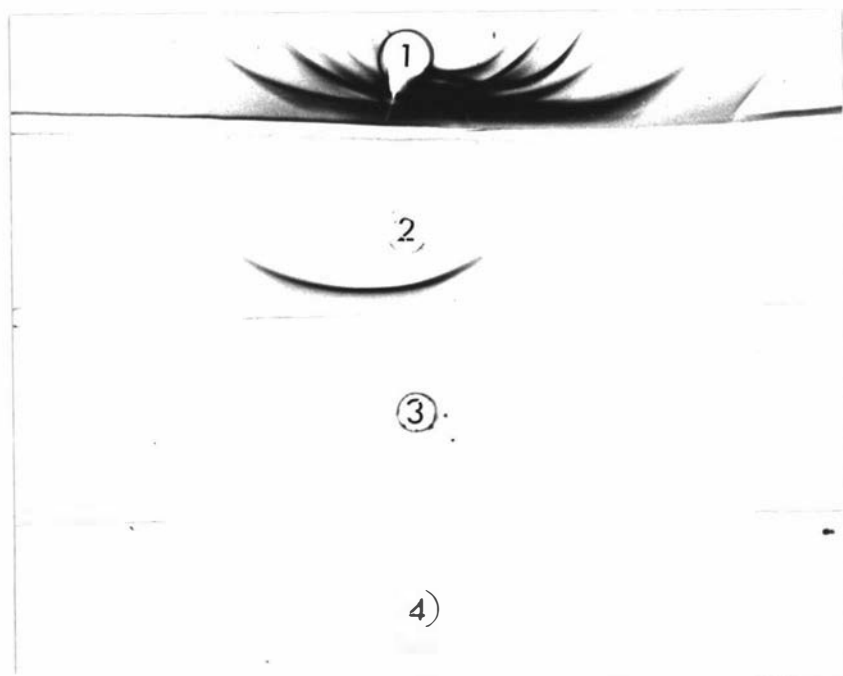
FIGURE 2.10a. Comparative IEP analysis of isolated immunoglobulins. Wells contain opossum serum (1), IgG.LA (Rivanol-derived, Fraction 1 of Fig. 2.3b) (2), sIgA (Fraction 1b of Fig. 2.9b) (3) and IgM (Fraction 1 of Fig. 2.8a) (4). Troughs contain rabbit anti-opossum serum. Cathode to left.

FIGURE 2.10b. Agar diffusion analysis of immunoglobulin isolates against anti-opossum serum. Wells contain anti-opossum serum (A), IgG.LA (1), IgM (2) and sIgA (3). Reactions of partial identity are seen.

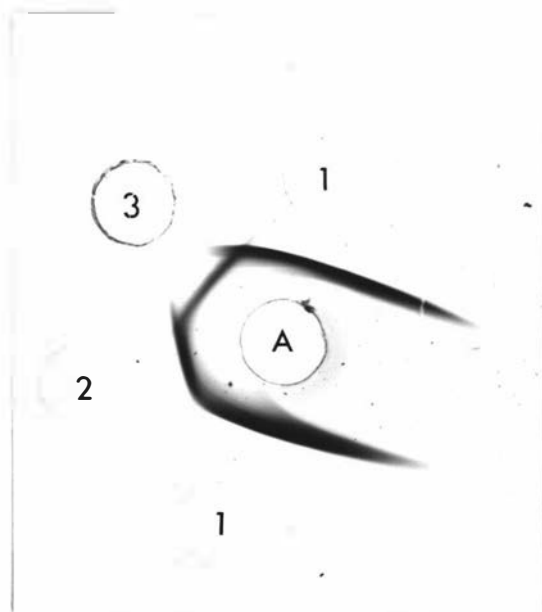
FIGURE 2.11. Comparative SDS-PAGE analysis of isolated immunoglobulins. a) IgG.LA (Rivanol-derived, Fraction 1a of Fig. 2.3b); b) IgM (Fraction 1 of Fig. 2.8a); c) sIgA (Fraction 1b of Fig. 2.9b).



2.10a



2.10b



2.11

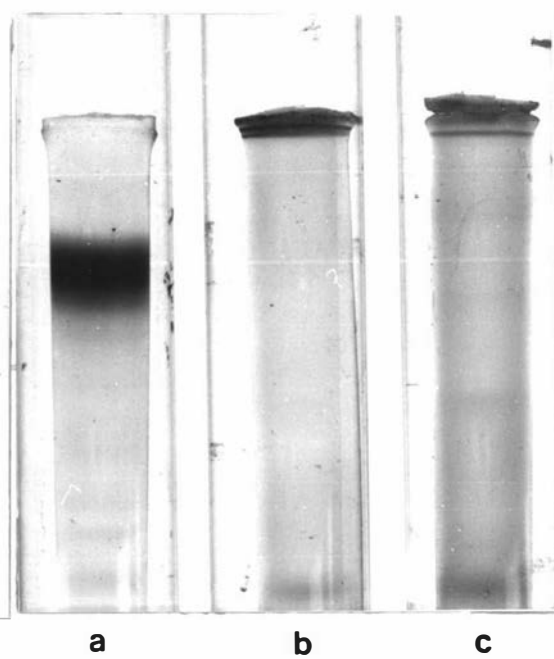


FIGURE 2.12a. IEP analysis of immunoglobulin class-specific antisera. Wells contain opossum sera. Troughs contain anti-IgG.LA (A), anti-sIgA (B) and anti-IgM (C). Cathode to left.

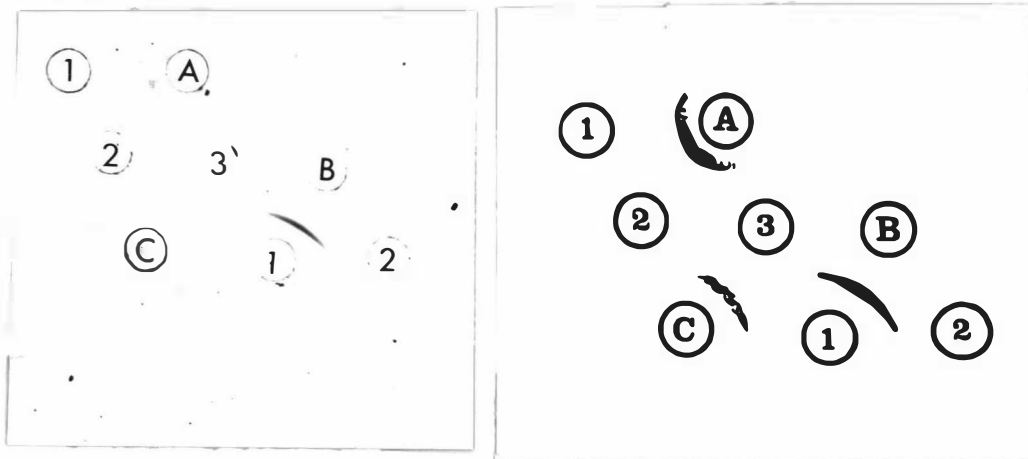
FIGURE 2.12b. Agar diffusion analysis of immunoglobulin class-specific antisera. Wells contain anti-IgG.LA (A), anti-IgM (B), anti-sIgA (C), IgM (1), IgG.LA (2) and sIgA (3).

FIGURE 2.13. Agar diffusion analysis of anti-sIgA (C) reacted against intestinal fluid (SE) and opossum serum (OS).

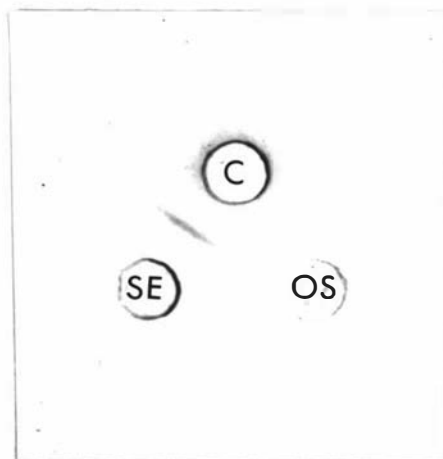
2-12a



2-12b



2-13



band with low gel penetration and up to 3 diffuse, barely discernible bands with high gel penetration (Fig. 2.11).

#### g. Analysis of anti-sIgA antisera

Antisera to sIgA were made by injecting rabbits with the precipitate which formed on adding rabbit anti-opossum IgG (non-absorbed and therefore capable of reacting with both light and heavy chains) to intestinal fluid. The antisera were absorbed with IgG.LA (Fraction 1a, Fig. 2.2b) until monospecific as judged by IEP (Fig. 2.12a) and agar diffusion (Fig. 2.12b). Fig. 2.12a shows the reaction of such an antiserum against whole opossum serum. A single arc of precipitation, formed by material having a different electrophoretic mobility from that of either IgG or IgM, is seen, its position being similar to that given by IgA in other species.

Further evidence of the ability of the anti-sIgA reagent to react with serum IgA is provided in Fig. 2.13. A line of apparent identity extends between the wells containing serum and intestinal fluid. No spur formation was seen indicating a possible lack of secretory component from the IgA isolated from intestinal fluid.

#### h. Measurement of serum levels of IgG and IgM

Fig. 2.14a shows a plot of the precipitation ring diameters formed when IgG standards were diffused in a gel containing specific anti-IgG.LA at a final dilution of 1:20. The appearance of the precipitation rings is shown in Fig. 2.14b. The plot constructed with precipitation ring diameters against IgG concentrations was used to determine the IgG levels of 27 opossum sera (Table 2.1). A mean value of 14.7 mg per ml of serum was established, the range being 13.8 to 19.0 mg per ml.

FIGURE 2.14a. A plot of the precipitation ring diameters (mm) given by standard IgG.LA concentrations in a gel containing specific anti-serum. The diameters shown are means from three experiments.

FIGURE 2.14b. Precipitation rings formed by IgG.IA standards and opossum serum IgG in single radial diffusion analysis. Top row: IgG.LA standards 2.5 to 20 mg per ml. Middle and bottom rows: serum aliquots.

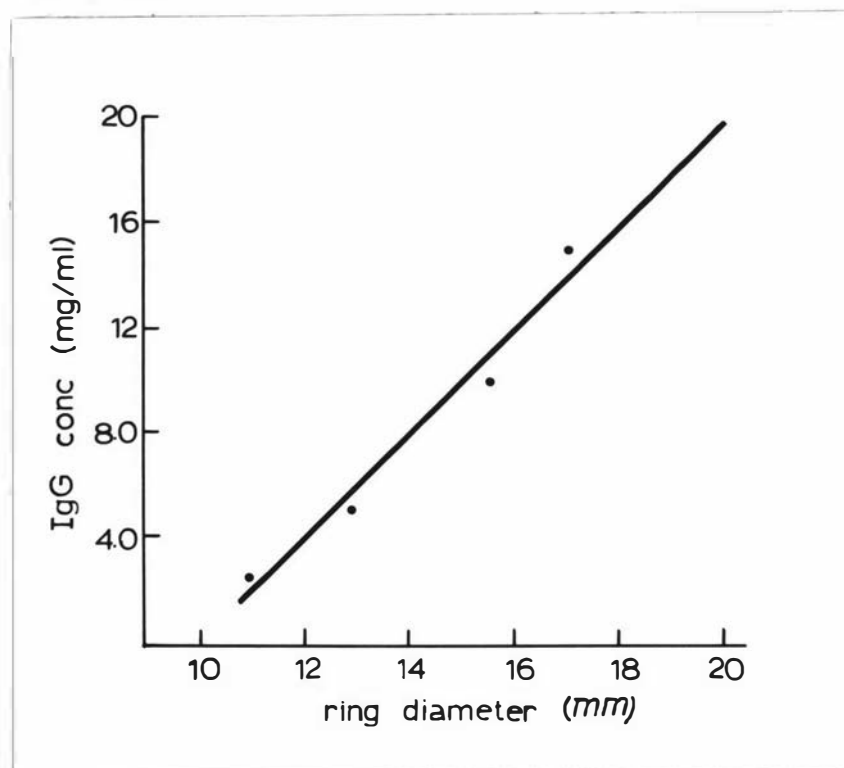
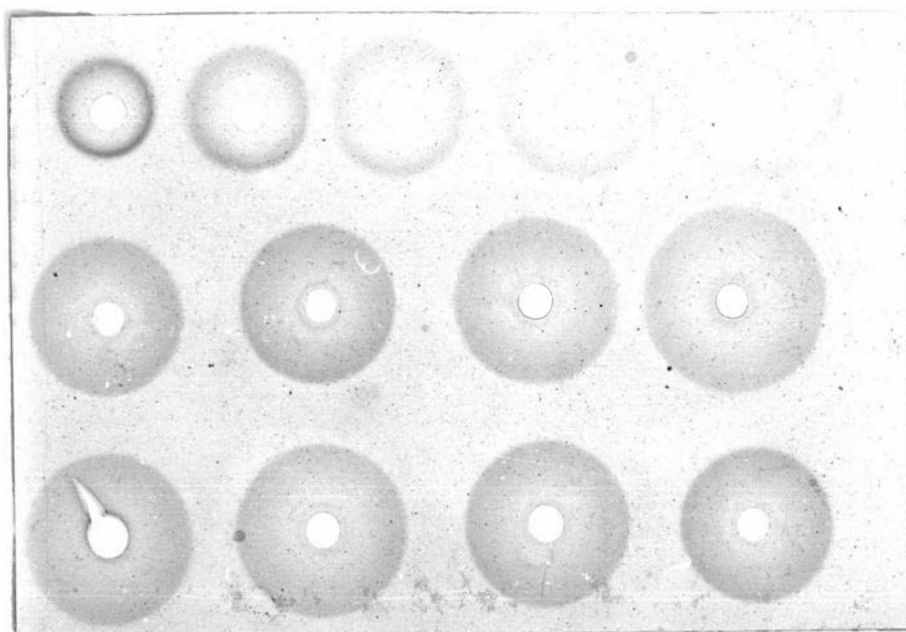
**2.14a****2.14b**

FIGURE 2.15a. A plot of the precipitation ring diameters (mm) given by standard IgM concentrations in a gel containing specific antiserum. The diameters shown are means from two experiments.

FIGURE 2.15b. Precipitation rings formed by IgM standards and opossum serum IgM in single radial diffusion analysis. Top row: serum aliquots. Bottom row: IgM standards 0.25 to 2 mg per ml.

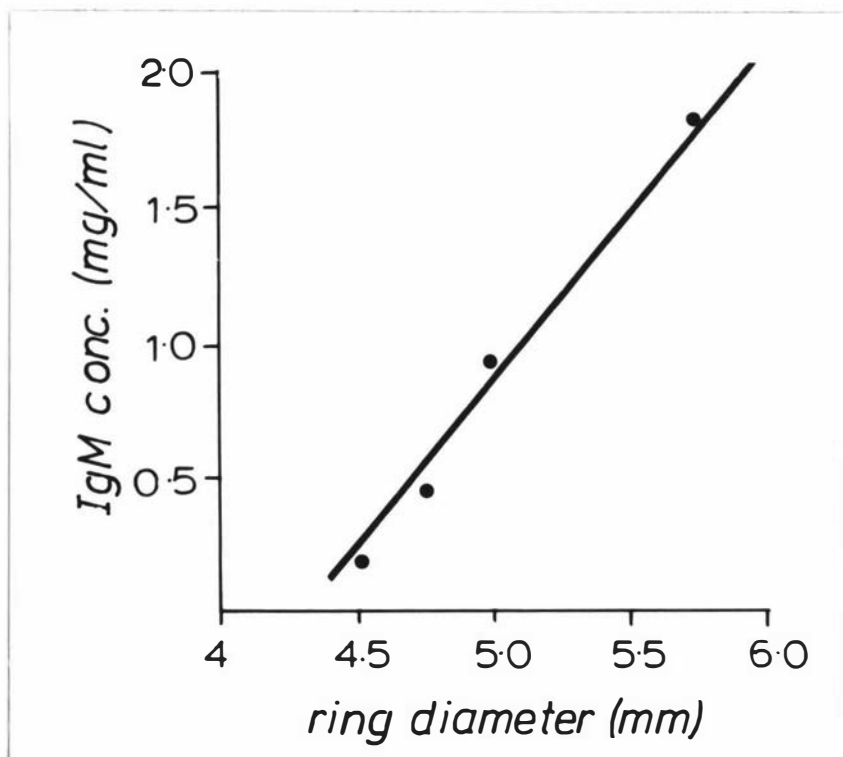
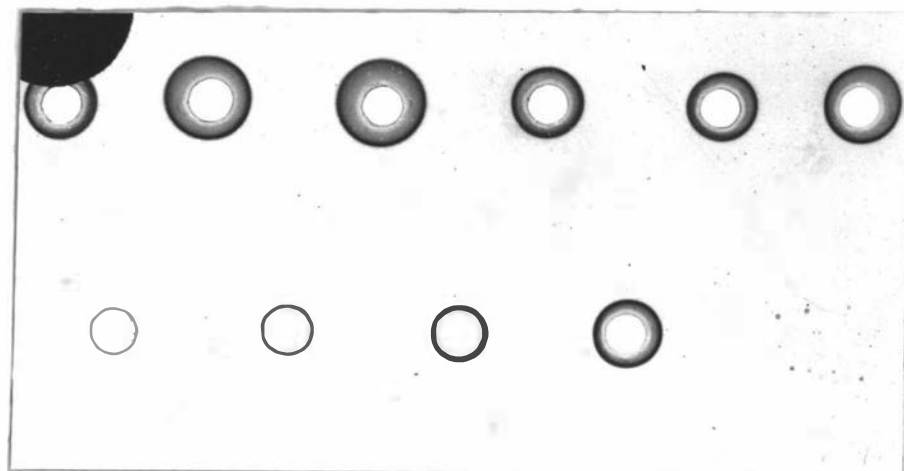
**2.15a****2.15b**



Table 2.1 IgG . LA and IgM serum levels in opossum  
determined by radial immunodiffusion analysis.

Immunoglobulin	mg per ml	
	Range	Mean $\pm$ S.E
IgG . LA	13.8 - 19.0	14.7 $\pm$ 0.4 (27) <sup>*</sup>
IgM	2.9 - 7.5	4.1 $\pm$ 0.3 (16)

\* Number in parentheses indicates number of sera examined.

The IgM serum levels of 16 animals were measured similarly (Figs. 2.15a and 2.15b; Table 2.1). A range of values from 2.9 to 7.5 mg per ml and a mean of 4.08 mg per ml of serum were recorded.

i) Molecular weight estimations

i) Intact IgG and secretory and serum IgAs

The migration in SDS-PAGE of the haemocyanin polymers used as standards is shown in Fig. 2.16a. Calibration curves of molecular weights versus relative mobilities are given for these polymers in Fig. 2.16b. These curves, constructed from the mean values from four runs, were used to calculate the molecular weights of intact IgG.LA, IgG.SA and serum and sIgAs. Two curves were plotted: the curve 'a' was plotted using the relative mobilities of 0.1 to 0.6 and was used to calculate the molecular weights of the two IgGs; curve 'b' was plotted using relative mobilities of 0.05 to 0.3, and was used to calculate the molecular weights of serum and sIgAs. Curve 'b' was constructed to allow a more precise calculation of the two IgAs by extending the relative mobility scale along the X axis.

IgG.LA was obtained by a combined Rivanol-DEAE-cellulose/Sephadex G-200 procedure, this material is seen as Fraction 1b of Fig. 2.2b. IgG.SA was obtained by Protein A-Sepharose fraction of opossum serum, this material is represented as Fraction 2 in Fig. 2.4b. The sIgA examined was derived from intestinal washings and is seen in Fraction 1c of Fig. 2.9b. The molecular weight of serum IgA, which was never satisfactorily isolated, was calculated using Fraction 3 of Fig. 2.4a. This was a Protein A-Sepharose column-derived material which, in addition to IgG and IgM, also contained IgA (Fig. 2.4c).

The position of the serum IgA migration band was deduced from knowing the migration behaviours of purified IgG and IgM. The SDS-PAGE mobilities of the immunoglobulins examined are shown in Fig. 2.16a.

Table 2.2 shows the molecular weights of the four intact immunoglobulins as determined by SDS-PAGE and also presents a molecular weight for IgM which is based on the calculated weights of its light and heavy chains (see next section), a pentameric structure being assumed. IgG.SA was found to have a molecular weight of  $150,000 \pm 4,000$  daltons, while IgG.LA had an estimated molecular weight of  $129,600 \pm 1,000$  daltons. Serum IgA and sIgA had molecular weights of  $175,000 \pm 5,000$  and  $303,800 \pm 10,000$  daltons, respectively. The molecular weight of IgM was calculated as 940,000 daltons.

ii) Heavy and light chains of IgG, IgM and IgA

A calibration curve based on the migration of standard proteins in SDS-PAGE is shown in Fig. 2.17a, the relative mobilities shown are the average from two electrophoretic runs. This curve was used to calculate the molecular weights of the heavy and light chains of IgG.SA, IgG.LA, IgM and serum and sIgA described in the previous section. The molecular weight of the alpha-chain of serum IgA was estimated from the SDS-PAGE of a mixture of IgG, IgM and IgA (Fraction 3 of Fig. 2.4b). The position of gamma and u chains in PAGE were established from analyses using reduced purified IgG and IgM; the position of alpha chain in a mixture of the three reduced immunoglobulins could then be determined. Fig. 2.17b shows the SDS-PAGE appearance of IgG.LA and IgM following their reduction to light and heavy chains.

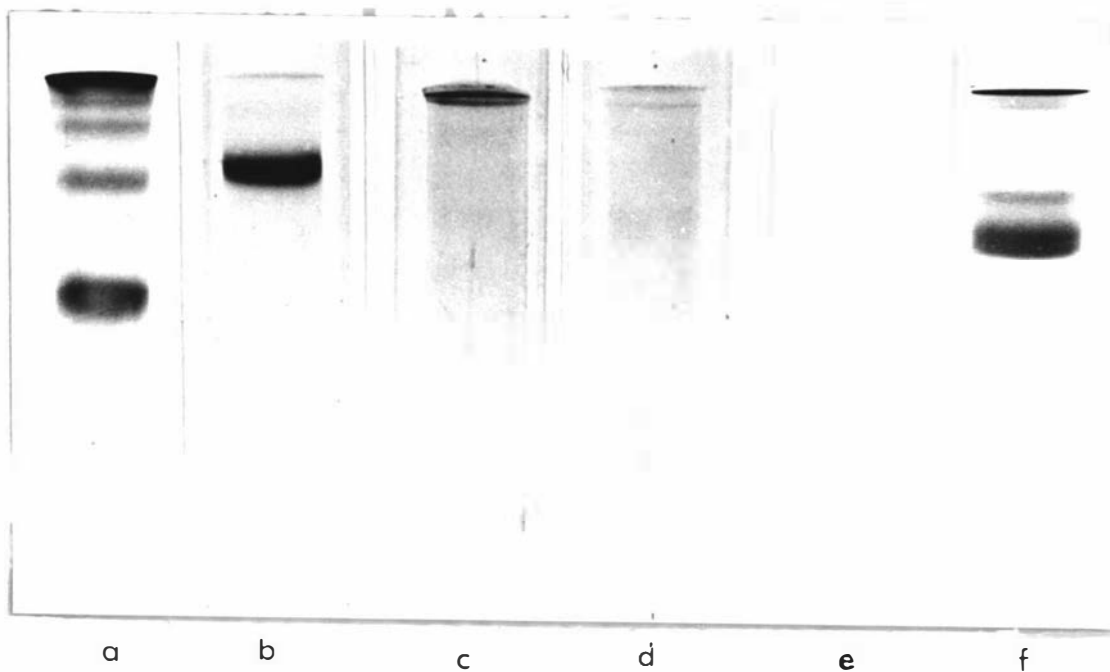
The molecular weight of the IgG.SA heavy chain was calculated to be  $50,300 \pm 500$  daltons, while the light

FIGURE 2.16a. SDS-PAGE analysis of haemocyanin polymers and isolated immunoglobulins used to determine molecular weights.

Gel a: Haemocyanin polymers;  
b: IgG.LA (Fraction 1b, Fig. 2.2b);  
c: IgM (Fraction 1a, Fig. 2.8a);  
d: sIgA (Fraction 1c, Fig. 2.9b);  
e: IgG.SA (Fraction 2, Fig. 2.4b);  
f: IgM (top), serum IgA, IgG.LA (bottom) (Fraction 3, Fig. 2.4b).

FIGURE 2.16b. A calibration curve obtained with haemocyanin polymers on SDS-PAGE. The relative mobilities shown (migration distance of protein/migration distance of tracking dye) are means from four analysis.

## 2.16a



## 2.16b

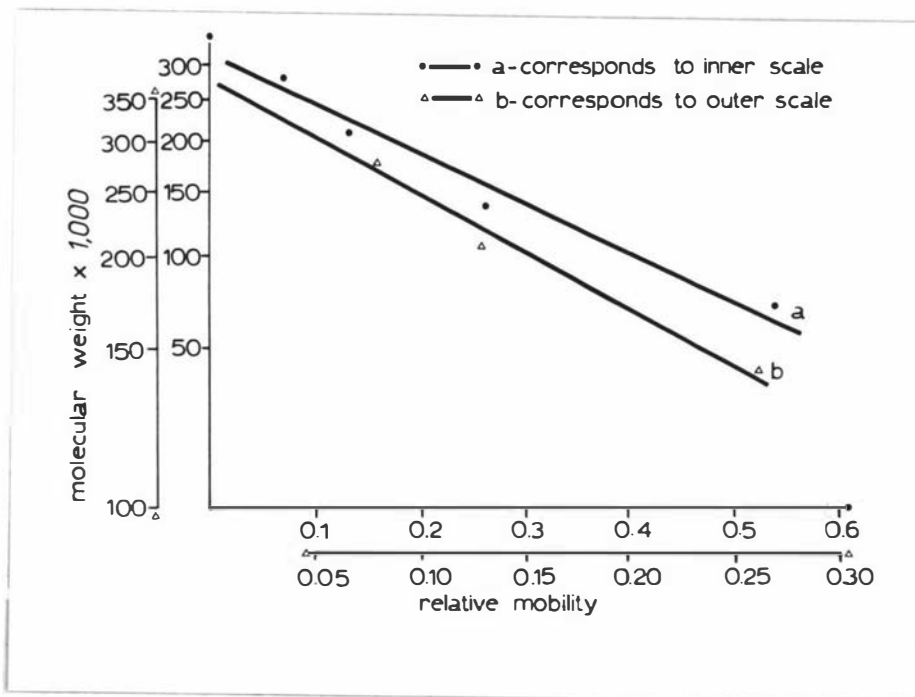


FIGURE 2.17a. A calibration curve obtained with protein standards on SDS-PAGE. The relative mobilities shown (migration distance of protein/migration distance of tracking dye) are average values from two analysis.

FIGURE 2.17b. SDS-PAGE analysis of 2-ME reduced IgG.LA (a) and IgM (b).

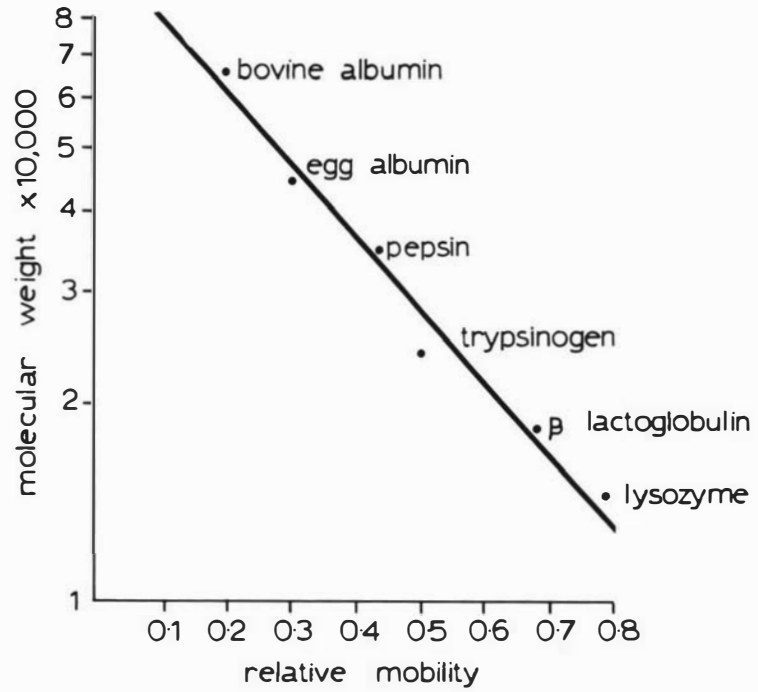
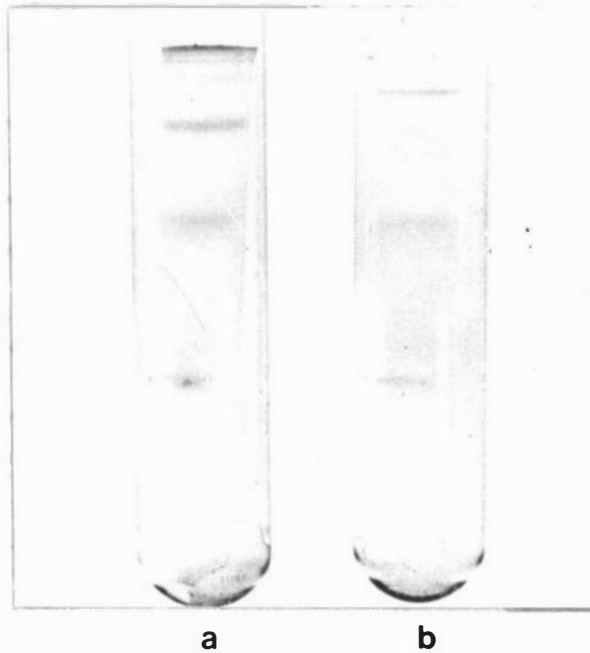
**2.17a****2.17b**

Table 2.2 Molecular weights of IgG.SA , IgG.LA and of serum and secretory IgAs determined by SDS-PAGE. The value for IgM was calculated from the molecular weights of the molecule's light and heavy chains, a pentameric structure being assumed.

Immunoglobulin	Molecular weight (daltons) <sup>a</sup>
IgG.SA	150,000 $\pm$ 4,000 ( 8) <sup>b</sup>
IgG.LA	129,600 $\pm$ 1,000 (12)
IgA, serum	175,000 $\pm$ 5,000 ( 5)
IgA, secretory	303,800 $\pm$ 10,000 ( 4)
IgM	940,000

a Results presented as mean values  $\pm$  standard error.

b Number in parentheses refers to number of estimations made.



chain of this immunoglobulin had a molecular weight of  $22,900 \pm 400$  daltons (Table 2.3). The molecular weights of the heavy and light chains of IgG.LA were  $47,900 \pm 600$  and  $21,300 \pm 500$  daltons, respectively. The IgM showed molecular weights of  $71,800 \pm 1,400$  daltons for its heavy chains and  $22,200 \pm 900$  daltons for its light chains. IgA from serum showed a molecular weight of  $58,000 \pm 2,000$  daltons for its heavy chains and  $22,800 \pm 700$  daltons for its light chains. The heavy and light chains of sIgA showed molecular weights of  $55,500 \pm 2,500$  and  $22,800 \pm 700$  daltons, respectively.

#### j. Carbohydrate analyses

IgG.LA and IgM were analysed for their carbohydrate contents, the results being given in Table 2.4. IgG.LA showed an average hexose content of 1.99 mg per 100 mg of protein. The hexosamine content of IgG.LA was found to be 0.83 mg per cent and the fucose content was 0.13 mg per cent. IgG.LA showed a sialic acid content of 1.37 mg per cent.

IgM had a somewhat higher carbohydrate content than did IgG.LA. The percentage of hexose in IgM was 3.71 mg. IgM also contained 2.56 mg per cent hexosamine, 1.03 mg per cent fucose and 1.91 mg per cent sialic acid.

#### k. Amino acid analyses

Table 2.5 shows the amino acid composition of IgG.LA and IgM. IgM has noticeably higher levels of histidine ( $\sim 5$  per cent) and arginine ( $\sim 8$  per cent) than does IgG.LA ( $\sim 2$  and  $\sim 4$  per cent, respectively) but a lower level of glutamic acid (IgM  $\sim 8$  per cent; IgG.LA  $\sim 11$  per cent).

Table 2.3 Molecular weight estimations determined by SDS-PAGE, of opossum immunoglobulin chains following reduction with 2-ME.

Immunoglobulin	Molecular weights (daltons) <sup>a</sup>	
	Heavy chain	Light chain
IgG.SA	50,300 $\pm$ 500	22,900 $\pm$ 400 ( 8) <sup>b</sup>
IgG.LA	47,900 $\pm$ 600	21,300 $\pm$ 500 (10)
IgA, serum	58,000 $\pm$ 2,000	22,800 $\pm$ 700 ( 2)
IgA, secretory	55,500 $\pm$ 2,500	22,800 $\pm$ 700 ( 2)
IgM	71,800 $\pm$ 1,400	22,200 $\pm$ 900 ( 5)

a Results presented as mean values  $\pm$  standard error.

b Number in parentheses refers to number of estimations made.

Table 2.4 Carbohydrate composition of IgG.LA and IgM.  
 Mean values and ranges of values are expressed  
 as mg of carbohydrate per 100 mg of protein.

Carbohydrate	Immunoglobulin	
	IgG.LA	IgM
Hexose	1.99(1.61-2.73) 6*	3.71(2.64-4.69) 3*
Hexosamine	0.83(0.67-1.11) 3	2.56(1.68-3.43) 3
Fucose	0.13(0.08-0.20) 3	1.03(0.60-1.45) 3
Sialic acid	1.37(0.96-2.17) 4	1.91(1.75-2.13) 3
Total	4.32	9.21

\* Number of estimations made (Note: The immunoglobulins analysed were isolated from pooled sera; the number of estimations refers to the number of different immunoglobulin isolates tested).

Table 2.5 Amino acid composition of IgG.LA and IgM.  
The values given are percentages of each amino acid recovered in respect to the total amino acid content.

Amino acid	IgG.LA	IgM
Lysine	6.02 <sup>a</sup> ± 0.28	5.37 <sup>b</sup> ± 0.88
Histidine	2.37 ± 0.80	4.78 ± 2.51
Arginine	3.86 ± 1.14	7.84 ± 2.39
Aspartic acid	9.02 ± 0.80	9.81 ± 2.03
Threonine	8.30 ± 0.30	7.18 ± 1.87
Serine	11.40 ± 0.45	11.04 ± 0.24
Glutamic acid	10.70 ± 0.79	8.39 ± 0.14
Proline	5.23 ± 0.06	5.21 ± 0.21
Glycine	6.89 ± 0.32	8.27 ± 0.82
Alanine	7.10 ± 0.14	6.86 ± 0.04
Cystine, Half	3.05 ± 0.26	2.40 ± 0.20
Valine	7.88 ± 0.21	7.03 ± 0.94
Methionine	0.96 ± 0.10	0.72 ± 0.02
Isoleucine	3.68 ± 0.06	3.88 ± 0.11
Leucine	6.61 ± 0.16	7.55 ± 0.98
Tyrosine	4.06 ± 0.14	3.51 ± 0.10
Phenylalanine	4.12 ± 0.05	4.40 ± 0.41
Total	101.25	104.25

a) Mean ± standard error from four estimations; b) mean ± standard error from two estimations.

## 2.4 Discussion

Three immunoglobulins have been isolated from the blood and intestinal fluid of non-immunized opossums. Despite their lack of antibody activity of any recognizable specificity they are considered to be analogous to eutherian IgG, IgM and IgA. This assertion is based on their having been isolated by methods known to isolate immunoglobulins, on their physical properties and on their antigenic relationship to each other.

Two forms of IgG were recognized initially by differences in their elution characteristics from both DEAE-cellulose and Protein A-Sepharose columns. These differences were reflected in the molecules' electrophoretic mobilities which led to their being designated IgG.LA and IgG.SA. Despite an apparent charge difference between the two forms, and repeated evidence of "splitting" of precipitation arcs in some circumstances, no antigenic differences between them could be detected by agar diffusion analyses.

To be successful these analyses rely on the detection of residual antibody activity in absorbed antisera. If this work were to be repeated the approach used by Bell et al. (1974a) of using anti-light chain antisera would be adopted. Using this technique the authors showed the complexity of the quokka's humoral immune system by demonstrating the occurrence in this species of separate IgG classes and subclasses. Obviously an alternative approach to that chosen here must be used to examine the complexity of the IgG class of immunoglobulins in T.vulpecula.

IgG.SA and IgG.LA were subsequently shown to differ also in their molecular weights. Using the mean molecular

weight values obtained by analyses of the intact molecules, IgG.SA was some 20,400 daltons heavier than IgG.LA. If the molecular weights estimated for the constituent heavy and light chains are considered, then this difference in mass could be reduced to 8,000 daltons. The more correct of these two values cannot be established. What is apparent is that both the heavy and the light chains of IgG.LA are not only lighter than those of IgG.SA, but also lighter than the corresponding chains of the other immunoglobulin classes. These data suggest that a deletion of material has occurred from one or other or both polypeptide chains in the IgG.LA molecule. Since IgG.SA and IgG.LA appeared antigenically the same, the lower molecular weight of IgG.LA might be due to that molecule's having a reduced amount of a component which is common to both IgG.LA and IgG.SA. Thus, a partial deletion of a repeating amino acid sequence from one or more constant domains of IgG.LA could be involved. In retrospect, a comparison of the carbohydrate and amino acid compositions of the two IgG forms may have shed some light on this question.

Neither transferrin nor haemopexin is precipitated by Rivanol (Putnam, 1960; Schultze and Heremans, 1966). Attempts to show that the contaminants seen in Rivanol-derived IgG isolates were one or other of these iron-binding proteins by the staining method of Uriel (1971) were unsuccessful, the technique proving to be non-specific in that most serum proteins became stained by this procedure.

Protein A, a cell wall component of Staphylococcus aureus, binds to the Fc regions of immunoglobulins and brings about their precipitation in agar diffusion systems (Forsgren and Sjoquist, 1966; Kronvall and Frommel, 1970). D.virginiana serum was the only one of

62 other mammalian species tested that failed to show this reaction which was originally thought to be mediated solely by IgG (Kronvall et al., 1970a). More recent evidence shows that protein A also binds to IgA and IgM (Harboe and Folling, 1974; Grov, 1976). Neither T.vulpecula serum nor IgG.IA precipitated with a commercial preparation of protein A in agar diffusion (results not presented). Nevertheless, when opossum serum was passed down a Protein A-Sepharose column IgG, IgM and IgA became bound to the matrix. It would seem, then, that binding between an immunoglobulin and protein A can occur without resulting in precipitation. Alternatively, the opossum immunoglobulins were binding to the Sepharose support rather than its coupled Protein A: free Sepharose could not be obtained in time to test this unlikely alternative. A considerable amount of opossum IgG failed to bind to the column and was eluted with the phosphate wash buffer. Similar findings have been described for sheep (Jonsson and Kronvall, 1974) and rabbit (Goding, 1976) IgGs. The possibility that the observed results were due to column overloading was checked by using 0.5 ml volumes of serum rather than the usual 5 ml volumes: the same elution patterns were obtained.

It is apparent that there is a subclass specificity in the protein A-immunoglobulin reaction. With human IgG, it has been shown that IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>4</sub> but not IgG<sub>3</sub> bind to Protein A (Kronvall and Williams, 1969). There is also a subclass specificity of human IgA in this respect with IgA<sub>2</sub> but not IgA<sub>1</sub> binding to protein A (Saltvedt and Harboe, 1976). However, there appears to be no simple relationship between an immunoglobulin subclass and its capacity to attach to protein A. Thus, Brunda et al. (1979) showed that while some human IgA<sub>1</sub> and IgA<sub>2</sub> immunoglobulins bound to the protein A others of both subclasses failed to do so. A similar situation apparently applies to opossum IgG.

Considerable variation in the molecular weights of immunoglobulins of, apparently, the same classes have been reported between different species. A detailed comparison of the molecular weights of opossum immunoglobulins with those of distantly related species would be tedious and unrewarding. Instead, Tables 2.6 and 2.7 present the mean molecular weights of intact immunoglobulin molecules and their heavy chains of selected species. It will be seen from these tables that the data provided for the opossum are in general agreement with those given for these other species. This is particularly true for IgM, although for IgG and IgA a greater range of values is apparent.

The molecular weight of the intestinal fluid secretory IgA is noticeably lower than those recorded for sIgA from other species listed both here and elsewhere. If a dimeric structure is assumed for opossum intestinal fluid IgA, then, using the molecular weights recorded for the molecules heavy and light chains, one could expect a molecular weight of some 313,000 daltons for the intact molecule, a figure in close agreement with the 303,800 daltons calculated. A range of molecular weights has been recorded for mammalian sIgA and extends from 357,000 daltons, for dogs to 420,000 daltons, for humans (Bjork and Lindh, 1974). If opossum sIgA can be said to have a molecular weight of around 310,000 daltons, then it is some 47,000 to 110,000 daltons lighter than sIgA from other species. The molecular weight of free secretory component ranges from 60,000 daltons, for rabbits (O'Daly and Cebra, 1971) to 110,000 daltons for humans (Brandtzaeg, 1974). The difference in the molecular weight of opossum sIgA and that of other animals approximates that of secretory component. It will be recalled that agar diffusion studies failed to demonstrate secre-



Table 2.6 The molecular weights of immunoglobulins of selected species.

Species	IgM	IgG	IgA
Chicken ( <u>Gallus domesticus</u> )	880-890 <sup>1,a</sup>	178 <sup>a</sup>	sIgA: 350-360 <sup>b</sup> 300-500 <sup>c</sup>
Echidna ( <u>O.paradoxus</u> )	950 <sup>d</sup>	150 <sup>d</sup> 177 <sup>e</sup>	-
Australian brush-tailed opossum ( <u>T.vulpecula</u> )	940	IgG.SA:150 IgG.LA:130	s IgA: 304 IgA: 175
Quokka ( <u>S.brachyurus</u> )	-	IgG <sub>1</sub> : 176 <sup>f</sup> 240 <sup>g</sup> IgG <sub>2</sub> : 136 <sup>f</sup> 130 <sup>g</sup>	s IgA: 200 <sup>h</sup> IgA: 150 <sup>h</sup>
Human	950 <sup>i</sup>	IgG <sub>1</sub> : 146 <sup>j</sup> IgG <sub>3</sub> : 165 <sup>j</sup>	s IgA: 380 <sup>j</sup> IgA: 160 <sup>j</sup>

1 Values given as m.w.  $\times 10^{-3}$  daltons.

a) Leslie and Clem (1969); b) Leslie and Martin (1973);  
c) Watanabe and Kobayashi (1974), Watanabe et al. (1975);  
d) Atwell et al. (1973); e) Marchalonis et al. (1974a);  
f) Lynch and Turner (1974a); g) Bell et al. (1974a); h)  
Bell et al. (1974b); i) Edelman and Gall (1969); j)  
Nisonoff et al. (1975).

Table 2.7 The molecular weights of heavy chains of immunoglobulins of selected species.

Species	IgM	IgG	IgA
Chicken ( <u>G. domesticus</u> )	62.6 <sup>1, a</sup> 70.0 <sup>b</sup>	62.0-66.0 <sup>a</sup>	-
Echidna ( <u>O. paradoxus</u> )	69.0 <sup>c</sup>	40.0 <sup>c</sup> 61.0 <sup>d</sup>	-
Australian brush-tailed opossum ( <u>T. vulpecula</u> )	71.8	IgG.SA: 50.3 IgG.LA: 47.9 55.7 <sup>e</sup>	s IgA: 55.5 IgA: 58.0
Human	70.0 <sup>f</sup> 72.0 <sup>g</sup>	53.0 <sup>f</sup> 51.0-60.0 <sup>g</sup>	64.0 <sup>f</sup> 52.0-58.0 <sup>g</sup> 65.0 <sup>h</sup>

1) Values given as m.w.  $\times 10^{-3}$  daltons.

a) Hersh and Benedict (1976); b) Leslie and Clem (1969);  
c) Atwell et al. (1973); d) Marchalonis et al. (1978);  
e) Atwell and Marchalonis (1975); f) Edelman and Gall  
(1969); g) Nisonoff et al. (1975); h) Cohen and Milstein  
(1967).

tory component on intestinal fluid sIgA. Precedent exists for the occurrence in secretions of IgA devoid of secretory component. Watanabe and Kabayashi (1973) and Watanabe et al. (1975) identified IgA in numerous secretions e.g., tears, saliva, bile, intestinal fluid, oviduct mucus, etc., of chickens and found that only intestinal IgA was associated with secretory component. The IgAs in secretion which did not show secretory component were generally in polymeric forms and had molecular weights of 800,000 to 900,000 daltons. Birds and mammals have both evolved from reptilian stock. Birds have both a dimeric form of IgA complete with secretory component and polymeric forms of IgA devoid of secretory component: eutherians have only a dimeric secretory IgA which is complete with secretory component. It may therefore be phylogenetically acceptable for marsupials, which diverged from a very early eutherian stock (Clemens, 1968), to show a dimeric IgA lacking a secretory component in its intestinal fluid. The further examination of opossum body fluids, other than intestinal secretions, for IgA and secretory component is obviously required. Repeated attempts to isolate IgA from serum by the method of Tomasi and Bienenstock (1968), using zinc sulphate precipitation, were unsuccessful. The isolated material when examined in IEP invariably contained some IgG and other contaminating serum proteins.

There appeared to be more IgG.LA than IgG.SA in opossum serum, an impression gained by the relative amounts of each judged by optical density analyses of material eluting from DEAE-cellulose and Protein A-Sepharose columns. For this reason emphasis was placed on IgG.LA in determining serum levels of the two forms and defining carbohydrate and amino acid compositions.

Although marked differences in the carbohydrate composition of the various immunoglobulin classes have been described, the functional significance of these components is not known. They are not needed for the antibody activities of the molecule (Putnam, 1977) but may play a role in the various functions of the Fc region, notably in secretion from plasma cells (Litman, 1976). The values for carbohydrates given here are derived from analyses using immunoglobulins isolated from different serum pools. The wide ranges of values given for these individual carbohydrates of IgG.LA and IgM are disturbing, but similar ranges have been described for other species and have been found acceptable. These ranges probably indicate a degree of imprecision in the colourimetric methods used; unfortunately more precise methods of carbohydrate determination were not available.

The total carbohydrate content of IgG.LA resembled those reported for the lower molecular weight immunoglobulins of chicken (Leslie and Clem, 1969), D.virginiana (Rowlands, 1976), quokkas (Bell et al., 1974a) and humans (Chaplin et al., 1965). The low concentration of fucose relative to those of the other carbohydrate is consistent with the findings in Chondrichthyes, Osteichthyes, Amphibia, reptiles, birds and mammals (Acton et al., 1972).

The carbohydrate content of opossum IgM was higher than that of IgG.LA and resembled that of the macroglobulins of the Horned shark, Heterodontus francisci (Frommel et al., 1971), the Paddle fish, Polyodon spathula (Pollara et al., 1968), the Bull frog, Rana catesbiana (Marchalonis and Edelman, 1966b), the American opossum (Rowlands, 1976) and man (Edelman and Gall, 1969). The carbohydrate composition of immunoglobulins, both IgG and IgM, appears to be a phylogenetically stable feature.

The serum level of IgG.LA was close to that recorded for eutherians, including man (Preval, 1978): that of opossum IgM (4.1 mg per ml) being much higher than the mean value of 0.92 mg per ml recorded for man, but comparable to those recorded for cattle (Duncan et al., 1972) and pig (Setcavage and Kim, 1976) IgMs. The wide range of values recorded for opossum IgM may well reflect inadequacies in the test system.

The amino acid compositions of opossum IgG.LA and IgM were in general agreement with those recorded for lower vertebrates (Marchalonis and Edelman, 1966b; Atwell and Marchalonis, 1975) and higher mammals (Crump-ton and Wilkinson, 1963; Chaplin et al., 1965). The ratio of leucine to isoleucine was about 2:1 in both opossum immunoglobulin classes. A similar situation is seen in other mammals whereas in the immunoglobulins of amphibians and chickens these two amino acids occur in approximately equal amounts (Atwell and Marchalonis, 1975). Although this feature seems to be a dividing line between mammals and lower vertebrates, its functional significance is not known. The similarities of the carbohydrate and amino acid compositions of immunoglobulins from diverse phylogenetic species reinforces the notion that these immunoglobulins arose from a common precursor gene (Hill et al., 1966).

The results presented here and elsewhere (Yadav, 1973b; Bell et al., 1974a,b) clearly indicate that the immunoglobulins of Australian marsupials are similar in their complexity to those of eutherians.

## CHAPTER 3

### IDENTIFICATION AND DISTRIBUTION OF B LYMPHOCYTES

#### 3.1 Introduction

Lymphocytes differ in their functions, distribution and surface markers and several classes of these cells are now recognised. Of these, the most studied are the T and B lymphocytes and considerable information is available on their activities, especially in mice and humans (for review, see Katz, 1977).

T and B cells fulfill complementary biological roles. T cells are concerned with the regulation of immune activity (Gershon, 1976), direct cytotoxicity (Cerottini and Brunner, 1974; Engers and MacDonald, 1976), mixed lymphocyte reactivity (Weber, 1974) and delayed type hypersensitivity (Tigelaar and Gorczynski, 1974; Gorczynski and Tigelaar, 1975; Dennert and Hatlen, 1975) while B cells mediate humoral immunity (Davies et al., 1967; Mitchell and Miller, 1968). Despite this division in immune capabilities, co-operation between the two cell types is required for most antibody responses (Claman et al., 1966; Davies et al., 1967).

T and B cells differ in their circulation patterns, T cells being relatively mobile between blood and lymphoid tissues while B cells are relatively sessile within lymphoid tissues (Bell, 1977). They also show different distributions within the lymph node and spleen (Howard, 1972; Sprent, 1973; Goldschneider and McGregor, 1973) and have different life spans (Howard, 1972; Sprent and Basten, 1973).

The two cell types can be distinguished by the presence of membrane-associated receptors and antigens

(Aiuti et al., 1974; Katz, 1977; Gelfand and Dosch, 1978; Stites, 1978). In the mouse, T lymphocytes carry Thy.1 (Raff, 1971), a surface antigen which also occurs on certain non-lymphoid cells (Reif and Allen, 1964; Raff, 1970). Murine B cells have surface markers that are generally lacking on T cells. These include readily detectable, surface membrane immunoglobulin (SmIg) (Raff et al., 1970; Unanue et al., 1971), a receptor for the third component of complement (Bianco et al., 1970; Dukor et al., 1971) and a receptor for the Fc region of reacted IgG (Basten et al., 1972c; Paraskevas et al., 1972).

The identification of T and B cells in other species relies on the detection of surface markers analogous to those found on mouse lymphocytes. While human T cells have a surface antigen that distinguishes them from B cells (Owen and Fanger, 1974; Bobrove et al., 1974), a more convenient characteristic of this cell type is its ability to form rosettes with SRBC (Jondal et al., 1972). Human B lymphocytes, in common with mouse B cells, show SmIg (Froland et al., 1971; Wilson and Nossal, 1971) and have receptors for the Fc portion of reacted IgG (Dickler and Kunkel, 1972) and for complement components (Bianco et al., 1970).

In contrast to the extensive literature dealing with the lymphocytes of eutherians there is no published information on these cells of metatherian species. This chapter describes the identification of B cells in T. vulpecula and presents some data on their distribution between blood and certain lymphoid organs.

### 3.2 Materials and methods

#### a. Harvesting of lymphocytes

Blood lymphocytes

Blood was collected into 10 ml evacuated tubes containing heparin (Becton and Dickinson) by cardiac puncture in animals anaesthetised with halothane (Chapter 2). Mononuclear cells were harvested by the method of Robinson and Lertratanakul (1975). The blood was centrifuged at 1750 g for 30 min at room temperature. The buffy coat was collected and diluted with twice its volume of PBS containing 100 units of preservative-free heparin (Sigma) per ml. The diluted cell suspension was layered onto a Ficoll-Hypaque gradient (Lymphoprep, Nyegaard) using a cell suspension to Ficoll-Hypaque ratio of 4:3, and centrifuged at 750 g for 30 min at room temperature. The interface layer of cells was harvested, and washed twice with PBS-heparin. The cells were resuspended in 10 ml of Eagle's minimal essential medium (MEM) supplemented with 10 per cent heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) foetal calf serum (FCS, Flow Laboratories) containing 100 IU of penicillin, 100  $\mu\text{g}$  of kanamycin and 100  $\mu\text{g}$  of streptomycin per ml of medium (MEM-FCS). The cell suspension was then transferred to a plastic petri dish and incubated at  $37^{\circ}\text{C}$  for 30 min in an atmosphere of 5 per cent  $\text{CO}_2$ -in-air to remove monocytes which adhere to the dish surface (Horwitz and Garrett, 1977) and to free lymphocytes of passively acquired immunoglobulin (Kurnick and Grey, 1975; Lobo *et al.*, 1975). The non-adherent cells were then washed from the petri dish with MEM-FCS and pelleted by centrifugation at 350 g for 10 min. The cells were counted (Appendix III) and resuspended to the required concentration in MEM-FCS. The percentage of viable cells was established by trypan blue exclusion (Appendix III), 200 cells being counted.

#### Spleen lymphocytes

Anaesthetised opossums were exsanguinated by section of a jugular vein and their spleens removed asepti-



cally into MEM-FCS. The spleens were diced and the pieces forced through a sterile 40-mesh stainless steel sieve. A cell suspension was prepared in MEM-FCS by the method of Shortman et al. (1972). Large debris was removed by layering the cell suspension onto 3 ml of FCS and leaving it at room temperature for 15 min to allow the larger cell clumps to settle. Fine debris and cell fragments were then removed by layering the MEM-FCS over fresh FCS and centrifuging at 500 g for 7 min at 4°C. To free the cells of contaminating erythrocytes, the pelleted material was resuspended in 3 ml of cold, 0.17M NH<sub>4</sub>Cl and held at 4°C for 10 min. One ml of FCS was then layered beneath the cell suspension and the tube centrifuged at 500 g for 7 min at 4°C. The pelleted cells were washed once with MEM and resuspended in this medium. Monocytes were removed by incubation in a plastic petri dish as described above and the lymphocytes were harvested by centrifugation at 350 g for 10 min at 4°C. The cells were tested for viability by trypan blue exclusion (Appendix III) and resuspended to the required concentration in MEM-FCS.

#### Thymus lymphocytes

Thymus lymphocytes were prepared as described for spleen lymphocytes. The cells were tested for their viability and adjusted to the required cell concentration in MEM-FCS.

#### Bone marrow lymphocytes

Femurs and humeruses were cleaned of tissues and transected at both ends. Cold MEM-FCS was syringed through the bone cavity and the mixture of bone marrow and medium was collected. The cell suspension was left at room temperature for 10 min to allow large clumps to settle. The supernatant was then layered onto a Ficoll-

Hypaque gradient and centrifuged at 750 g for 20 min at room temperature. The interface layer of cells was harvested and washed twice with PBS-heparin. Monocytes were removed by incubating the cells in a plastic petri dish, the non-adhering cells being harvested and counted as described above. The lymphocytes were tested for viability and resuspended to the required cell concentration in MEM-FCS.

b. Removal of monocytes

The extent to which the incubation of cell suspensions in plastic petri dishes was effective in removing monocytes was assessed by doing differential counts of smears of blood mononuclear cells prior to and following the incubation step. The smears were stained with MacNeal's stain (Appendix III) and the percentages of monocytes were calculated after counting at least 200 mononuclear cells.

c. Fractionation of lymphocytes on nylon wool columns

Enriched population of B and T lymphocytes were obtained by fractionating Ficoll-Hypaque-derived mononuclear cells from blood on nylon wool columns using the methods of Greaves and Brown (1974) and Roy *et al.* (1976). Monocytes were removed from the cell suspension prior to its fractionation as described. Six hundred mg of nylon wool (Leukopak, Fenwal Laboratories) were packed into a 10 ml syringe barrel and washed ten times alternately with 0.2N HCl and distilled water. MEM-FCS, at 37°C, was run through the column until there was no visible difference in colour between the MEM-FCS coming off the column and the starting medium, indicating that the nylon wool was adjusted to the correct pH. The column was incubated with MEM-FCS at 37°C for 30 min before being loaded with approximately  $10^8$  lymphocytes in 2 ml of medium. After incubation at 37°C for 30 min the non-adherent cells were eluted at

room temperature with 20 ml of medium over a period of 15 min. The eluted cells were pelleted by centrifugation in MEM-FCS, resuspended in 2 ml of this medium and passed down a second column containing 300 mg of nylon wool which had been equilibrated as described above. After incubation for 30 min at 37°C the non-adherent cells were eluted as described previously.

Adherent cells were recovered from the nylon wool. After flushing each column with 100 ml of MEM-FCS, the nylon wool was removed, teased apart in MEM-FCS and the freed cells collected by centrifugation.

The percentage of viable cells in both cell populations was assessed by trypan blue exclusion and the cells resuspended to the required cell concentration in MEM-FCS.

d. Demonstration of surface membrane immunoglobulin (SmIg)

Indirect immunofluorescent staining was used to detect the presence of SmIg and to determine the class of immunoglobulin involved. The technique used was based on the methods of Moller (1961), Pernis et al. (1970), Johnson (1977) and Zan-Bar et al. (1978).

Preparation of antisera to opossum immunoglobulins

Opossum gammaglobulin was obtained by mixing equal volumes of opossum serum and 90 per cent saturated ammonium sulphate at room temperature for 4 hr. The resulting precipitate was collected by centrifugation and dissolved in distilled water to give a final volume equal to that of the initial serum sample. This cycle was repeated three times. The final precipitate was dissolved in PBS to 1/10 of the volume of the original serum sample.

Rabbit antisera to the opossum gammaglobulin and to opossum IgG (IgG.LA, Rivanol-derived) and IgM (Starch block electrophoresis-derived) were prepared and analysed by IEP as described in Chapter 2.

#### Labelling of lymphocytes

Two hundred  $\mu$ l of lymphocyte suspension containing ca.  $4 \times 10^6$  cells were mixed with 20  $\mu$ l of rabbit anti-opossum gammaglobulin. When lymphocytes were examined for the presence of either IgG or IgM on their membranes, the appropriate specific antiserum was used in place of the anti-opossum gammaglobulin reagent. The lymphocytes were allowed to react with the antisera at 4°C for 30 min. They were then washed three times with PBS containing 0.02 per cent sodium azide. The washed lymphocytes were reacted with 10  $\mu$ l of a fluoresceine-conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories) which had been absorbed with opossum liver powder (100 mg of liver powder per ml of antiserum) at 37°C for 30 min. Unreacted conjugate was removed by washing the cells three times with cold PBS containing 0.02 per cent sodium azide. The cell pellet was resuspended in 3 drops of a mixture of equal volumes of PBS and glycerol. One drop of the suspension was placed on a microscope slide, mounted with a coverslip and sealed with lacquer. Control experiments were carried out in which lymphocytes were stained with the fluorescein conjugate without prior treatment with the rabbit anti-opossum immunoglobulin sera.

Slides were examined in a fluorescence microscope equipped with a HBO 50 mercury lamp. Lymphocytes showing fluorescence were considered as positive cells. At least 200 cells were counted from each of three slides which were routinely prepared for each animal. The mean

value derived from these three slides was used to establish the range of values given in Table 3.1.

e. Demonstration of Fc receptors (FCR)

The method used to detect FcRs was that of Sjoberg and Inganas (1979) and involved using latex particles coated with opossum IgG. A 0.4 per cent suspension of latex particles (average diameter 0.8  $\mu\text{m}$ ) in a solution of opossum IgG at a concentration of 20 mg per ml in PBS was prepared and incubated at 37°C for 24 hr. The IgG used was obtained from serum by Rivanol precipitation (Rejnek et al., 1973) as described in Chapter 2. The particles were then washed three times with PBS and suspended to give a 0.2 per cent solution in 2.5 per cent PSA in PBS containing 0.02 per cent sodium azide. If it was necessary for the IgG-coated latex (IgG-latex) particles to be stored at 4°C, they were sonicated for 5 sec before use to disperse any aggregated material. Fifty  $\mu\text{l}$  of the IgG-latex particles suspension were mixed with 50  $\mu\text{l}$  of lymphocyte suspension containing ca.  $4 \times 10^6$  cells per ml. The mixture was centrifuged at 200 g for 6 min to achieve a close association between the particles and the lymphocytes and held at room temperature for 15 min. The cells were then resuspended using a Pasteur pipette. A drop of this suspension was placed on a microscope slide and mounted with a coverslip. Cells binding three or more IgG-latex particles were considered positive for FcRs. Lymphocytes with a clump of latex particles at one point of the cell surface were not considered positive. At least 200 cells were counted from each of three slides which were routinely prepared for each animal. The mean value derived from these slides was used to establish the range of values given in Table 3.1. Control assays were made in which the latex particles were initially incubated in a BSA-PBS solution rather than the IgG-PBS mixture.

#### f. Demonstration of complement receptors (CR)

CRs were detected by using a zymosan-complement complex as described by Kajdacsy-Balla and Mendes (1976) and Sher and McIntyre (1977). Zymosan A particles (Sigma), derived from Saccharomyces cerevisiae, were suspended in MEM at a concentration of 0.38 mg per ml and incubated at 37°C for 1 hr with an equal volume of guinea pig serum as a complement source. The complement-coated zymosan (ZC) particles were then washed three times in MEM-FCS at 200 g for 5 min and resuspended in MEM-FCS to their original volume. Two hundred  $\mu$ l of a lymphocyte suspension containing  $2 \times 10^6$  cells per ml were added to 200  $\mu$ l of ZC suspension and the mixture incubated at 37°C for 1 hr. After incubation the tube was centrifuged at 200 g for 5 min. One drop of Giemsa stain was added and the cell pellet was resuspended using a Pasteur pipette. A drop of the suspension was placed on a microscope slide, mounted with a coverslip and examined for rosettes. A rosette was defined as a lymphocyte with three, or more, adherent ZC particles. Lymphocytes showing ZC aggregates accumulated at a single location on the cell surface were not considered as positive. At least 200 cells were counted from each of three slides which were routinely prepared for each animal. A mean value was derived from these slides which was used to establish the range of values given in Table 3.1. A control consisting of zymosan particles in MEM-FCS was used.

### 3.3 Results

#### a. Viability of lymphocytes

The viability of isolated lymphocytes, tested by trypan blue exclusion, always exceeded 95 per cent.

b. Removal of monocytes

The suspension of blood mononuclear cells from the Ficoll-Hypaque gradients contained 9 to 11 per cent monocytes. After incubation on plastic petri dishes this level was reduced to about 1 per cent.

c. Demonstration of SmIg

Analysis of antisera

Rabbit antisera to opossum gammaglobulin, IgM and IgG were analysed by IEP against opossum serum (Fig.3.1). The anti-gammaglobulin serum gave prominent precipitation arcs in the gamma and beta regions. The anti-IgM and anti-IgG antisera, appropriately absorbed, each showed single precipitation arcs in the regions where IgM and IgG appear on IEP analysis.

Quantitation of SmIg-positive cells

The mean percentages and the ranges of values of SmIg positive lymphocytes in blood, spleen, bone marrow and thymus are given in Table 3.1; the values quoted in the text are means. The fluorescence seen on the cells varied from discrete patches and crescents to an even distribution (Fig. 3.2). Bone marrow showed the highest proportion of SmIg-positive cells (49.3 per cent) while none were detected in the thymus. Forty two per cent of spleen lymphocytes were positive for this marker compared with 17.3 per cent of blood lymphocytes. Nylon wool fractionation of blood lymphocytes gave a non-adherent population which was depleted of SmIg-positive cells (3.2 per cent) and an adherent population enriched for these cells (62.2 per cent). Controls consistently showed less than 1 per cent of cells fluorescing.

When the SmIg isotype on blood lymphocytes was investigated 14.8 per cent of the lymphocytes reacted

FIGURE: 3.1 Immunelectrophoretic analysis of rabbit antisera used to detect surface membrane immunoglobulin on opossum lymphocytes. Wells contain opossum serum. Troughs contain antisera to opossum IgM (A), opossum IgG (B), opossum gammaglobulin (C) and opossum serum (D).

FIGURE: 3.2 Demonstration of indirect immunofluorescence of lymphocyte surface membrane immunoglobulin showing different patterns of fluorescence. Lymphocytes treated with rabbit anti-opossum gammaglobulin and with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate.



3.1



3.2

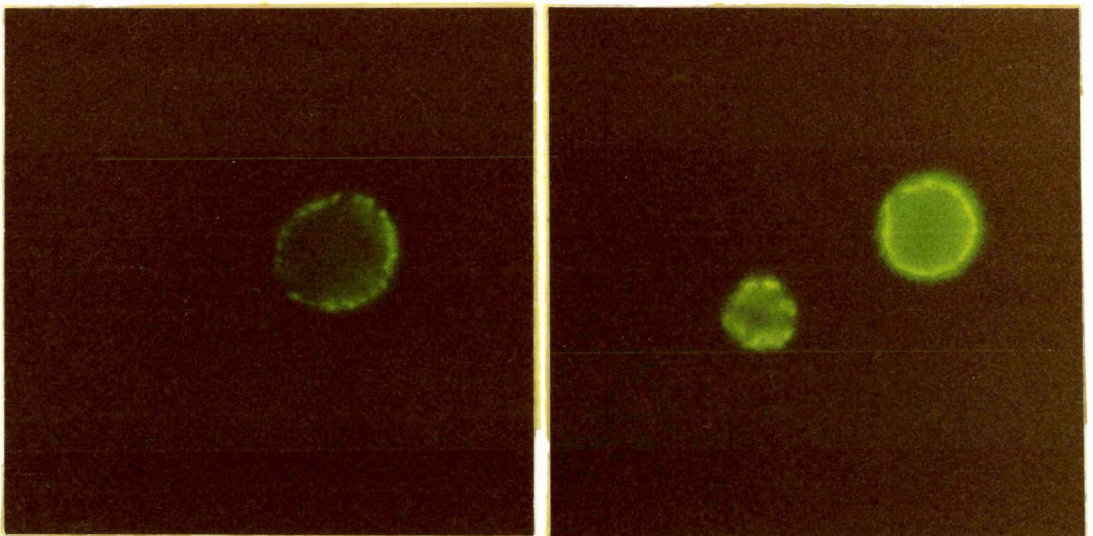
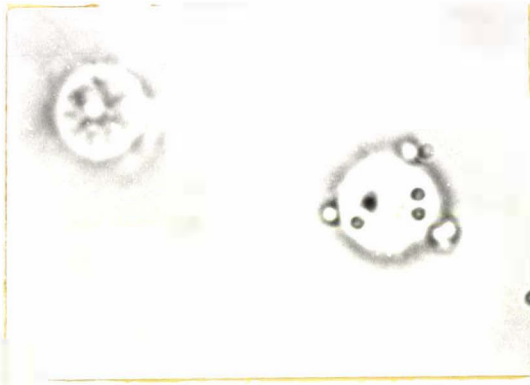


FIGURE: 3.3 Demonstration of Fc receptors on opossum lymphocytes using IgG-coated latex particles.

FIGURE: 3.4 Demonstration of complement receptors on opossum lymphocytes using a Zymosan-complement rosette method. Lymphocytes stained with Giemsa stain.

**3.3**



**3.4**

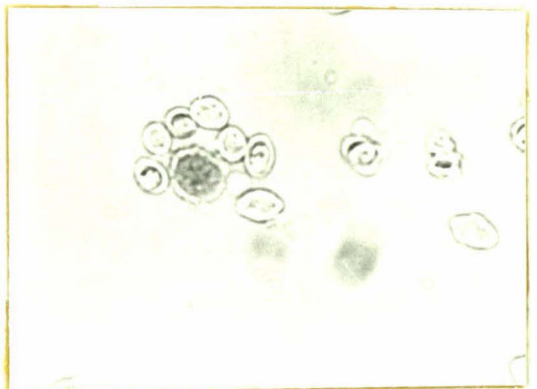
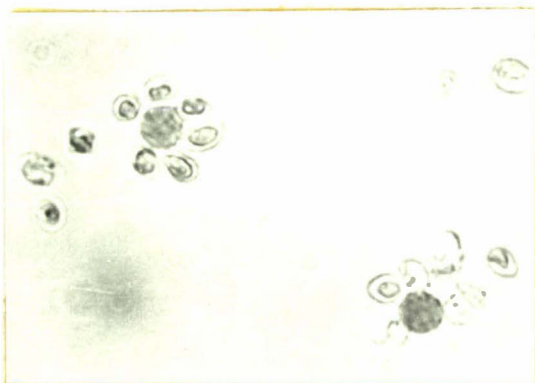


Table 3.1 B cell markers on opossum lymphocytes from various sources

Source	Markers		
	SmIg <sup>1</sup>	FcR <sup>2</sup>	CR <sup>3</sup>
Blood			
Unfractionated	17.3 <sup>a</sup> (13.0-22.0) 4 <sup>b</sup>	16.8 (13.0-24.5) 3	15.9 (10.5-24.5) 10
Non-adherent (T)	3.2 ( 1.0- 7.0) 5	1.5 ( 0.0- 3.0) 2	2.3 ( 1.0- 5.0) 6
Adherent (B)	62.2 (53.0-72.0) 5	25.7 (20.5-31.0) 2	51.3 (40.0-65.0) 6
Spleen	42.0 (36.0-46.0) 3	42.5 (42.0-43.0) 2	38.6 (27.0-48.0) 4
Bone marrow	49.3 (43.0-53.5) 3	14.0 (12.0-14.0) 2	11.8 ( 9.0-15.0) 3
Thymus	0.0 2	4.0 ( 2.0- 6.0) 2	1.8 ( 1.0- 2.5) 3

1) SmIg, Surface membrane immunoglobulin; 2) FcR, Fc receptor; 3) CR, complement receptor; a) mean percentages; b) Number of animals examined; Figures in parentheses show ranges of values.

with anti-IgM (Range 11.0 to 21.0 per cent, from 5 experiments) while 4.8 per cent reacted with anti-IgG (Range 4.0 to 7.0 per cent, from 4 experiments).

d. Demonstration of FcR

The binding of IgG-coated latex particles to opossum lymphocytes is shown in Fig. 3.3. The proportions of unfractionated blood and spleen lymphocytes bearing FcR were similar to those showing SmIg (Table 3.1). Fractionation of blood lymphocytes into T and B cell populations and examination of these cells for FcRs gave a somewhat surprising result. While non-adherent cells showed an expected low level of such cells (1.5 per cent) adherent cells were, on average, only 25.7 per cent positive for this receptor. Thymus cells showed a low level (4.0 per cent) of cells with this B-cell marker. There was a marked difference in the percentage of cells which were FcR-positive (14.0 per cent) in the bone marrow population and those which had been seen to be SmIg positive (49.3 per cent). Less than 1 per cent rosettes were found in control preparations.

e. Demonstration of CR

The results obtained from CR analyses using the zymosan-complement system are given in Table 3.1. The rosettes that formed were readily identifiable (Fig. 3.4). The levels of unfractionated blood and spleen lymphocytes which were CR-positive (15.9 and 38.6 per cent, respectively) were similar to those positive for SmIg and FcRs. The B-cell nature of the CR-positive cell was indicated by the results using nylon wool fractionated cells. Non-adherent cells showed a mean of 2.3 per cent positive cells while the adherent population was 51.3 per cent positive. Bone marrow lymphocytes showed a lower proportion of CR-bearing cells

(11.8 per cent) than did either cells from blood or spleen. Thymus cells showed 1.8 per cent CR-positive cells. Controls using zymosan particles alone gave less than 1 per cent positive cells in all instances.

### 3.4 Discussion

The results presented here have demonstrated the presence on opossum lymphocytes of markers which, in eutherians, are believed to be more characteristic of B cells than T cells. Of the three markers studied perhaps only stable, readily demonstrable SmIg would be acknowledged as being typical of B cells (Aiutii et al., 1974) since FcRs and CRs can be found on normal (Anderson and Grey, 1974; Basten et al., 1975; Arbeit et al., 1976) and activated T cells (Yoshida and Andersson, 1972; Gyongyossy et al., 1975; Arnaiz-Villena et al., 1975) and also on non-lymphoid cells (Huber et al., 1968; Reynolds et al., 1975; Gupta et al., 1976). Even SmIg may not be unique to B cells as this marker has been claimed to occur on T cells (Feldmann, 1972, 1974; Marchalonis et al., 1975) and on mouse (Warr et al., 1978) and human (Haegert et al., 1978) "null" lymphocytes.

Not all "B cells" show the presence of all three markers simultaneously (Basten et al., 1972a,b; Dickler and Kunkel, 1972; Dickler et al., 1975; Tursi et al., 1978) and considerable overlapping of subpopulations positive for one or more receptors probably occurs. Furthermore it should be emphasised that the lymphocyte surface membrane is a fluid mosaic (Singer and Nicholson, 1972) and the likely influence that the dynamic state of this structure plays on the presentation of receptors must be remembered.

In Table 3.2 a comparison is made of the ranges of values recorded for SmIg-positive lymphocytes in opossums, mice and humans. Two points emerge from this table. Firstly, there are marked variations in the values recorded for a given species. Some of these differences will be due to variations between individual animals, others are undoubtedly due to differences in techniques and the interpretation of results (Warner, 1974; Kurnick and Grey, 1975; Lobo et al., 1975). Secondly, the levels of SmIg-positive cells recorded for opossums are in general agreement with those given for the other two species, a possible exception to this is the somewhat higher proportion of these cells seen in opossum bone marrow than in bone marrow of either human or mouse origin.

It is generally agreed that IgM is the immunoglobulin most frequently identified on human lymphocytes, some 60 per cent of SmIg-positive cells carrying this isotype (Pernis et al., 1971; Froland and Natvig, 1972, 1973). The presence of IgD on the surface of human lymphocytes has also been reported (van Boxel et al., 1972; Knapp et al., 1973; Fu et al., 1974). Rowe et al. (1973) and Knapp et al. (1973) using human blood lymphocytes and Brandon et al. (1979) using mouse spleen lymphocytes have reported the concomitant existence of IgD and IgM on the same lymphocytes. The simultaneous occurrence of IgG and IgM on individual lymphocytes in mice (Greaves, 1971; Abney et al., 1978) and rabbits (Bona et al., 1972) has also been reported.

In the present study 14.8 per cent of cells reacted with anti-IgM and 4.8 per cent with anti-IgG. The total percentage of cells showing SmIg was 17.3. From these data it was calculated that 66 per cent of the SmIg-bearing cells were likely positive for IgM, the remainder showing IgG. These results are in close agreement

Table 3.2 The percentages of SmIg-positive lymphocytes in various tissues of opossum, mouse and human origin.

Lymphocyte source	Species		
	Opossum	Mouse	Human
Blood			
Unfractionated	13 - 22 <sup>*</sup>	14 - 28 <sup>a</sup>	8 - 30 <sup>b</sup>
Non-adherent (T)	1 - 7	7 <sup>c</sup>	1 - 11 <sup>d</sup>
Adherent (E)	53 - 72	86 <sup>e</sup>	73 - 97 <sup>f</sup>
Spleen	36 - 46	40 - 49 <sup>g</sup>	21 - 39 <sup>h</sup>
Bone marrow	43 - 54	23 - 62 <sup>i</sup>	22 - 23 <sup>j</sup>
Thymus	0	1 - 2 <sup>k</sup>	1 - 2 <sup>l</sup>

\* Values are ranges in percentages.

a) Rabellino et al. (1971), Warner (1974); b) Ross et al. (1973), Dickler et al. (1974), Warner (1974), Forni and Pernis (1975), Grey et al. (1975), Winchester et al. (1975); c) Handwerger and Schwartz (1974); d) Froland and Natvig (1973), Wisloff and Froland (1973), Jondal et al. (1973), Greaves and Brown (1974), Tursi et al. (1978); e) Handwerger and Schwartz (1974) f) Jondal et al. (1973), Greaves et al. (1974b) g) Rabellino et al. (1971), Dickler and Sachs (1974); h) Ross et al. (1973); i) Ryser and Vassalli (1974), Yang et al. (1978); j) Fauci (1975), Husz et al. (1977); k) Rabellino et al. (1971), Lamelin et al. (1972); l) Ross et al. (1973).



with those obtained for human blood lymphocytes (Fro-land and Natvig, 1973).

Most, but not all, B lymphocytes have receptors for the Fc portion of reacted IgG. A range of 64 to 96 per cent has been given for SmIg-positive lymphocytes carrying this receptor in murine and human systems (Dickler and Kunkel, 1972; Forni and Pernis, 1975; Ramasamy and Lawson, 1975). Again, this marker is not restricted to B lymphocytes as both normal (Anderson and Grey, 1974; Basten et al., 1975; Arbeit et al., 1976) and activated (Yoshida and Andersson, 1972) mouse T cells have been shown to have FcRs for various immunoglobulin classes. Pang and Wilson (1978) have shown subpopulations of lymphocytes bearing receptors of varying affinity for reacted Fc and have pointed out that different techniques may detect different Fc-reactive populations. The percentages of FcR-bearing lymphocytes in the tissues of opossum, mouse and human origin are compared in Table 3.3. It can be seen that the values given for the three species are in general agreement, although the values are noticeably higher in mice than for the other two species, with respect to bone marrow.

Bone marrow cells from mice and humans tend to lack FcRs and CRs, these markers being associated with more mature B cells (Basten et al., 1972b; Ryser and Vassalli, 1974; Husz et al., 1977; Yang et al., 1978). These findings are reflected in Table 3.1, which indicates that FcRs and CRs develop on opossum lymphocytes only after the migration of these cells from the bone marrow. SmIg, however, is readily seen on these cells and the value of 49.3 per cent for SmIg-positive cells in this environment is in close agreement with the 50 per cent recorded by Ryser and Vassalli (1974) for mouse bone marrow cells.

Table 3.3 The percentages of Fc receptor-positive lymphocytes in various tissues of opossum, mouse and human origin.

Lymphocyte source	Species		
	Opossum	Mouse	Human
Blood			
Unfractionated	13 - 25 <sup>*</sup>	15 - 23 <sup>a</sup>	12 - 22 <sup>b</sup>
Non-adherent (T)	0 - 3	-	2 - 3 <sup>c</sup>
Adherent (B)	21 - 31	-	18 - 24 <sup>d</sup>
Spleen	42 - 43	39 - 58 <sup>e</sup>	-
Bone marrow	12 - 14	21 - 33 <sup>f</sup>	9 - 14 <sup>g</sup>
Thymus	2 - 6	0 - 2 <sup>h</sup>	0 - 2 <sup>i</sup>

\* Values are ranges in percentages.

a) Basten et al. (1972a), Ryser and Vassalli (1974);  
 b) Dickler and Kunkel (1972), Dickler et al. (1974), Kurnick and Grey (1975), Abdou et al. (1976), Sjoberg and Inganas (1979); c) Greaves et al. (1974b), Tursi et al. (1978); d) Jondal et al. (1973), Greaves et al. (1974b), Abdou et al. (1976), Tursi et al. (1978);  
 e) Basten et al. (1972a), Dickler and Sachs (1974); f) Basten et al. (1972a); g) Abdou et al. (1976); h) Basten et al. (1972a), Dickler and Sachs (1974); i) Dickler et al. (1973).

Nylon wool-adherent blood lymphocytes (B cells) of opossums were considerably less FcR-positive than they were for either SmIg or CRs. A similar result has also been described in humans by Tursi et al. (1978) who found that only 78 per cent of the SmIg-positive cells also had FcRs. A subpopulation of B cells lacking FcRs may also occur in opossums.

The proportion of B cells having CRs may vary with the species concerned. Ross et al. (1973) reported that while all CR-positive cells were also SmIg-positive, only 50 per cent of SmIg-positive cells had CRs in a human lymphocyte population. Table 3.4 shows that similar ranges of values have been recorded for CR-positive lymphocytes in opossum, mouse and human lymphoid tissues. In some cases, human blood for example, a wide range of results has been recorded which may be due to variations in the assay system used. The relatively low levels of CR-positive cells in opossum bone marrow as compared to the level of SmIg-positive cells in this site has already been commented on.

In this study attention has been focussed solely on the identification of opossum B lymphocytes. A reliable T cell marker for this species is not available (Southern, personal communication). Rodents and humans have lymphoid cells which are negative for most B and T cell surface markers (Greenberg et al., 1973; Chess et al., 1975) and Froland and Natvig (1973) claim that up to 20 per cent of human blood lymphocytes fall within this category. Whether or not comparable numbers of such cells occur in opossum blood is not known; if they do then the relatively low percentage of SmIg-positive cells in the nylon wool adherent population might be explained. The approach of Warr et al. (1978) of using a phylogenetically distant species in which to raise

Table 3.4 The percentages of complement receptor-positive lymphocytes in various tissues of opossum, mouse and human origin.

Lymphocyte source	Species		
	Opossum	Mouse	Human
Blood			
Unfractionated	11 - 25 <sup>*</sup>	15 - 30 <sup>a</sup>	10 - 39 <sup>b</sup>
Non-adherent (T)	1 - 5	6 <sup>c</sup>	1 - 9 <sup>d</sup>
Adherent (B)	40 - 65	63 <sup>e</sup>	49 - 69 <sup>f</sup>
Spleen	27 - 48	25 - 65 <sup>g</sup>	20 - 31 <sup>h</sup>
Bone marrow	9 - 15	5 - 8 <sup>i</sup>	5 - 13 <sup>j</sup>
Thymus	1 - 3	0 <sup>k</sup>	0 - 2 <sup>l</sup>

\* values are ranges in percentages.

a) Ross et al. (1976); b) Ross et al. (1973), Abrahamsohn et al. (1973), Handwerger and Schwartz (1974), Abdou et al. (1976), Kajdacsy-Balla and Mendes (1976); c) Handwerger and Schwartz (1974); d) Jondal et al. (1973), Greaves et al. (1974b), Tursi et al. (1978); e) Handwerger and Schwartz (1974); f) Jondal et al. (1973), Greaves et al. (1974b), Tursi et al. (1978); g) Raff et al. (1970), Ross et al. (1976); h) Silveira et al. (1972), Ross et al. (1973); i) Bianco et al. (1970), Ryser and Vassalli (1974), Yang et al. (1978); j) Bianco et al. (1970), Dukor et al. (1971), Abdou et al. (1976), Husz et al. (1977); k) Silveira et al. (1972); l) Ross et al. (1973), Silveira et al. (1972).

anti-immunoglobulin antisera could be rewarding in this respect.

With the facilities available it was not possible to examine simultaneously the cells from a single animal for all three markers. Neither do the results explore the likelihood of day-to-day variation of a given marker in an individual animal. Both of these aspects might be profitably examined.

In summary, it can be stated that recognised B cell markers have been demonstrated on opossum lymphocytes. Both the proportion of cells showing these markers and their tissue distribution are broadly similar in opossums and eutherian species.

## CHAPTER 4

### THE IN VITRO RESPONSE OF LYMPHOCYTES TO VARIOUS MITOGENS

#### 4.1 Introduction

Lymphocytes cultured in the presence of antigens (Lycette and Pearmain, 1965), anti-lymphocyte antibodies (Grasbeck et al., 1964), anti-immunoglobulin reagents (Sell and Gell, 1965a,b; Sieckmann et al., 1978), Fc fragments (Berman and Weigle, 1977; Morgan and Weigle, 1979) or mitogens (Oppenheim and Rosenstreich, 1976) undergo blastogenesis and produce either lymphokines (David, 1966; Lawrence and Landy, 1969; Granger, 1972) or immunoglobulins (Andersson et al., 1972c; Kearney and Lawton, 1975a,b; Hammerling et al., 1978). Materials which are mitogenic for lymphocytes include plant lectins (Lis and Sharon, 1977), microbial products (Clagett and Engel, 1978) and chemicals (Diamanstein et al., 1973; Coutinho et al., 1974; Novogrodsky, 1976).

Some mitogens act selectively on either T or B cells and in doing so they provide convenient probes for identifying these cells in the absence of other markers. Furthermore, subsets of T and B cells can be identified by their reactivities to certain mitogens (Janossy and Greaves, 1971; Greaves and Janossy, 1972; Andersson et al., 1972b; Peavy et al., 1974).

While there is an extensive literature on the mitogen-induced responses of eutherian lymphocytes, particularly those of mice and man, information concerning lower vertebrates is relatively sparse. Lymphocytes from cyclostomes (Olson, 1967; Du Pasquier, 1976a), cartilaginous fishes (Lopez et al., 1974), bony fishes (Etlinger et al., 1975, 1978), amphibia (Wright and Cooper, 1978), reptiles (Cuchens et al., 1976) and birds

(Evans and Ivanyi, 1974; Waltenbaugh et al., 1977) all respond to one or more mitogens. Results from investigations of such responses have assisted in following the phylogeny of lymphocyte heterogeneity.

Of the metatherians, the Australian quokka (Setonix brachyurus) and tammar (Macropus augenii) (Ashman et al., 1972, 1976, 1977; Ashman and Keast, 1976) and the American opossum (D. virginiana) (Fox et al., 1976) have been studied in this respect. The dose response curves to Con.A in the quokka and the tammar (Ashman et al., 1976) were found to be similar to those recorded for hamster lymphocytes (Singh and Tevethia, 1972) while their responses to PWM resembled those of murine cells (Janossy and Greaves, 1971). Because of the similarities between these marsupials and eutherian mammals in their responses to mitogens, Ashman et al. (1976) have suggested that functional elements of the cellular immune response may have evolved along very similar lines in these groups of animals.

The limited information available on the responses of metatherian lymphocytes to mitogens prompted an investigation of the effects of a number of such substances on opossum lymphocytes. The aims of this study were twofold. First, to determine whether or not opossum B lymphocytes could be stimulated by recognized eutherian B cell mitogens. Data so obtained might indicate any similarities or differences in lymphocyte cell membrane structure and function between these mammalian groups. Second, to rank opossum B cell-stimulating mitogens in order of their abilities to induce DNA synthesis with a view to undertaking a study of the morphology and immunoglobulin secreting capabilities of polyclonally stimulated B cells from T. vulpecula.

## 4.2 Materials and Methods

### a. Collection of lymphocytes

Mononuclear cells were harvested from opossum blood and depleted of monocytes by techniques described in Chapter 3. The lymphocytes were cultured either unfractionated or after fractionation into B and T cell populations on nylon wool columns (Chapter 3). Lymphocyte viability was measured by trypan blue exclusion (Appendix III).

### b. Culture conditions

In a previous study in which four media were assessed for their abilities to maintain opossum lymphocyte viability in culture, Eagle's minimal essential medium (MEM, GIBCO) supplemented with 10 per cent foetal calf serum (FCS) was shown to be as satisfactory as any other tested (Southern, personal communication). Consequently MEM containing 10 per cent heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) FCS (Flow Laboratory) and enriched with glutamine (1 ml of 200 mM per 100 ml of medium) was used routinely. The medium also contained 100 IU penicillin, 100 ug of kanamycin and 100 ug of streptomycin per ml.

Lymphocytes were cultured in Micro Test II tissue culture plates (No. 3041, Falcon Plastics). Triplicate cultures, each containing  $2 \times 10^5$  lymphocytes in 200 ul volumes, were prepared. Concentrations of the various mitogens in 5 to 20 ul volumes of MEM-FCS were added as required. Mitogen-free cultures were used as controls. The culture plates were incubated in a humid atmosphere of 5 per cent  $\text{CO}_2$ -in-air at  $37^{\circ}\text{C}$  for various times (see below).



c. Mitogen stimulation

The following mitogens were used over a range of concentrations (see Results):

Insoluble Concanavalin A (Insol.Con.A) (Sigma): Concanavalin A (Con.A) is a soluble product of the jack bean, Canavalia ensiformis, and is mitogenic for T cells (Greaves and Roitt, 1968a; Janossy and Greaves, 1971). When rendered insoluble, the lectin is said to activate predominantly B cells (Andersson et al., 1972 a,b). The preparation used here consisted of Con.A bound to Agarose beads at a concentration of 300 mg of Con.A per gm of Agarose. The Insol.Con.A was suspended in MEM-FCS at a stock concentration of 1 mg of mitogen per ml.

Pokeweed mitogen (PWM) (Sigma): PWM, from the tuber of Phytolacca americana, is mitogenic for both B and T cells (Janossy and Greaves, 1971; Shortman et al., 1973). One mg of PWM per ml of MEM-FCS was prepared from the lyophilised powder.

Lipopolysaccharide B (LPS) (Difco): The LPS used was from E.coli 055:B5. This B cell mitogen (Peavy et al., 1970; Andersson et al., 1972b) was prepared at an initial concentration of 10 mg per ml of MEM-FCS. This material was held in a boiling water-bath for 60 min prior to use in order to inactivate the endotoxin (Andersson et al., 1972c).

Purified protein derivative (PPD): This B cell mitogen (Pearmain et al., 1963; Sultzer and Nilsson, 1972) was provided as a neutralised, freeze-dried powder by the Central Veterinary Laboratory, Weybridge, England. One mg of PPD was dissolved in 1 ml of MEM-FCS.

Dextran sulphate ( $\text{DxSO}_4$ ) (Sigma): Described by Gronowicz and Coutinho (1974) as a B cell mitogen,  $\text{DxSO}_4$  was prepared as a stock solution containing 10 mg per ml of MEM-FCS.

Protein A (Prot.A) (Pharmacia): One mg of Prot.A of Staphylococcus aureus origin, was dissolved in 1 ml of MEM-FCS. Prot.A has been described as a B cell mitogen (Forsgren et al., 1976; Moller and Landwall, 1977).

Lanotoside C (Lan.C) (Calbiochem): Hammarstrom and Smith (1979) reported that Lan.C, a Digitalis glycoside, was mitogenic for B cells. Lan.C was prepared at an initial concentration of 10 mg per ml of MEM-FCS.

Purified phytohaemagglutinin (PHA) (Wellcome): PHA from Phaseolus spp. is a recognized T cell mitogen (Nowell, 1960; Robbins, 1964). The reagent was dissolved in MEM-FCS at a concentration of 1 mg per ml.

#### d. Measurement of mitogen-induced DNA synthesis

Twenty-four hours before being harvested, the cultures were each pulsed with 0.2 uCi of (methyl- $^3\text{H}$ ) thymidine (specific activity 5 Ci/mmol) (The Radiochemical Centre) in 20 ul of PBS. At the end of the pulse period, the cells were harvested in a multiple cell harvester (Dynatch Mini Mash, Cooke) onto glass microfiber discs (GF/C, Whatman) which were then dried overnight at 37 $^{\circ}\text{C}$ . The dried filters were placed in scintillation vials containing 5 ml of a toluene-based scintillation fluid (Appendix II) and counted in a liquid scintillation spectrophotometer (Beckman). The counts per minute recorded for the mitogen stimulated cultures were divided by those recorded for the mitogen-free cultures to establish a 'stimulation index' (SI).

The means and standard errors were calculated for the SIs obtained from repeat experiments.

The following experiments were done:

1. Determination of lymphocyte viability in culture.

Blood lymphocytes were cultured in MEM-FCS and their viability was assessed daily for 7 consecutive days by trypan blue exclusion (Appendix III). Triplicate cultures were established. In a single experiment to determine the relative merits of homologous and heterologous serum supplements on maintaining cell viability, heat-inactivated opossum serum (10 per cent, final concentration) replaced FCS.

2. Determination of optimum culture times and mitogen concentrations for blood lymphocytes.

Lymphocytes were cultured in triplicate in each of the concentrations of the mitogens used. The cultures were pulsed for 24 hr after 1,2,3,5 and 7 days of culture. The cells were harvested and their incorporated radioactivity measured.

3. The response of blood B and T cells to selected mitogens.

The results of the above experiment allowed the mitogens to be ranked in order of their mitogenicity. The three most effective were used at optimal concentrations for optimal culture times to stimulate cultures of blood-derived B and T cells. Triplicate cultures were established and were pulsed and harvested as described above.

4. Stimulation of lymphocyte cultures with either washed, commercial Insol.Con.A (Insol.Con.A-W) or with laboratory-prepared Insol.Con.A (Insol.Con.A-LP).

Results from the previous experiment showed that the Insol.Con.A used stimulated T lymphocytes to a much greater degree than B lymphocytes. A possible explanation for this unexpected finding could have been the presence of free, soluble Con.A in the reagent used. Two approaches were used to examine this possibility. Firstly, the commercial material was washed three times with MEM before use. Secondly, freshly prepared Insol. Con.A was prepared by adsorbing Con.A (Sigma) onto carrier particles by the methods of Greaves and Bauminger (1972) and Andersson and Melchers (1973).

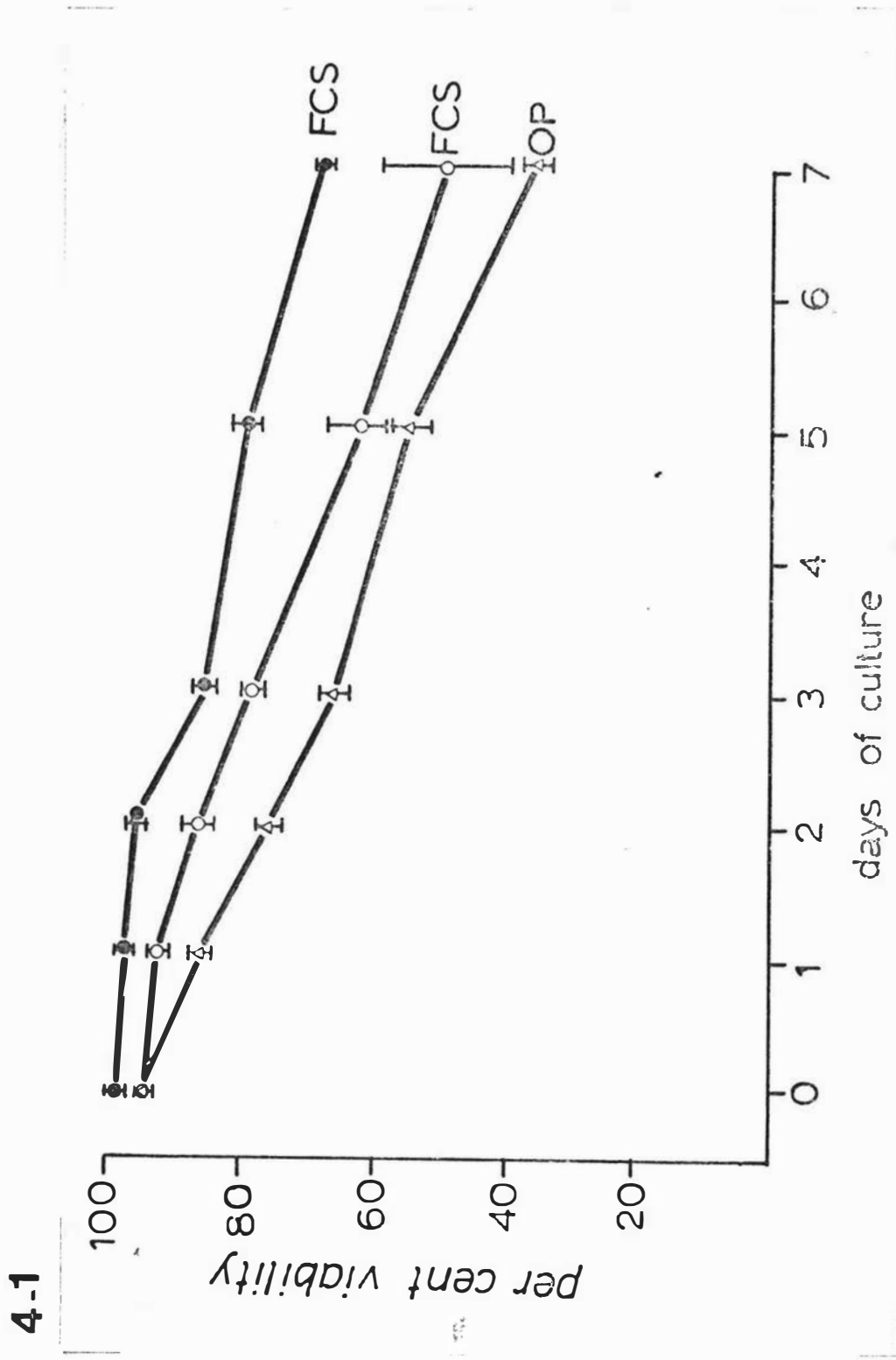
Ten mg of soluble Con.A (Sigma) were dissolved in 1 ml of 0.1M carbonate buffer, pH 8.5 (Appendix II) containing a 10 molar excess of alpha methyl-D-mannoside. One mg of cyanogen bromide-activated Sepharose beads (Pharmacia) was added and the mixture left at room temperature for 30 min. Unbound proteins were removed by washing with 0.1M carbonate buffer, pH 8.5, and PBS. The Sepharose-Con.A conjugate was equilibrated with MEM-FCS by incubation with this medium twice at 37°C for 24 hr. Dilutions of packed, Insol.Con.A-LP were prepared and used for mitogen stimulation studies.

#### 4.3 Results

##### a. Blood lymphocyte viability in culture

The viability of blood lymphocytes cultured in media supplemented with either FCS or homologous serum is shown in Fig. 4.1 which presents the results of two experiments in which FCS was used and one using opossum serum. In this experiment, as in all others, the lympho-

FIGURE: 4.1 The viability of opossum blood lymphocytes cultured in Eagle's minimal essential medium supplemented with either 10 per cent foetal calf serum (FCS) or 10 per cent opossum serum (OP). Results are given as mean percentages of triplicate cultures  $\pm$  standard error.



cytes were at least 95 per cent viable when the cultures were established. After 3 days of culture, the duration of many of the experiments described in this chapter, lymphocyte viability in the media supplemented with FCS was between 80 to 90 per cent, while the viability in cultures containing opossum serum was around 65 per cent. After 7 days these values fell to 50 to 70 per cent and 36 per cent respectively. The use of opossum serum did not appear to offer any advantage over the more readily available, standard FCS which was used in all subsequent experiments.

b. Determination of optimum culture times and mitogen concentrations

The SIs obtained by varying both the concentration of each mitogen and the duration of cell culture are shown in Tables 4.1a to 4.1g. This information is summarised in Table 4.2 which shows the optimal concentration of each mitogen and the culture period needed with this concentration to give a maximum SI. The values in Tables 4.1a to 4.1g are based on single experiments involving triplicate cultures for each mitogen concentration/time of culture combination. The values in Table 4.2 are again based on the results of triplicate cultures but are from repeated experiments using selected combinations of mitogen concentrations and culture times.

Insol.Con.A was the most potent mitogen giving an average SI of  $146.06 \pm 35.32$ , when used at a concentration of 50 ug per  $10^6$  cells over a 3 day culture period. PWM and LPS gave SIs of  $61.41 \pm 17.96$  and  $16.18 \pm 1.45$ , respectively, while the SI of PPD was  $3.21 \pm 0.16$ . The optimum concentration of PWM and LPS were 5 and 250 ug per  $10^6$  cells, respectively. These concentrations were most effective using a 3 day culture period for PWM and a 2 day culture period for LPS. Prot.A,  $\text{DxSO}_4$  and Lan.C gave SIs of less than 2.00.

Table 4.1 Mitogenic stimulation of opossum blood lymphocytes with various mitogens at different concentration and cultured for different days.

a) Insoluble Con.A : ug per 10 <sup>6</sup> cells						
Day	1	5	10	25	50	100
1	2.10*	4.66	6.64	20.43	9.96	4.71
2	3.59	9.91	18.71	26.71	71.81	83.30
3	1.17	2.12	9.83	111.27	<u>184.30</u>	180.16
5	2.23	5.49	7.82	12.71	34.40	30.12

b) Pokeweed Mitogen : ug per 10 <sup>6</sup> cells						
Day	1	5	10	25	50	100
1	5.02	7.29	4.21	2.22	1.99	1.02
2	7.69	13.60	6.33	3.21	1.32	0.97
3	47.72	<u>52.51</u>	11.79	8.58	3.91	2.99
5	3.28	15.97	2.81	1.49	1.26	0.67

c) Lipopolysaccharide : ug per 10 <sup>6</sup> cells						
Day	10	50	100	250	500	1000
1	1.92	4.00	5.32	7.17	5.12	2.92
2	5.81	7.93	11.58	<u>13.53</u>	3.76	2.88
3	1.76	8.43	10.96	10.66	6.04	1.98
5	2.17	2.56	2.99	1.67	1.00	0.25

\* Stimulation index (cpm mitogen-treated cultures/cpm control) from triplicate cultures from one experiment. Maximum stimulation index obtained at optimal concentration and optimal culture period is underlined.



Table 4.1 (contd).

Day	d) Purified Protein Derivative : ug per $10^6$ cells					
	1	5	10	25	50	100
1	0.78	1.80	1.98	2.02	2.12	2.00
2	0.77	1.79	2.09	2.78	2.91	2.72
3	1.53	1.90	2.43	3.18	<u>3.36</u>	0.64
5	0.71	1.52	1.97	2.12	2.57	2.02

Day	e) Staphylococcal Protein A : ug per $10^6$ cells					
	1	5	10	25	50	100
1	0.83	0.98	1.01	1.31	1.71	0.83
2	0.71	0.99	1.02	1.23	1.11	0.93
3	0.58	0.62	0.79	<u>1.95</u>	1.65	1.43
5	0.78	1.02	1.43	1.61	1.60	1.21

Day	f) Dextran sulphate : ug per $10^6$ cells					
	10	50	100	250	500	1000
2	1.00	0.82	0.63	0.58	0.68	0.68
3	1.07	0.70	0.38	0.39	0.63	0.55
5	0.77	<u>1.37</u>	0.53	0.51	0.50	0.58
7	1.02	1.20	0.98	0.70	0.50	0.45

Day	g) Lanotoside C : ug per $10^6$ cells					
	10	50	100	250	500	1000
1	0.51	0.53	0.71	0.83	0.88	0.90
2	0.41	0.62	0.65	0.81	0.93	0.98
3	0.52	0.41	0.61	0.78	0.87	<u>1.44</u>
5	0.70	0.82	0.91	0.93	0.93	0.99

Table 4.2 Maximum stimulation indices recorded for opossum blood lymphocytes when cultured with selected doses of mitogens for selected periods.

Mitogen	ug per $10^6$ cells	Days cultured	Stimulation index <sup>*</sup>
Insol.Con.A	50	3	146.06 $\pm$ 35.32 (4) <sup>**</sup>
PWM	5	3	61.41 $\pm$ 17.96 (3)
LPS	250	2	16.18 $\pm$ 1.45 (4)
PPD	50	3	3.21 $\pm$ 0.16 (2)
Prot.A	25	3	1.95 $\pm$ 0.14 (1)
DxSO <sub>4</sub>	50	5	1.80 $\pm$ 0.43 (2)
Lan.C	1000	3	1.44 $\pm$ 0.40 (1)

\* Stimulation index = cpm mitogen-stimulated cultures / cpm control cultures, maximum values given as means  $\pm$  standard errors.

\*\* Figures in parentheses refer to number of experiments.

c. The responses of blood B and T lymphocytes to selected mitogens.

Insol.Con.A, PWM and LPS, the mitogens showing maximum SIs for unfractionated blood lymphocyte cultures, were used to stimulate cultures of B or T lymphocytes in order to determine which population was responding. The results are presented in Table 4.3. Insol.Con.A and PWM were more efficient at stimulating T cells than B cells. When Insol.Con.A was used, the ratio of the SI for T cells (391.05) to that of B cells (42.59) was 9.18; for PWM this value was  $45.98/4.65 = 9.97$ . LPS stimulated B cells more than it did T cells; the ratio of T cell SI (7.13) to that of B cells (19.52) was 0.36 for this mitogen. B and T cell cultures were also stimulated with PHA. Preliminary experiments using unfractionated blood lymphocyte cultures showed that maximum stimulation was achieved with 50 ug PHA per  $10^6$  cells in 3 day cultures. When this combination of mitogen concentration and culture period was applied to T and B cell cultures the ratio of SI for T cells to SI for B cells was  $436.05/16.52 = 26.39$ .

d. The responses of blood B and T lymphocytes to Insol.Con.A, Insol.Con.A-W and Insol.Con.A-LP.

The mitogenic effects of the three Insol.Con.A preparations are shown in Table 4.4. In a single experiment all three stimulated T cells considerably more than B cells. The commercial material (Insol.Con.A) gave the highest SI T cells to SI B cells ratio (22.21) and Insol.Con.A-LP the lowest (6.5). These results show that the high level of T cell stimulation seen initially with Insol.Con.A (Table 4.3) was unlikely to be due to the presence of soluble Con.A in the reagent used.

Table 4.3 Stimulation indices recorded for opossum blood B and T lymphocytes cultured with optimal concentrations of mitogens for optimal culture periods.

Mitogen	ug/10 <sup>6</sup> cells	Stimulation index <sup>*</sup>	
		B cells	T cells
Insol. Con. A	50	42.59 ± 3.92 (5) <sup>a</sup>	391.05 ± 128.22 (6)
PPM	5	4.65 ± 1.05 (4)	45.98 ± 11.32 (4)
LPS	250	19.52 ± 3.23 (4)	7.13 ± 1.30 (3)
PHA	50	16.52 ± 8.29 (2)	436.05 ± 164.50 (2)

\* Stimulation index (cpm mitogen-stimulated cultures / cpm control culture), maximum values shown as means ± standard errors.

a--Numbers in parentheses show number of experiments.

Table 4.4 Stimulation indices of blood B and T lymphocytes stimulated with three different preparations of Insol.Con.A.

Mitogen	Lymphocytes	
	B cells	T cells
Insol.Con.A	13.81 $\pm$ 0.47	306.50 $\pm$ 15.38 <sup>*</sup>
Insol.Con.A-W <sup>1</sup>	13.36 $\pm$ 0.37	210.70 $\pm$ 6.35
Insol.Con.A-LP <sup>2</sup>	6.24 $\pm$ 0.92	38.99 $\pm$ 8.94

\* Stimulation index (cpm mitogen-stimulated cultures / cpm control culture), maximum values shown as means  $\pm$  standard errors, from triplicate cultures from one experiment.

1) Washed Insoluble Con.A; 2) Laboratory prepared Insol. con.A.

#### 4.4 Discussion

The results presented in this chapter show that opossum blood lymphocytes respond to substances which are recognised as being mitogenic for eutherian lymphocytes. Of the materials tested for this effect, Insol. Con.A, PWM and LPS, in that order, were the most effective at inducing DNA synthesis in cultures of unfractio- nated lymphocytes. Consequently these substances were used to stimulate cultures of B or T cells in order to determine which lymphocyte subset was responding.

Insol.Con.A consistently induced more than twice as much DNA production by cultured lymphocytes than did PWM. When an analysis was made of the cell type respon- sible for this activity it was found to reside predomi- nantly in T lymphocyte as these cells generally incorpo- rated about 9 times more tritiated thymidine than did B cells. This is in contrast to the findings of Andersson et al. (1972b) and Greaves and Janossy (1972) who showed that mouse thymus cells failed to react to either locally concentrated or insolubilised Con.A. The effect that insolubilisation of a mitogen has on its capacity to stimulate T cells is somewhat controversial. Basham and Waxdal (1977) maintain that Succinyl-Con.A cross-linked into an insoluble aggregate stimulates DNA synthesis equally as well in both T and B cells of mouse origin. Similarly, Greaves and Bauminger (1972), in mice, and Michlmayr et al. (1975), in humans, showed that inso- lubilised PHA stimulated both B and T cells, although B cells were more reactive than T cells. Since it was adequately demonstrated that the opossum T cell stimu- lation was not caused by soluble Con.A being present in the reagents used, it must be concluded that T.vulpecula T cells are stimulated by Insol.Con.A to a much greater degree than are B cells.

It was also a consistent finding that T cell cultures were more responsive to the mitogen than were cultures of unfractionated lymphocytes, the difference being greater than could be accounted for by a simple increase in the number of T cells available. Hersh et al. (1974) and Bona et al. (1976) have proposed a role for suppressor B cells in PHA- and PPD-stimulated cultures. The present results suggest that a similar suppressor population may occur in opossum lymphocytes.

The efficiency of nylon wool fractionation in providing essentially T or B cell populations in this work is difficult to assess. Cells bearing surface immunoglobulin occur at a frequency of 1 to 7 per cent in the non-adherent, presumably T cell fraction (Chapter 3). In the absence of a T cell marker it is impossible to estimate the number of these cells in the nylon wool-adherent, surface immunoglobulin-enriched population. An assessment of this fractionation method was attempted by culturing both cell populations with PHA. While Greaves et al. (1974a) and Geha et al. (1974) have provided evidence that purified B lymphocytes of human origin are not stimulated by PHA, others report the contrary (Phillips and Roitt, 1973; Chess et al., 1974a,b). The 27-fold difference in T cell-associated DNA synthesis over that of B cells would argue in favour of a successful T/B cell separation by the technique used.

The response of opossum blood lymphocytes to PWM resembles those of eutherian species to this mitogen (Janossy and Greaves, 1971; Greaves and Janossy, 1972; Waxdal and Basham, 1974; Janossy and Greaves, 1975; Waxdal et al., 1976; Saxon et al., 1977) but differ markedly to the response of the metatherian quokka. Ashman et al. (1976, 1977) reported that the quokka response to PWM was independent of the mitogen dose. In

this study the optimum dose of PWM was 5 ug per  $10^6$  cells and any higher concentration markedly reduced DNA synthesis, a situation comparable to that reported for mouse (Janossy and Greaves, 1971) and D. virginiana lymphocyte cultures (Fox et al., 1976). Opossum T lymphocytes were stimulated by PWM some 10 times more effectively than B cells, similar results having been reported for eutherians (Janossy and Greaves, 1975; Weksler and Kuntz, 1976; Moretta et al., 1979). PWM activation of B cells is thought to be largely dependent on T cell help (Fauci et al., 1976; Johnsen and Madsen, 1979), a finding which explains the relatively low SIs of the B cell cultures.

The need for monocytes in the PWM-driven activation of eutherian lymphocytes is controversial, some workers describing an essential role for these cells (Finkelman and Lipsky, 1979; Rosenberg and Lipsky, 1979) while others do not (Fauci et al., 1976; Saxon et al., 1977). In four experiments, the results of which are not presented here, it was seen that the level of DNA synthesis in monocyte-depleted cultures was no less, and was frequently greater, than in cultures containing monocytes. However, a more stringent depletion of monocytes would be needed before a conclusion could be drawn with certainty on this point.

Although the response of unfractionated lymphocytes to LPS was considerably less than to the other two mitogens, LPS was the only one to stimulate B cells more than T cells. LPS has been shown to induce DNA synthesis in B cells and is thought to have no effect on T lymphocyte cultures (Peavy et al., 1970; Andersson et al., 1972b; Grey et al., 1972). In cultures containing both types of lymphocytes LPS may act on T cells by way



of a soluble mediator elaborated by B cells (Elfenbein et al., 1973). Furthermore, Kunori and Ringden (1979) studying human blood lymphocyte subpopulations, have shown that a small proportion of erythrocyte-rosette forming cells (presumably T cells) were also activated along with B cells. The authors have suggested that this T cell proliferation could be secondarily induced by LPS-activated B cells.

But in this study, T cell cultures stimulated with LPS showed SIs of around 7, a value roughly one-third of that recorded for B cell cultures. The low level of surface immunoglobulin-positive cells in the T cell fractions has been commented on. It must be concluded, then, that either opossum T cells respond to LPS or that a population of surface immunoglobulin negative cells in T.vulpecula is stimulated by this mitogen. The SIs of opossum blood lymphocyte and of B lymphocyte cultures stimulated with LPS were similar, a finding also reported for human blood lymphocytes (Ringden et al., 1977). This may reflect a degree of T helper cell activity in the cultures of mixed lymphocyte nature, as reported for mouse (Narcross and Smith, 1977; Goodman and Weigle, 1979), rabbit (Shinohara and Kern, 1976) and human (Finkelman and Lipsky, 1979) lymphocytes.

Although PPD is acknowledged generally as being a potent B cell mitogen for mice and guinea pig lymphocytes (Sultzer and Nilsson, 1972; Andersson et al., 1979), cultures of unfractionated opossum blood lymphocytes showed a comparatively low SI to this material. To determine if this result could have been due to a lack of mitogenicity of the PPD available, cultures of rabbit and mouse spleen-derived B cells were stimulated with the PPD (data not presented). The results were equivocal in that rabbit B cells responded well (SI 54.00) while mouse B

cells responded poorly (SI, 1.4). The low response of the mouse cells to PPD could be due to strain variation (Gronowicz and Coutinho, 1975) or variations in different batches of PPD (Andersson et al., 1979). The exorbitant price of commercially available PPD precluded further experiments to determine the effect of this material on opossum lymphocytes. However, the failure to detect a proliferative effect of PPD on T.vulpecula lymphocyte cultures is in agreement with the findings of Fox et al. (1976) who showed that lymphocytes from normal D.virginiana also failed to react to this mitogen.

Protein A is mitogenic for human (Rodney et al., 1972) and mouse (Moller and Landwall, 1977) B cells. Some activity on T cell populations, which acted synergistically on B cell responses, has also been reported (Sakane and Green, 1978). It is a potent mitogen for canine blood lymphocytes, being more effective than either PHA or Con.A in stimulating unfractionated lymphocyte populations (Betton et al., 1980). Neither Prot.A,  $DxSO_4$  nor Lan.C showed any obvious mitogenicity in the few experiments in which they were used in this investigation. Whether or not these negative results indicate a genuine failure of opossum lymphocytes to respond to these mitogens cannot be judged at this stage.

In most of the experiments reported here lymphocytes from different individuals have been used on each occasion. A study of possible day-to-day variations within an individual animal over a period of time might provide interesting data.

In summary, it can be said that the responsiveness of opossum lymphocytes to the three mitogens used resembles those described for human (Peavy et al.,

1970; Keightley et al., 1976; Smith et al., 1979), mouse (Janossy and Greaves, 1971; Kearney and Lawton, 1975a) and other eutherian (Bryant et al., 1973; Harvey et al., 1974; Mumford et al., 1975) lymphocytes.

## CHAPTER 5

### POLYCLONAL B CELL ACTIVATION:

#### ULTRASTRUCTURE OF TRANSFORMED CELLS

##### 5.1 Introduction

Lymphocytes proliferate and enlarge into blast cells when stimulated with mitogens in vitro. Two blast forms are recognised, their occurrence depending on the mitogen used. T cell mitogens induce blasts showing numerous mitochondria, free ribosomes and well developed Golgi apparatuses (Farnes et al., 1964; Biberfeld, 1971; Janossy et al., 1972; Shands et al., 1973). Blasts induced by B cell mitogens show, in addition to these features, the development of endoplasmic reticula with numerous associated polyribosomes (Janossy et al., 1972; Janossy and Greaves, 1975; Pages et al., 1978). PWM which stimulates both T and B cells (Waxdal and Basham, 1974) induces the formation of both types of blasts (Movat and Fernando, 1965; Bach and Reyes, 1976).

In this chapter the ultrastructure of non-stimulated opossum blood lymphocytes is compared with those of cells stimulated in vitro for varying periods with either LPS, PWM or PHA.

##### 5.2 Materials and Methods

###### a. Collection of lymphocytes

Lymphocytes were harvested from blood, tested for viability and counted as described in Chapter 3. Cells from the thymus and bone marrow of one animal were also examined.

b. Cell culture

The lymphocytes were suspended in culture medium (Chapter 4) at a concentration of approximately  $5 \times 10^6$  cells per ml. One ml amounts of lymphocyte suspension were cultured in 35x10 cm plastic tissue culture dishes (No.3001, Falcon Plastics) at  $37^{\circ}\text{C}$  in 5 per cent  $\text{CO}_2$ -in-air. Cultures were fed daily with 90 ul of supplemented medium.

c. Mitogens

The cultures were stimulated with either LPS, PWM or PHA at concentrations of 250 ug, 5 ug or 50 ug per  $10^6$  cells respectively (Chapter 4). LPS was detoxified in a boiling water bath for 60 min prior to use (Anderson et al., 1972c). Cultures lacking mitogens served as controls. The cultured cells were harvested for electron microscopy after the incubation times shown in the results.

d. Electron microscopy

Cell pellets were prepared by the method of Douglas et al. (1967a). The cells were collected by scraping the surfaces of the culture dishes with a plastic policeman and washed twice with MEM-FCS, centrifugation being at 225 g for 10 min. The cell pellet was resuspended in a drop of medium and taken up into a haematocrit tube and centrifuged at 15,000 g for 3 min. The tube was cut at the interface of the cell pellet and the supernatant, and the cells placed in Karnovsky's fixative (Appendix II) for 4 hr. The fixed cells were then extruded from the haematocrit tube and washed with cold phosphate buffer, 0.1M, pH 7.2 (Appendix II) and post-fixed in 1 per cent osmium tetroxide for 30 min at  $4^{\circ}\text{C}$ . The cells were then dehydrated in increasing concentrations of ethanol from

25 per cent to absolute. They were stained with 1 per cent uranyl acetate at the 75 per cent ethanol stage. Final dehydration was carried out with 100 per cent propylene oxide. Infiltration and embedding of the cells were achieved by treating the cells initially with a 25 per cent epoxy resin mixture (Appendix II) in propylene oxide and then with a 100 per cent epoxy resin mixture. The resin was polymerised at 60°C for 36 hr.

Thick and thin sections were cut on a microtome (Ultratome III, LKB). After trimming the block, sections of 0.5 to 1.0  $\mu\text{m}$  thickness were cut, picked up on a microscope slide, stained with toluidine blue/basic fuchsin stain (Appendix II) on an 80°C hot plate and examined. Areas with numerous cells were selected, trimmed and thin sections were cut and floated onto carbonised collodion-coated grids. The thin sections were stained with uranyl acetate-lead citrate stain (Appendix II) and examined in a EM-200 Philips electron microscope.

### 5.3 Results and Discussion

The electron micrographs shown are sections of individual cells and as such cannot be used either to measure cell diameters or to gauge accurately nuclear morphology. It must also be pointed out that the sections have been chosen to show certain features of the cells. This raises the question as to whether or not they are "typical" of a given preparation. The heterogeneity of lymphocytes and their dynamic nature must result in a range of cell types being present in any population. Difficulties also arise in the interpretation of thin sections. For example, Figs. 5.1 to 5.3 are representatives of a non-incubated, non-stimulated starting population. Fig. 5.2 shows a cell with a simple structure. The nucleus appears round and central to the cell. The rim of cytoplasm shows only

some scattered ribosomes, a few vacuoles and the suggestion of slight endoplasmic reticulum development. A more elaborate cytoplasmic architecture is seen in the cell in Fig. 5.3. Here the cytoplasm is occupied by numerous mitochondria, abundant ribosomes, some of which are aggregated, and traces of endoplasmic reticulum. There is a Golgi zone evident with centrioles, microtubules and vesicles. In addition the nucleus appears to be eccentric and indented. However, had this cell been sectioned in the plane A-B as indicated it could have appeared very similar to that shown in Fig. 5.2.

With this reservation in mind it is difficult to be convinced that resting B and T cells can each be recognized by their unique architectures. Thus B lymphocytes of mice and humans are said to have "clear" cytoplasm containing numerous organelles whereas T cells of these species have "dense" cytoplasm lacking in structural features (Matter *et al.*, 1972; Toma *et al.*, 1977; Le Bouteiller *et al.*, 1978). In addition, the nuclei of B cells have been described as being more irregular and having more indentations than those of T lymphocytes (Olah *et al.*, 1975; Le Bouteiller *et al.*, 1978). If the plane of sectioning can decide the apparent nuclear outline then an extremely large sample of cells would be needed to confirm or refute this suggestion. Certainly Shohat *et al.* (1973) could not distinguish mouse spleen B and T cells from each other on similar criteria. When opossum blood lymphocytes were 'classified' on their nuclear outlines little over half of them (56 per cent) were found to be T cells while 30 per cent of thymic lymphocytes failed to fit this T cell description.

Cells cultured for 24 hr, either in the absence or presence of mitogens appeared generally similar (Figs. 5.4 to 5.6). Their cytoplasm were usually simple and showed scattered mitochondria and numerous ribosomes

which were occasionally aligned on sparse endoplasmic reticulum. There was frequently evidence of Golgi zones though to a variable extent.

After 2 days of culture the mitogen-treated cells seemed to show an increased complexity of their cytoplasm when compared with the controls. The control lymphocytes (Fig. 5.7) were similar in appearance to those described from human blood lymphocyte cultures (Douglas et al., 1967a) and mouse (Shands et al., 1973) and Leopard frog spleen cultures (Wright et al., 1979). Both the LPS- and the PWM-stimulated cells (Figs. 5.8 and 5.9) showed same degree of activation. Their cytoplasm to nucleus ratios appeared greater than that of the controls and there were signs of increased cytoplasmic activity. Particularly prominent was the development of numerous mitochondria with obvious cristae, a feature probably reflecting an increased energy demand by these cells. The LPS-treated cells may have shown some advance in endoplasmic reticulum development. Prominent nucleoli were seen in many of the mitogen-treated cells.

Even more marked were the changes seen in cells cultured for 3 days. Many of the control cells (Fig. 5.10) showed evidence of increased cytoplasmic activity particularly in the numbers of enlarged mitochondria and the appearance of centrioles and Golgi zones. The stimulus for this activity may have been provided by the foetal calf serum present in the medium (Shigi et al., 1977). Cells stimulated with PWM varied in ultrastructure from seemingly resting cells (Fig. 5.11) to those showing intense cytoplasmic activity. Thus, Fig. 5.12 shows a cell with a marked re-arrangement of nuclear material



and a complex cytoplasm. The cytoplasm contains enlarged mitochondria, frequently showing a loss of cristae, and dilated endoplasmic reticula. While numerous polyribosomes are seen associated with the endoplasmic reticula many appear to be free in the cytoplasm (Fig. 5.13). While the eccentric nucleolus of this cell and also its chromatin distribution resemble those of the LPS-stimulated cell shown in Fig. 5.14, the two cells differ appreciably in their overall appearance and their relationship to each other is not clear.

The PWM-stimulated cell in Fig. 5.12 is different in ultrastructure to both the LPS-stimulated cell in Fig. 5.14 and the PHA-induced thymus cell in Fig. 5.24. PWM is undoubtedly a potent B and T cell stimulator in opossums (Chapters 4 and 6) but whether or not the cell in Fig. 5.24 is being driven towards a B or T blast, or indeed, has already peaked in its activity, cannot be decided.

Plasma cells were first seen in LPS-treated cultures after 2 days of incubation, their numbers seeming to increase over the following 24 to 48 hr. By day 4, approximately 26 per cent of the cells were identified as belonging to the plasma cell series. Both in their frequency and ultrastructure these cells resembled those described in LPS-activated mouse spleen cell cultures (Gudat et al., 1970; Shands et al., 1973; Shohat et al., 1973). The LPS-induced plasmacytes had eccentric nuclei which frequently showed prominent nucleoli (Fig. 5.14). The cytoplasm of these cells contained enlarged mitochondria and numerous cytoplasmic vacuoles in addition to extensive endoplasmic reticula and associated polyribosomes (Figs. 5.15 and 5.16). Most of the cells appeared to resist LPS stimulation and were presumably T cells and LPS-non-responsive B cells (Janossy and Greaves, 1975).

The plasma cell of Fig. 5.14 should be compared with the cell shown in Figs. 5.25 and 5.26. The latter cell, seen in a bone marrow preparation, is clearly of the same lineage as that in Fig. 5.14 but the two differ in appearance, a likely explanation being that they represent different developmental stages.

Five day cultures contained, in addition to the blast forms described above, cells with numerous large mitochondria and, frequently, vacuoles and electron-dense bodies (Fig. 5.17 to 5.19). The general appearance of these cells was similar to that of the thymus cell blasts in Fig. 5.24.

After 7 days the cultures contained considerable amounts of cell debris. The control cells were frequently vacuolated, the vacuoles containing small inclusions (Fig. 5.20). Plasmacytes were evident in the LPS-stimulated cultures, the nuclei in many instances being largely euchromatin while the cytoplasm was rich in laminated endoplasmic reticula and contained many mitochondria (Figs. 5.21 and 5.22). PWM-stimulated cells frequently contained numerous small vacuoles (Fig. 5.23) and there was evidence of uropod formation by these cells. Uropod formation in PHA- and Con.A-stimulated cultures has been reported from other species (McFarland and Schechter, 1970; Toma et al., 1977; Le Bouteiller et al., 1978; Wright et al., 1979). It has been suggested that uropods are involved in cell-to-cell interactions (Biberfeld, 1971) and may also serve as areas specialised for endocytosis (Rosenthal and Rosenstreich, 1974). The ultrastructural features of the uropods seen in the opossum lymphocyte cultures were essentially the same as those described in human blood lymphocyte cultures (Rosenthal and Rosenstreich, 1974).

These results show that the ultrastructural features of both resting and mitogen-stimulated blood lymphocytes of T.vulpecula are analogous to those seen in mice (Janossy and Greaves, 1975; Pages et al., 1978) and man (Douglas et al., 1967a,b). Stimulation of the lymphocyte cultures with a known B cell mitogen certainly gave rise to characteristic B cell blast forms. Overall the results further support the notion that metatherian and eutherian immune functions are similar and that they are based on cells of closely similar, if not identical, structure.

FIGURES 5.1 to 5.26 are electron micrographs of sections of opossum lymphocytes. The following abbreviations are used to label the ultrastructural features of these cells.

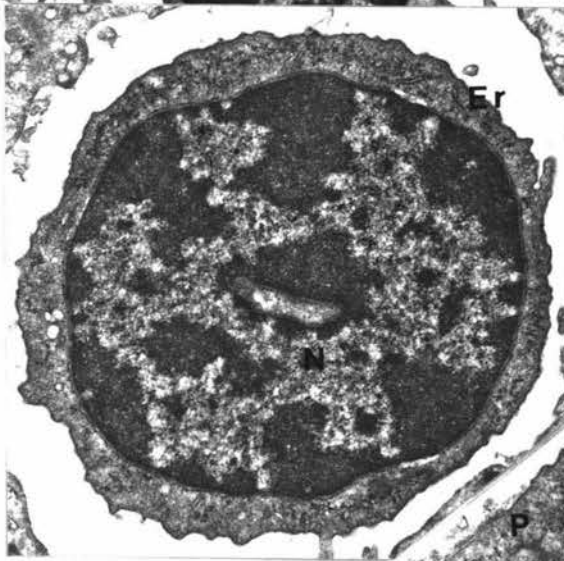
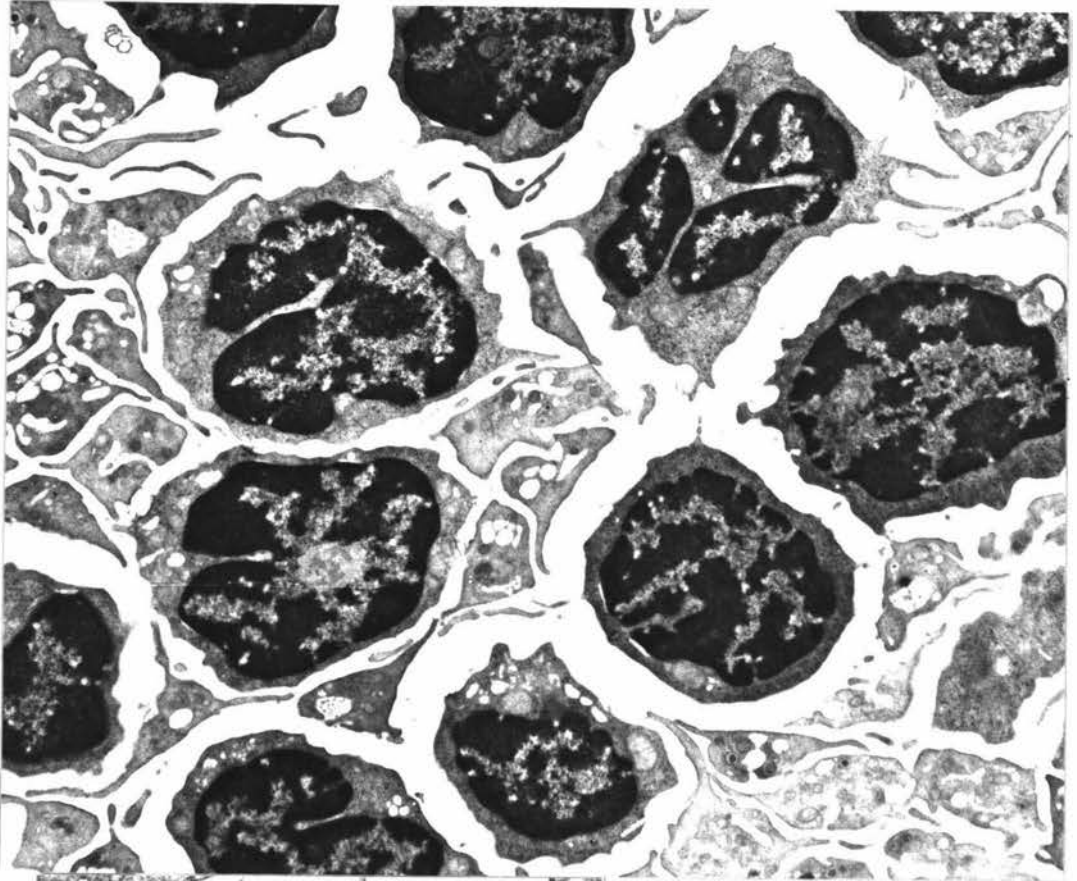
C	Centriole
Ed	Electron dense lysosome-like bodies
Er	Endoplasmic reticulum
G	Golgi apparatus
Ig	Immunoglobulin
M	Mitochondria
Mt	Microtubules
N	Nucleus
Nu	Nucleolus
P	Polyribosomes
R	Ribosomes
V	Vacuole
Vs	Vesicle

FIGURE 5.1 Opossum blood lymphocytes. Normal.  
X6,400

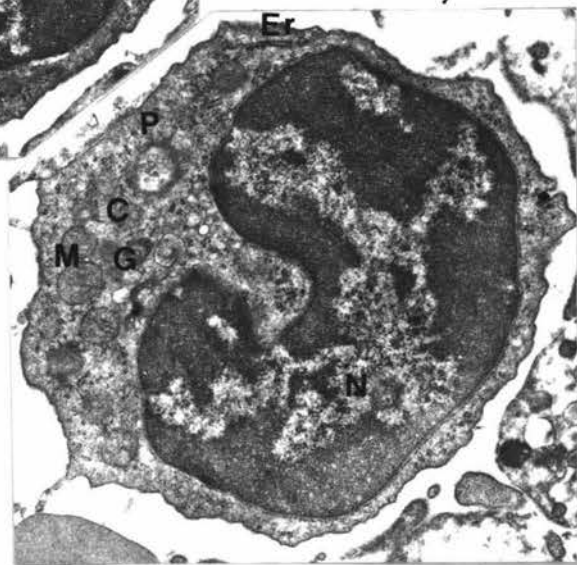
FIGURE 5.2 Opossum blood lymphocyte. Normal.  
X13,500

FIGURE 5.3 Opossum blood lymphocyte. Normal.  
X13,500

5.1



5.2



5.3

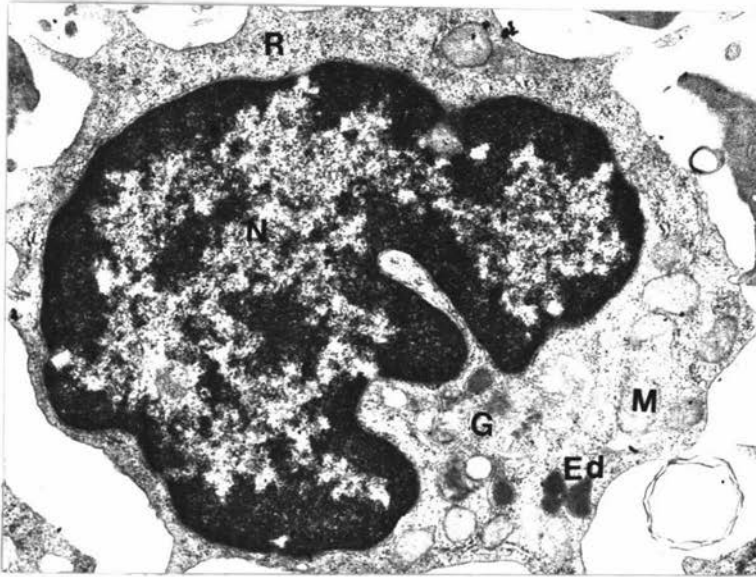
A

B

FIGURE 5.4 Opossum blood lymphocyte. 1 day culture,  
control. X 13,500

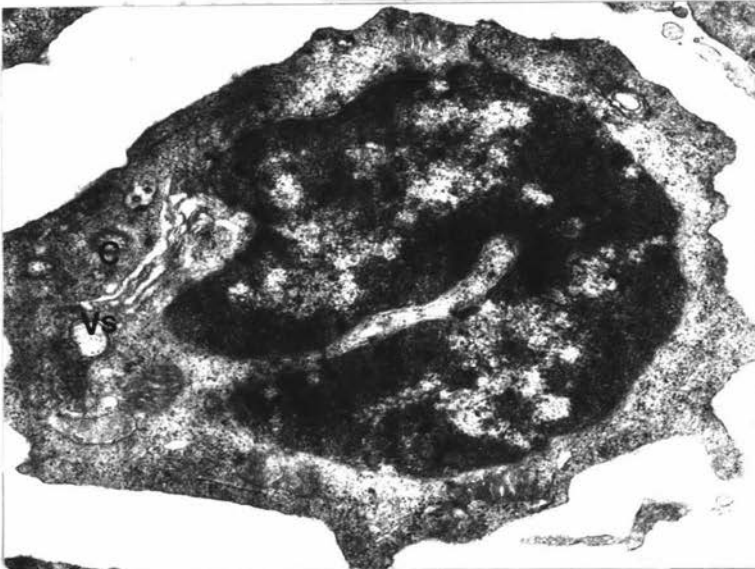
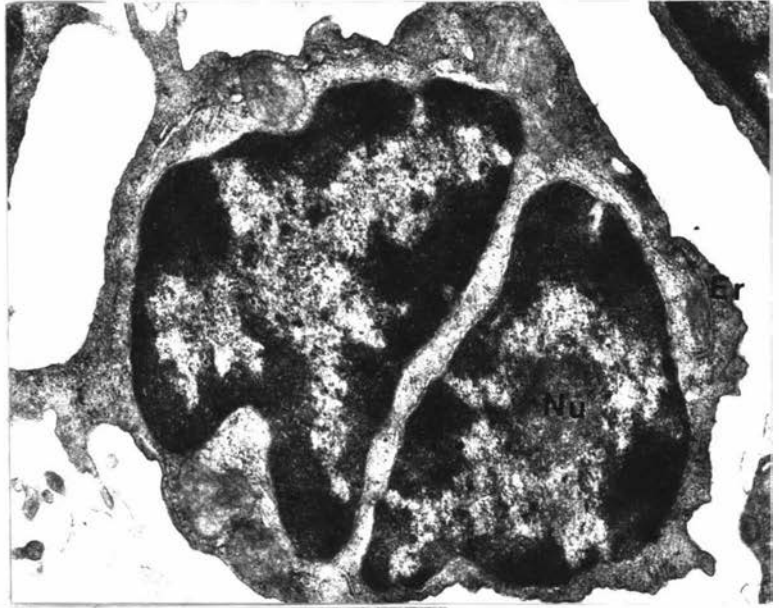
FIGURE 5.5 Opossum blood lymphocyte. 1 day culture,  
stimulated with LPS. X 17,500

FIGURE 5.6 Opossum blood lymphocyte. 1 day culture,  
stimulated with PWM. X 22,500



5.4

5-5



5.6



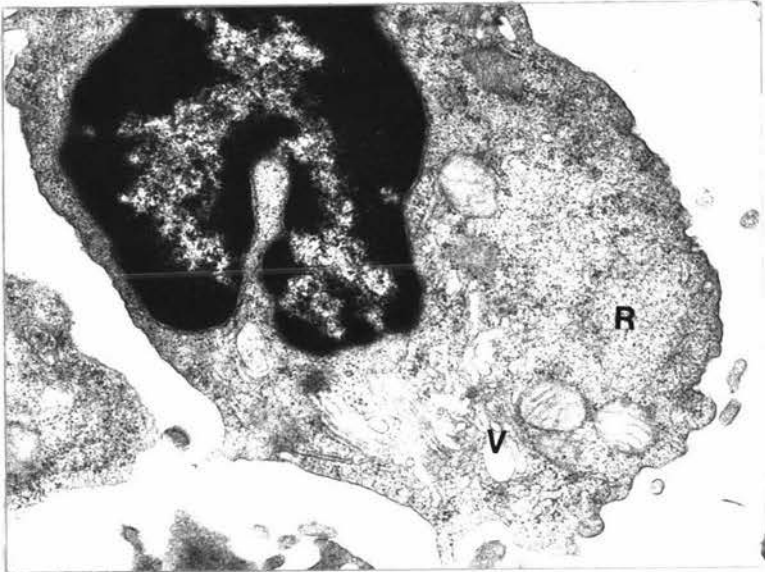
FIGURE 5.7 Opossum blood lymphocyte. 2 day culture, control. X 17,500

FIGURE 5.8 Opossum blood lymphocyte. 2 day culture, stimulated with LPS. X 17,500

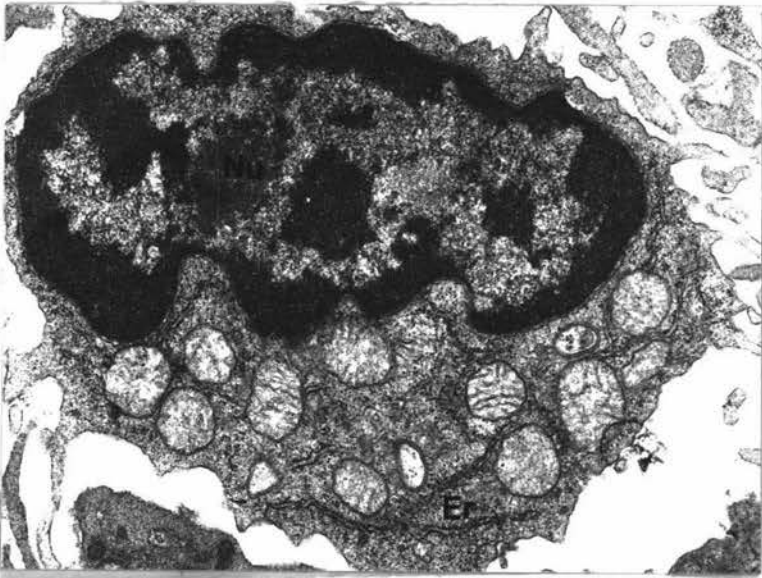
FIGURE 5.9 Opossum blood lymphocyte. 2 day culture, stimulated with PWM. X 17,500



5-7



5-8



5-9

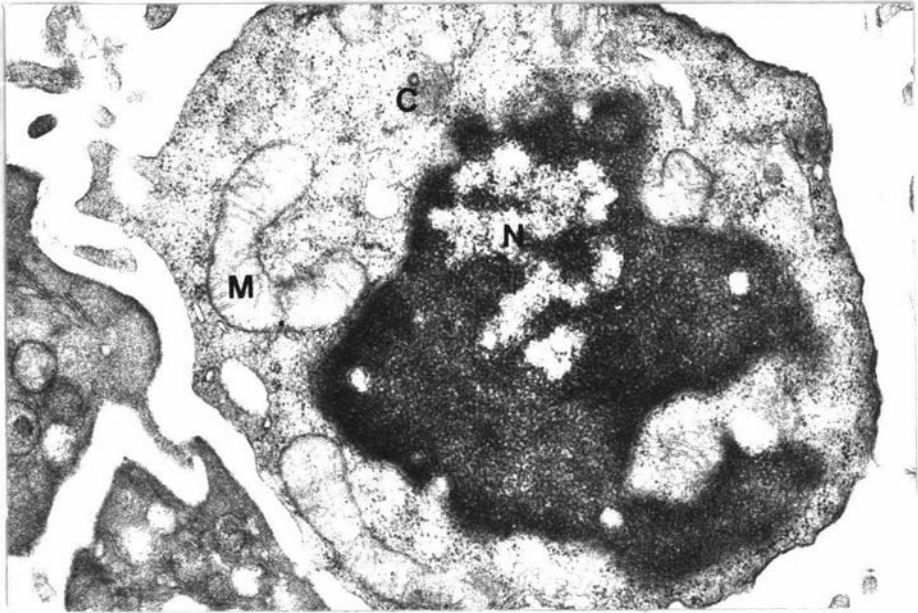
FIGURE 5.10 Opossum blood lymphocyte. 3 day culture, control. X 22,500

FIGURE 5.11 Opossum blood lymphocyte. 3 day culture, stimulated with PWM. X 8,100

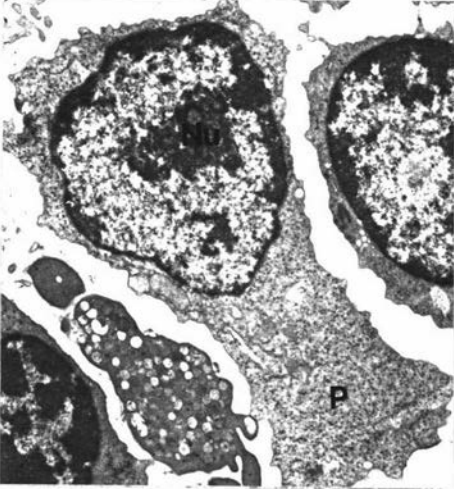
FIGURE 5.12 Opossum blood lymphocyte. 3 day culture, stimulated with PWM. X 13,500

FIGURE 5.13 Higher magnification of part of the cytoplasm of blast cell in Fig.5.12 showing enlarged mitochondria and dilated rough endoplasmic reticulum. X 61,000

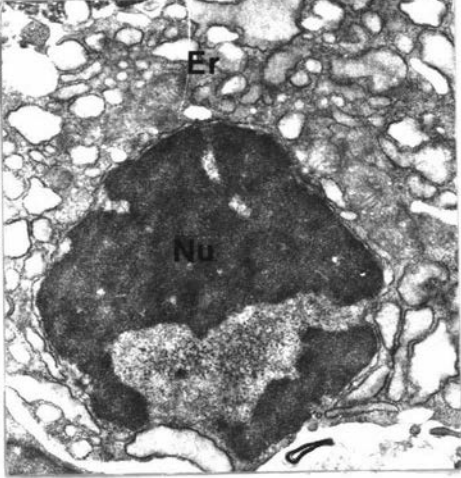
5.10



11



12



5.13

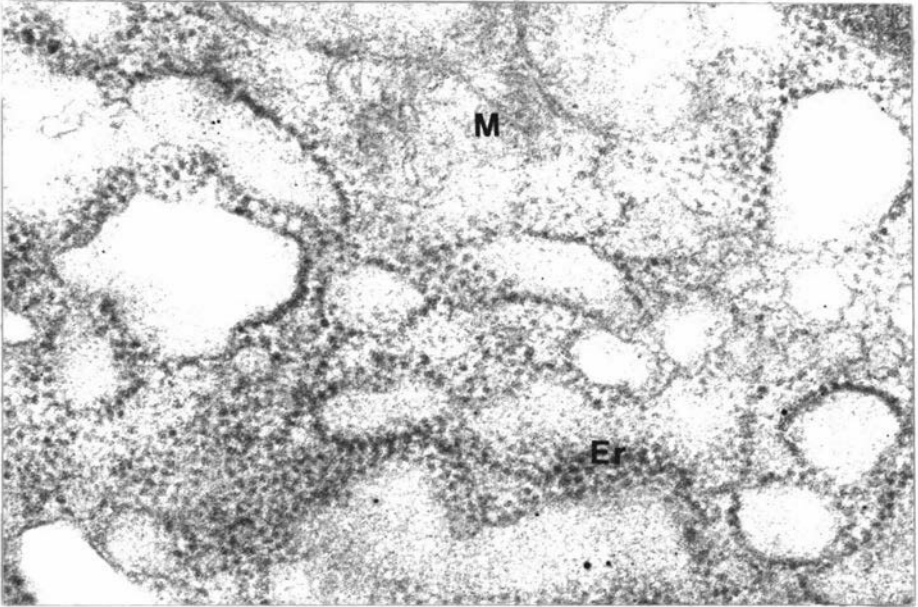
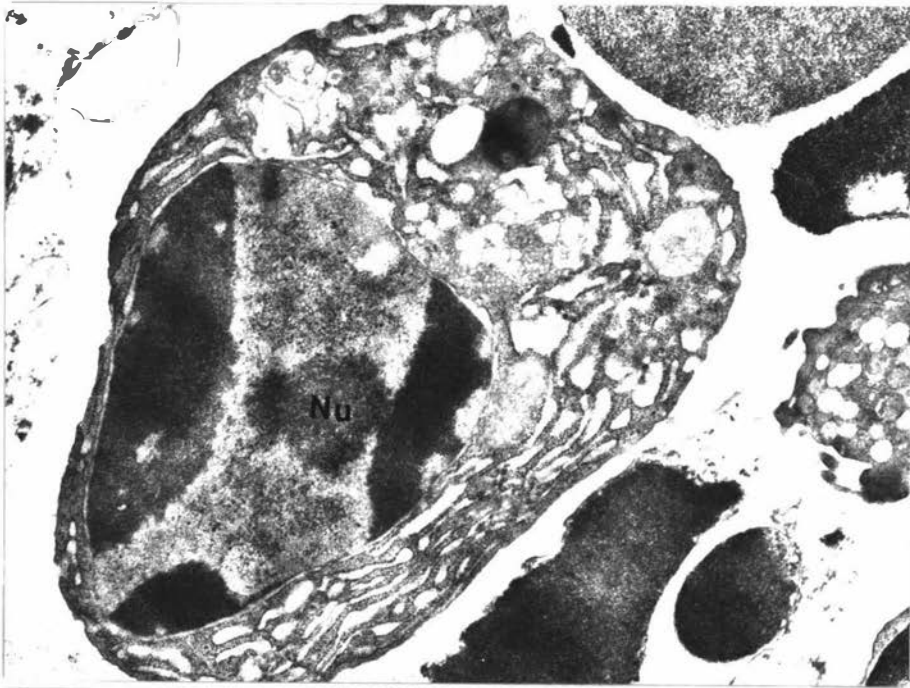


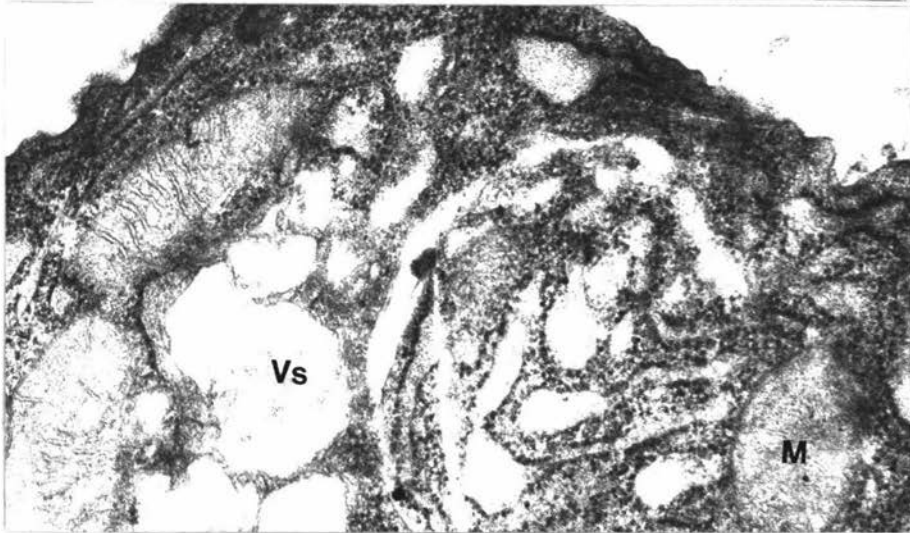
FIGURE 5.14 Opossum blood lymphocyte. 3 day culture, stimulated with LPS. X 13,500

FIGURE 5.15 Higher magnification of part of the cytoplasm of an LPS-stimulated blast cell cultured for 3 days, showing cytoplasmic vesicles (Vs), mitochondria (M) and dilated rough endoplasmic reticulum (Er). X 46,000

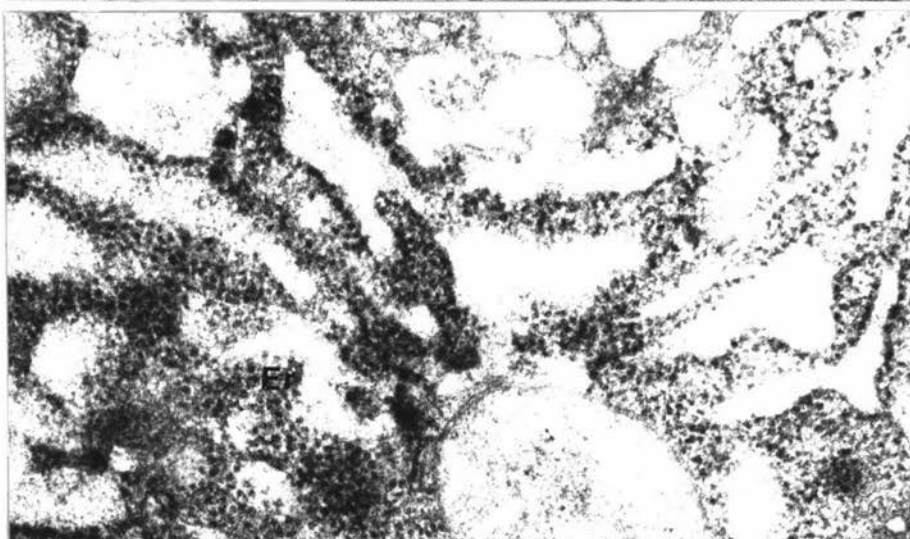
FIGURE 5.16 Higher magnification of part of the cytoplasm of the blast cell shown in Fig. 5.14 showing enlarged mitochondria (M) with loss of cristae and enlarged rough endoplasmic reticulum (Er). X 46,000



5.14



5.15

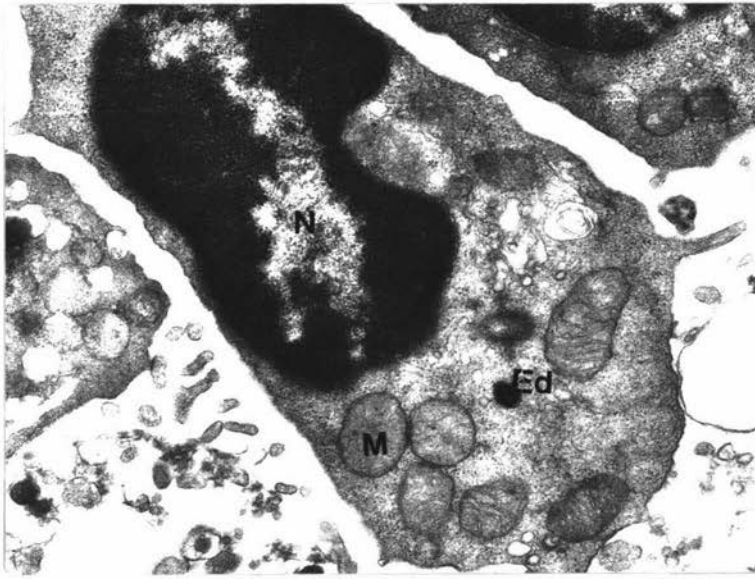


5.16

FIGURE 5.17 Opossum blood lymphocyte. 5 day culture,  
control. X 22,500

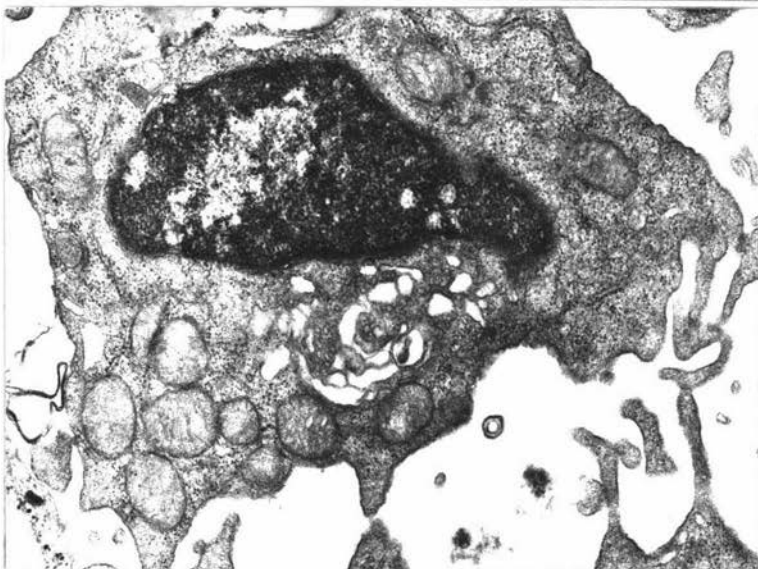
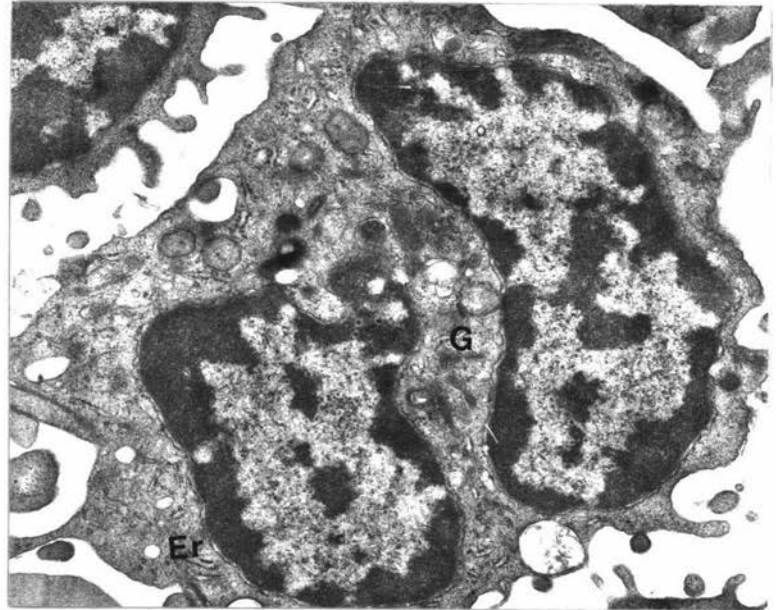
FIGURE 5.18 Opossum blood lymphocyte. 5 day culture,  
stimulated with LPS. X 13,500

FIGURE 5.19 Opossum blood lymphocyte. 5 day culture,  
stimulated with PWM. X 22,500



5-17

5-18



5-19



FIGURE 5.20 Opossum blood lymphocyte. 7 day culture, control. X 13,500

FIGURE 5.21 Part of a blast cell from a 7 day culture stimulated with LPS showing euchromatin nucleus and cytoplasm showing enlarged mitochondria (M), rough endoplasmic reticulum (Er), numerous polyribosomes (P) and cytoplasmic vacuoles and vesicles. X 17,500

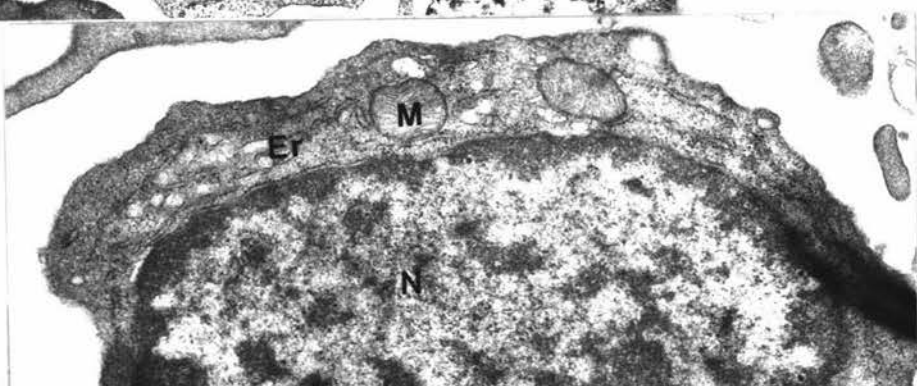
FIGURE 5.22 Part of the cytoplasm of a plasma cell following stimulation with LPS for 7 days showing enlarged mitochondria, rough endoplasmic reticulum and cytoplasmic vacuoles. X 46,000

FIGURE 5.23 Part of a blast cell from a 7 day culture stimulated with PWM showing euchromatin nucleus. Cytoplasm showing well developed Golgi zone, mitochondria, numerous cytoplasmic vacuoles and vesicles, polyribosomes and rough endoplasmic reticulum. X 17,500

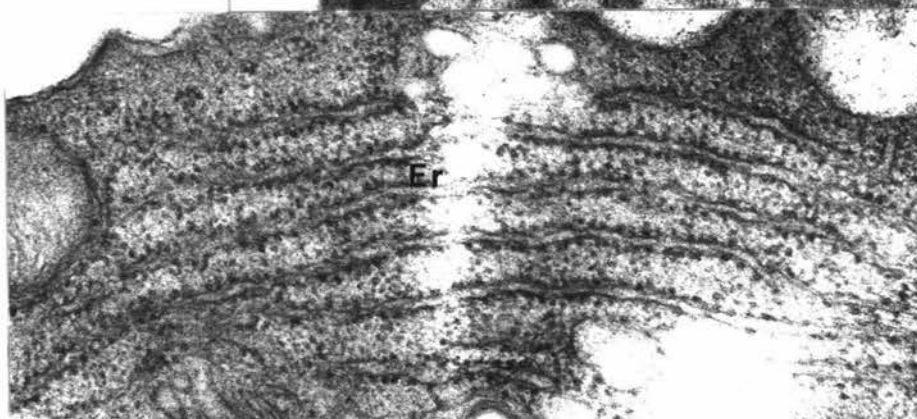


5-20

5.21



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5-23

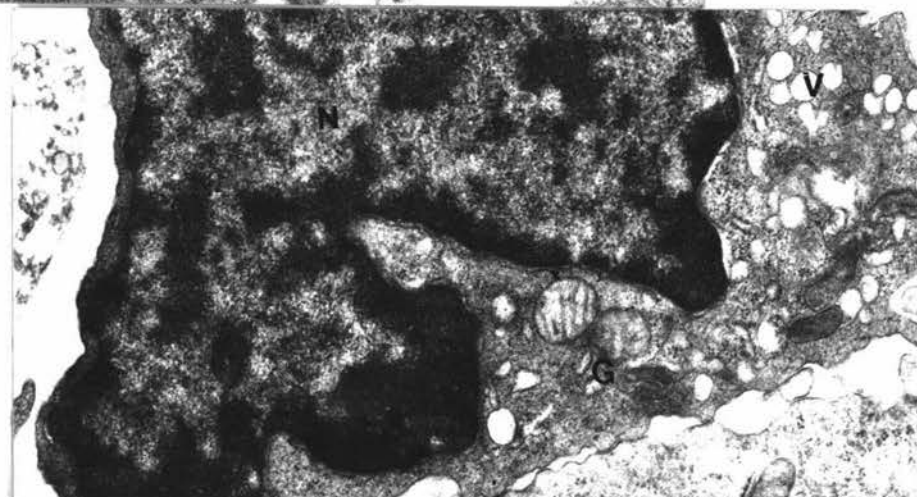
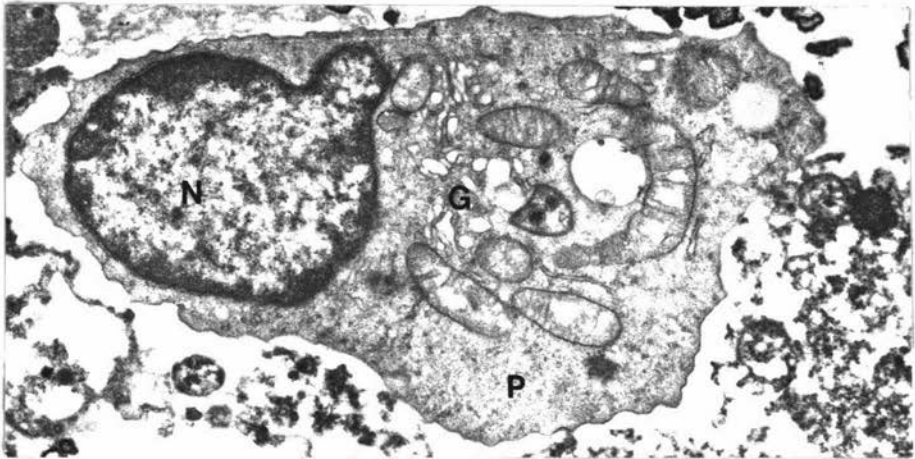


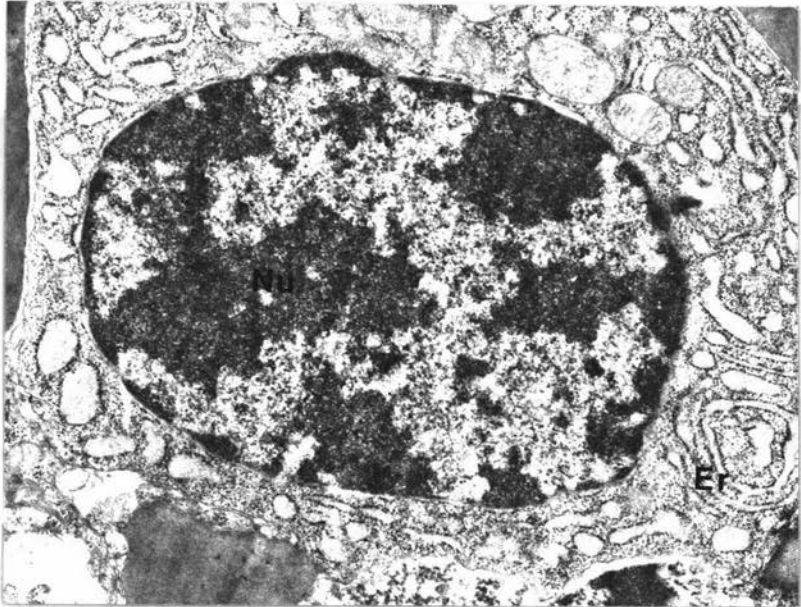
FIGURE 5.24 Opossum thymus lymphocyte. 7 day culture, stimulated with PHA. X 10,500

FIGURE 5.25 Opossum bone marrow plasma cell.  
X 13,500

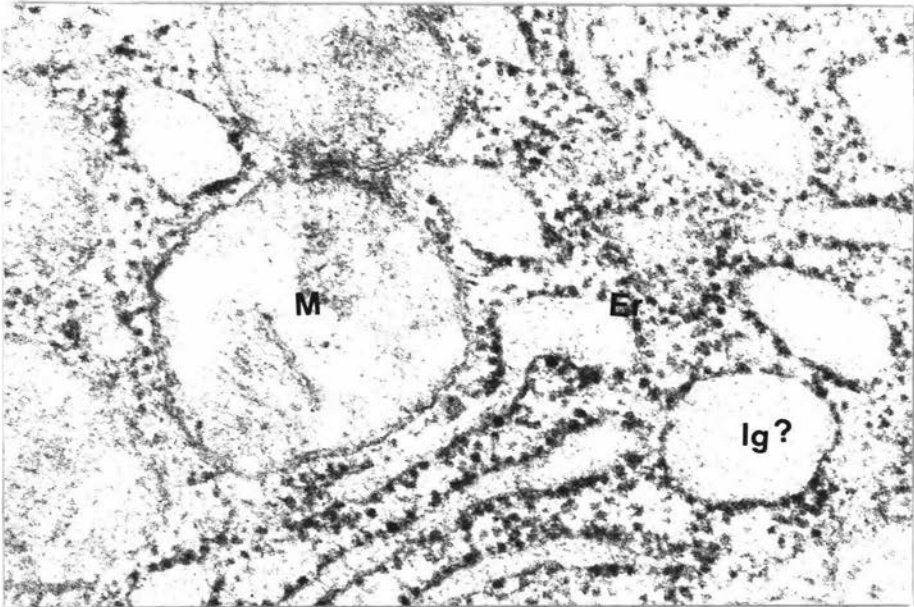
FIGURE 5.26 Higher magnification of part of the cytoplasm of the cell shown in Fig. 5.25 showing enlarged mitochondria (M) with loss of cristae and enlarged rough endoplasmic reticulum (Er) containing electron opaque material, probably immunoglobulin (Ig?). X 61,000



5-24



5-25



5-26

## CHAPTER 6

### POLYCLONAL B CELL ACTIVATION: AN ANALYSIS OF THE IMMUNOGLOBULINS SYNTHESISED

#### 6.1 Introduction

B cells are lymphocytes that are capable of synthesising immunoglobulins. Pre-B cells, already committed to the synthesis of a single idiotype (Gathings et al., 1977), are found in the foetal liver and adult bone marrow of humans (Hayward and Lawton, 1977). These cells develop into small ( $\sim 7\mu\text{m}$ ), mature, resting B lymphocytes displaying surface immunoglobulin (Froland et al., 1971). Exposure of resting B cells to suitable mitogens results in their progression to fully differentiated plasma cells which are characterized by the presence of extensive rough endoplasmic reticula and Golgi apparatuses (Geha et al., 1973). Many substances are capable of inducing the in vitro transformation of small lymphocytes to activated B cells (Moller, 1979). The use of such "polyclonal B cell activators" (PBAs) (Greaves et al., 1974a) has facilitated the functional analysis of B lymphocyte populations since cells activated by PBAs appear to act in the same manner as do antigen-driven cells.

Different PBAs are known to activate different subpopulations of B cells which may be at various stages of development and in different lymphoid tissues. This is seen in murine (Diamanstein et al., 1974; Gronowicz and Coutinho, 1975; Melchers et al., 1975) and human systems (Andersson et al., 1977). In mice, for example, dextran sulphate acts on relatively immature B cells and induces the production of DNA rather than of immunoglobulin, whereas PPD acts on mature B cells and the synthesis of immunoglobulin exceeds that of DNA (Gronowicz and Coutinho, 1975).

Species differ in their responses to individual PBAs. While LPS (Andersson et al., 1972c; Grey et al., 1972), enterotoxin B (Janossy and Greaves, 1975), dextran sulphate (Dorris et al., 1974), flagellin and levan (Coutinho and Moller, 1973a), and pneumococcal polysaccharide III (Coutinho and Moller, 1973b) effectively stimulate murine B cells, especially those from the spleen, they fail to stimulate human blood B cells. A water-soluble component from the cell wall of several *Nocardia* species (Bona et al., 1974, 1979) and Epstein-Barr virus (Bird and Britton, 1979) act as PBAs for human lymphocytes.

B cell activation induced by PBAs is best studied by measuring immunoglobulin production rather than DNA synthesis since immunoglobulin production is limited to this cell type. The immunoglobulin produced can be measured in a number of ways. Intra-cytoplasmic accumulations of immunoglobulin can be detected by fluorescent antibody techniques (Cooper et al., 1971) while secreted material can be measured either by radioimmunoassay techniques (Waldmann et al., 1974) or by its incorporation of an isotype-labelled precursor provided in the culture medium (Melchers, 1970; Parkhouse and Melchers, 1971). Alternatively, immunoglobulin production can be measured as specific antibody using variations of the original plaque-forming cell technique (Jerne and Nordin, 1963). Antibody to both SRBCs and soluble antigens can be assessed sensitively by these methods (Chiorazzi et al., 1979).

In this study the levels of total, 19S and 7S proteins produced by mitogen-stimulated cultures of blood lymphocytes were measured. Both secreted and intracellular proteins were examined: secreted proteins were measured in cell-free culture supernatants and intracellular

proteins in the lysates of cultured lymphocytes. The de novo synthesised proteins were measured by their incorporation of isotope-labelled leucine which was provided in the medium. The presence of newly synthesised IgM and IgG was demonstrated by precipitating them with immunoglobulin class-specific antisera.

## 6.2 Materials and Methods

### a. Collection of lymphocytes

Lymphocytes were harvested from blood, tested for viability and counted as described in Chapter 3. The cells were suspended in culture medium at a concentration of approximately  $5 \times 10^6$  per ml.

### b. Culture conditions

The medium used was leucine-free Eagle's MEM (GIBCO) supplemented with glutamine, antibiotics and heat-inactivated foetal calf serum at the concentrations given in Chapter 4. One ml amounts of the lymphocyte suspension were cultured in 35x10 mm plastic tissue culture dishes (No.3001, Falcon Plastics) at 37°C in 5 per cent CO<sub>2</sub>-in-air. All the cultures were made in duplicate and control cultures lacking mitogens were established routinely. When culture supernatants were to be examined for the presence of secreted proteins the cultures were fed daily with 10 uCi of L-(4,5-<sup>3</sup>H) leucine (100 Ci per mmol, The Radiochemical Centre) (Andersson et al., 1972c). When the cells were to be examined for intracellular proteins the cultures were pulsed with 60 uCi of the isotope 4 hr before being harvested (Andersson et al., 1974; Choi, 1977).

### c. Mitogenic stimulation

The cultures were stimulated with either LPS (Difco), PWM (Sigma) or Insol.Con.A (Sigma) at concentrations of

250 ug, 5 ug and 50 ug per  $10^6$  cells respectively, these concentrations having induced maximum stimulation in previous experiments (Chapter 4). The LPS was detoxified in a boiling water bath for 60 min prior to use (Andersson et al., 1972c).

d. Measurement of secreted proteins

Secreted proteins were detected by the method of Andersson et al. (1972c).

i) Total secreted proteins

The cultures were harvested on the third day when LPS was the mitogen used and on the fourth day when PWM or Insol.Con.A was used. Lymphocytes were dislodged from the culture dishes by scraping the surfaces with a plastic policeman and the culture fluid and suspended cells collected. Each dish was then rinsed with 1 ml of MEM-FCS and these washings were added to the harvested cell suspensions. The lymphocytes were pelleted by centrifugation at 200 g for 10 min at 4°C. The supernatant was further centrifuged at 1,000 g for 20 min at 4°C to ensure complete removal of cells and debris. One ml of 10 per cent trichloroacetic acid (TCA) in water was added to 200 ul of the recentrifuged supernatant. The resulting precipitate was collected on glass microfibre filters (GF/C, Whatman) and washed three times with 1 ml amounts of 10 per cent TCA. The filters were dried overnight at 37°C and placed in 5 ml of scintillation fluid (Appendix II). The radioactivity of the precipitate was then measured. The results are expressed both as counts per min (cpm) per culture and stimulation indices (SI). The cpm per culture were arrived at by multiplying by 10 the cpm recorded for the 200 ul sample counted. The cpm of the duplicate test cultures were totalled and an average obtained; the data from the control cultures were



also treated in this way. A SI was obtained by dividing the cpm of mitogen-stimulated cultures by the cpm of the controls. Results were tested by analysis of variance on logged data. Means were tested a posteriori by least significant range method (Sokal and Rohlf, 1969).

ii) 19S and 7S secreted proteins

The remaining 1.8 ml of culture supernatant was mixed with 500 ul of carrier opossum serum and the mixture precipitated with an equal volume of saturated ammonium sulphate at 4°C overnight. The precipitate was collected by centrifugation, dissolved in 2 ml of distilled water and re-precipitated with an equal volume of saturated ammonium sulphate for 3 hr at 4°C. This precipitate was dissolved in 1 ml of distilled water and dialysed against 0.15M sodium chloride overnight to remove excess ammonium sulphate. This material was then applied to a Sephadex G-200 column (40cm x 3cm) and eluted with 0.1M Tris-HCl-NaCl buffer, pH 8.0. Two ml fractions were collected, their optical densities being measured at 280 nm. 400 ul of each of the fractions were mixed with 5 ml of scintillation fluid and counted in a liquid scintillation counter. The recorded cpm were converted to the cpm per total volume of the fraction and the data analysed and presented as described above.

e. Measurement of intracellular proteins

Intracellular proteins were analysed using a slight modification of the methods of Andersson et al. (1974) and Choi (1977).

i) Total intracellular proteins

Lymphocyte cultures were harvested as described. The cells were pelleted, washed once with MEM and then lysed with 3.5 ml of 0.5 per cent Triton X-100 (Primal,

Rohm and Haas) in Tris-HCl buffer, pH 7.6, 0.05M. After being held at room temperature for 30 min this mixture was centrifuged at 150,000 g for 1 hr at 4°C. The supernatant was dialysed overnight against PBS and centrifuged at 20,000 g for 2 hr. Two hundred ul of this material were precipitated with 1 ml of 10 per cent TCA, filtered and washed with 10 per cent TCA as described previously. The radioactivity of the precipitate was determined following its suspension in 5 ml of scintillation fluid. The data are analysed and presented as described above.

ii) 19S and 7S Intracellular proteins

The remaining 1.8 ml of the cell lysate was mixed with 500 ul of opossum serum which acted as a carrier. The mixture was subjected to ammonium sulphate precipitation and gel filtration on Sephadex G-200. The radioactivity of each fraction was then determined as described for the analysis of secreted immunoglobulins. The data are analysed and presented as described above.

f. The identification of immunoglobulins in the intracellular proteins.

The presence of in vitro synthesised IgM and IgG in the cell lysates was established by their precipitation with immunoglobulin class-specific antisera. The preparations of these antisera have been described in Chapter 2. 100 ul amounts of the cell lysates, which have been dialysed against PBS, were mixed with equal volumes of the appropriate antisera and held at 4°C overnight. The precipitates that formed were collected on glass microfibre filters (GF/C, Whatman) and washed twice with PBS. The filters were dried and counted, the results being expressed as cpm per culture as described earlier. All samples were processed in duplicate.

The numbers of cultures examined are shown in Tables 6.1 and 6.2.

### 6.3 Results

#### a. Measurement of secreted proteins

The extent to which protein secretion by blood lymphocytes was stimulated by each of the three mitogens is shown in Table 6.1. In this table, as also in Table 6.2, total protein refers to material that was precipitated from culture supernatants by TCA and, therefore, includes not only 19S and 7S proteins but also proteins of lower molecular weights. The use of the designations "19S" and "7S" is based on the elution characteristics of IgM and IgG from Sephadex G-200. It is appreciated that 19S proteins conceivably can include proteins other than IgM, while 7S proteins may include proteins other than IgG.

Each of the mitogens induced the secretion of significant amounts of total protein, LPS being the most effective.

Table 6.1 also shows the relative amounts of 19S and 7S proteins secreted by the mitogen-stimulated and control cultures. The values in Table 6.1 (and Table 6.2) are based on the means of repeat experiments using cells from different animals. In these experiments the individual fractions forming either the 19S and 7S peaks were pooled and the total radioactivity of each peak measured. Fig. 6.1 is representative of these experiments and shows the spread of radioactivity within each peak from a single experiment for each mitogen. First peak material (Fractions 1 to 7) represents 19S proteins, second peak material (Fractions 8 to 14), 7S proteins.

Table 6.1 The incorporation of tritiated leucine into immunoglobulins secreted by control and mitogen stimulated blood lymphocytes.

Mitogen	Protein	cpm <sup>1</sup>	SI <sup>2</sup>	p <sup>3</sup>
LPS	Total	28.12 ± 4.70 (6) <sup>*</sup>	3.65	<0.01
	19S	2.76 ± 0.54 (5)	3.00	<0.01
	7S	1.09 ± 0.28 (3)	2.73	NS
PWM	Total	20.91 ± 1.46 (10)	2.72	<0.01
	19S	5.19 ± 1.00 (4)	5.64	<0.01
	7S	1.56 ± 0.20 (4)	3.90	<0.01
Insol.Con.A	Total	12.20 ± 1.71 (5)	1.58	<0.05
	19S	5.31 ± 1.21 (3)	5.77	<0.01
	7S	2.75 ± 0.95 (3)	6.88	<0.05
Control	Total	7.70 ± 0.96 (6)	1.00	
	19S	0.92 ± 0.10 (3)	1.00	
	7S	0.40 ± 0.14 (3)	1.00	

\* Figures in parentheses refer to number of cultures examined.

1 Mean counts per minute per culture  $\times 10^{-4}$  ± S.E.

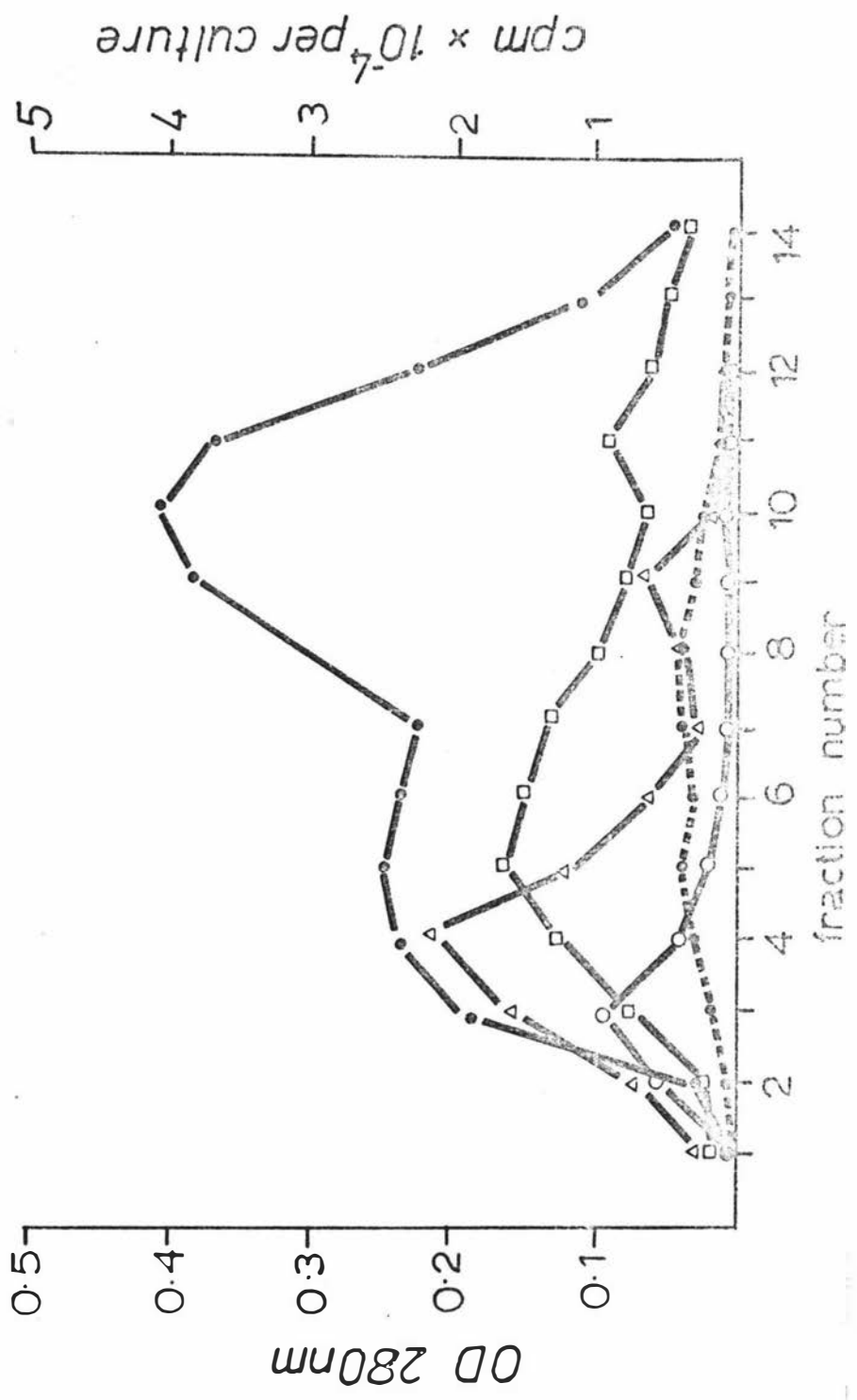
2 Stimulation index (cpm test/cpm control).

3 Statistical probability ( $p < 0.05$ )

NS Not significant.

FIGURE 6.1 Representative result of Sephadex G-200 filtration of ammonium sulphate precipitated supernatants of mitogen-stimulated and control cultures. Culture conditions and pulse period described in text.

- OD of normal (carrier) opossum serum.
- cpm per culture stimulated with LPS.
- △—△ cpm per culture stimulated with PWM.
- cpm per culture stimulated with Insol. Con.A
- cpm per culture, control.



If the levels of 19S proteins in the three mitogen-treated cultures are compared with that of the control then all three are seen to be significantly increased ( $p < 0.01$ ). The ranking of the mitogens in terms of their abilities to induce 19S protein synthesis was Insol.Con.A > PWM > LPS. If the synthesis of 7S protein is considered then PWM and Insol.Con.A significantly increased production ( $p < 0.01$  and  $p < 0.05$ , respectively) of this material; LPS failed to do so.

b. Measurement of intracellular proteins

Of the three mitogens, Insol.Con.A generated the highest levels of total intracellular proteins (Table 6.2). In all cases the intracellular total protein levels of mitogen-stimulated cultures were significantly higher than those of control cultures. Table 6.2 also shows the amounts of intracellular 19S and 7S proteins. Insol.Con.A, PWM and LPS were decreasingly effective, in that order, in inducing the production of these materials.

Fig. 6.2 shows typical results from a single experiment using each of the mitogens. The distribution of radioactivity between the fractions is shown and can be related to 19S (Fractions 1 to 7) and 7S (Fractions 8 to 14) proteins. The higher radioactivity generally seen with the intracellular proteins as opposed to the secreted proteins (Fig. 6.1) is apparent. Also seen in Fig. 6.2 is the higher level of 7S protein than 19S protein induced by PWM in this system; PWM was the only mitogen to show this effect.

c. A comparison of the amounts of secreted versus intracellular 19S and 7S proteins.

Table 6.3 re-presents data shown in Tables 6.1 and 6.2 to emphasise the relative amounts of secreted and

Table 6.2 The incorporation of tritiated leucine into intracellular immunoglobulins in control and mitogen stimulated blood lymphocytes.

Mitogen	Protein	cpm <sup>1</sup>	SI <sup>2</sup>	p <sup>3</sup>
LPS	Total	15.25 $\pm$ 0.49 (4) <sup>*</sup>	1.65	<0.01
	19S	8.20 $\pm$ 1.14 (3)	2.63	<0.05
	7S	3.77 $\pm$ 0.80 (3)	1.80	NS
PWM	Total	64.89 $\pm$ 7.04 (4)	7.01	<0.01
	19S	8.20 $\pm$ 1.55 (3)	2.63	<0.05
	7S	9.23 $\pm$ 0.55 (3)	4.40	<0.01
Insol.Con.A	Total	79.37 $\pm$ 3.71 (4)	8.57	<0.01
	19S	15.29 $\pm$ 4.90 (3)	4.90	<0.05
	7S	12.86 $\pm$ 6.12 (3)	6.12	NS
Control	Total	9.26 $\pm$ 0.61 (2)	1.00	
	19S	3.12 $\pm$ 0.22 (2)	1.00	
	7S	2.10 $\pm$ 0.07 (2)	1.00	

\* Figures in parentheses refer to number of cultures examined.

1 Mean counts per minute per culture  $\times 10^{-4} \pm$  S.E.

2 Stimulation index (cpm test/cpm control).

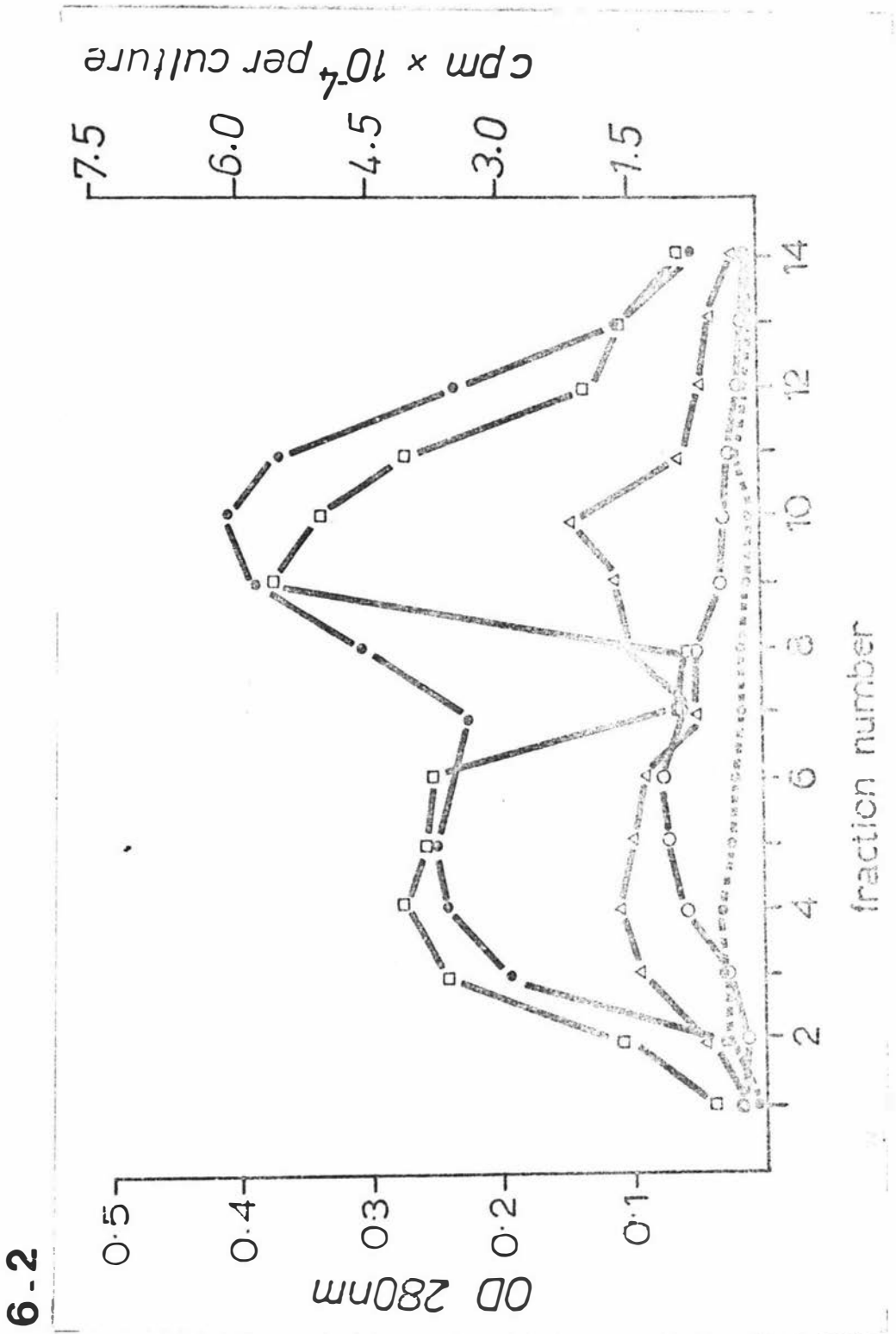
3 Statistical probability (p 0.05).

NS Not significant.



FIGURE 6.2 Representative result of Sephadex G-200 filtration of ammonium sulphate precipitated cell lysates from mitogen-stimulated and control cultures. Culture conditions and pulse period described in text.

- OD of normal (carrier) opossum serum.
- cpm per culture stimulated with LPS.
- △—△ cpm per culture stimulated with PWM.
- cpm per culture stimulated with Insol. Con.A.
- cpm per culture, control.



intracellular 19S and 7S proteins synthesised by the stimulated and control cultures. The ratios of the combined intracellular 19S and 7S protein values to those of the combined secreted 19S and 7S protein values were broadly similar for all cultures, including the controls, and ranged from 2.58 to 3.95. Table 6.3 presents the statistical significances of the individual results for secreted and intracellular proteins. Each mitogen induced significantly increased levels of 19S protein synthesis in the form of secreted and intracellular proteins. PWM induced significantly increased levels of both secreted and intracellular 7S proteins.

The amount of either secreted or intracellular protein detected is a measure of a mitogen's ability to elicit production of that particular protein. From these results it can be concluded that PWM is capable of stimulating a statistically significant increase in the synthesis and secretion of 19S and 7S proteins. Insol. Con.A also increased the levels of 19S and 7S proteins synthesised intracellularly and secreted although the increase in the level of intracellular 7S protein did not attain statistical significance, probably because of the wide variation in counts in the treated cultures. LPS treated cultures only showed significant increases in 19S protein synthesis and secretion.

d. Identification of immunoglobulins in the intracellular proteins

De novo synthesised isotope-labelled IgM and IgG were precipitated from cell lysates by their specific antisera. Table 6.4 shows the radioactivity associated with the precipitates and compares these values with those of the parent 19S and 7S fractions. The cpm of the precipitates were always considerably lower than those

Table 6.3 A comparison of the amounts of secreted and intracellular 19S and 7S proteins produced by blood lymphocytes in response to mitogen stimulation.

Mitogen	Protein	Radioactivity <sup>1</sup> , cpm x10 <sup>-4</sup>			
		19S	7S	19S+7S	Ic/S <sup>2</sup>
I.PS	Secreted	2.76 <sup>*</sup>	1.09 <sup>@</sup>	3.85	
	Intra-cellular	8.20 <sup>*</sup>	3.77 <sup>@</sup>	11.97	3.10
PWM	Secreted	5.19 <sup>*</sup>	1.56 <sup>*</sup>	6.75	
	Intra-cellular	8.20 <sup>*</sup>	9.23 <sup>*</sup>	17.43	2.58
Insol. Con.A	Secreted	5.31 <sup>*</sup>	2.75	8.06	
	Intra-cellular	15.29 <sup>*</sup>	12.86 <sup>@</sup>	28.15	3.49
Control	Secreted	0.92	0.40	1.32	
	Intra-cellular	3.12	2.10	5.22	3.95

1 Mean counts per minute per culture x10<sup>-4</sup>.

2 Ratio of total intracellular protein to total secreted protein.

\* Statistically significant (p < 0.05).

@ Statistically not significant.

Table 6.4 A comparison of the incorporation of tritiated leucine into the intracellular immunoglobulins in control and mitogen-stimulated blood lymphocyte cultures following Sephadex G-200 fractionation and subsequent immune precipitation.

Mitogen	19S from Sephadex	IgM from Imm.Pr. <sup>1</sup>	7S from Sephadex	IgG from Imm.Pr. <sup>1</sup>
Insol.Con.A	15.29 $\pm$ 4.90 <sup>2</sup>	3.73 $\pm$ 1.41	12.86 $\pm$ 6.12	3.96 $\pm$ 1.11
PWM	8.20 $\pm$ 1.55	2.99 $\pm$ 0.22	9.23 $\pm$ 0.55	2.73 $\pm$ 0.24
Control	3.12 $\pm$ 0.22	1.62 $\pm$ 0.28	2.10 $\pm$ 0.07	1.19 $\pm$ 0.26

1) Immune precipitation; 2) Counts per minute per culture  $\times 10^{-4}$ ; values given are means from two experiments  $\pm$  S.E.

of the 19S and 7S materials, a finding which probably reflects certain deficiencies in the method used. However, the results obtained did confirm that the 19S and 7S fractions contained IgM and IgG respectively.

#### 6.4 Discussion

In this chapter evidence is presented which shows that opossum lymphocytes stimulated in vitro with certain PBAs produce either IgM alone or IgM and IgG in significant amounts.

In preliminary experiments, the results of which are not presented, it was found that opossum blood lymphocyte cultures showed maximal intracellular total protein production and peak DNA synthesis on the same day, the mitogens used being PWM, LPS and Insol.Con.A. Consequently, it was decided that the culture times established in Chapter 4 would be appropriate for the present experiments. There is some discrepancy in the optimal culture periods recommended for determining immunoglobulin production. Thus Coutinho et al. (1973) and Janossy (1976) used 3 day cultures when studying the immunoglobulin synthesis of LPS-stimulated mouse lymphocytes while Andersson et al. (1972c) and Kunori et al. (1978), using the same system, found that maximum immunoglobulin synthesis took 4 to 5 days. Basham and Waxdal (1975) reported that maximum protein synthesis by mouse spleen cell cultures stimulated with PWM was seen on the fourth day of culture, whereas human blood lymphocyte cultures exposed to the same mitogen, show peak immunoglobulin synthesis between 5 and 7 days of culture (Choi and Good, 1977; Nespoli et al., 1978; Fauci et al., 1978).

The amount of total protein produced has been used in the past as a reference against which to estimate the relative amounts of 19S and 7S materials produced. Using

this approach Andersson et al. (1972c) employing a mouse spleen cell system and Symons and Lay (1978) using pig blood lymphocytes, both stimulated with LPS, showed that 12.8 and 13.0 per cent, respectively, of the total protein secreted was 19S material. For opossums this value was 9.8 per cent. Finkelman and Lipsky (1979) reported that, compared to control cultures, human spleen cells stimulated with LPS gave a SI of 4.4 for IgM secretion; the corresponding value for opossum blood lymphocytes was 3.0.

The intracellular protein levels were always considerably higher than those of the secreted proteins. This is not unexpected because of some dilution of the secreted material by culture medium. Furthermore, synthesis must precede secretion. Longer culture periods may have reduced the difference between the levels of the secreted and intracellular proteins.

The T cell-stimulating effects of PWM and Insol. Con.A (Chapter 4) are reflected in the levels of intracellular proteins induced by these mitogens. If the sum of the 19S and 7S values is calculated as a percentage of the total protein, then for LPS, a marginal T cell stimulator, this value is roughly 80 per cent; for PWM and Insol.Con.A it falls to about 12 and 35 per cent, respectively.

LPS induced the synthesis of higher levels of 19S than 7S proteins, results that agree with those obtained from eutherian species (Landy et al., 1965; Britton and Moller, 1968; Melchers and Andersson, 1973; Symons and Lay, 1978). However, it has been claimed recently that LPS promotes the synthesis, by mouse spleen cells, of more IgG than IgM (Gronowicz et al., 1979; Hepper et al., 1979). The latter authors point out that technical dif-

ferences, including cell density in culture, LPS variability, animal strain differences and the sensitivities of the assay systems, are important in determining the type and amount of immunoglobulin detected.

PWM induced the synthesis of significant amounts of secreted IgM and IgG, the level of IgM exceeding that of IgG. Nespoli et al. (1978) and Lanzavecchia et al. (1979) have described similar results for human blood lymphocytes. However, Finkelman and Lipsky (1979) have reported that human spleen cells stimulated with PWM secrete more IgG than IgM. When the intracellular proteins induced by PWM were investigated in this present work, it was seen that there was somewhat more 7S than 19S protein. Had a longer culture period been used with the opossum lymphocytes, a result comparable to that given by human spleen cells might have been seen. Mouse spleen cells are said to produce only IgM when stimulated with PWM (Parkhouse et al., 1972).

The highest levels of both secreted and intracellular 19S and 7S proteins were found in Insol.Con.A - stimulated cultures, a reflection perhaps of the potent mitogenic effect that this substance has for both opossum T and B lymphocytes. Unfortunately, in the case of intracellular 7S protein synthesis, large individual variations were seen between different cultures making the increased protein synthesis statistically not significant. Anderson and Melchers (1973) reported that locally concentrated Con.A stimulates only IgM production by cultures of mouse B cells. Whether or not opossum B cells, perhaps with T cell help, produce significant levels of 7S proteins in response to Insol.Con.A cannot be resolved at present.



The results from a limited number of experiments confirmed that the cell lysates contained IgM and IgG. The cpm of the precipitates were markedly lower than those of the parent material from which they were derived. Two reasons can be advanced to account for this. Firstly, the precipitation would have been more efficient had varying amounts of the specific antisera been added to the constant volume of cell lysates. Secondly, the method involved a single precipitation step when ideally a second precipitation, using an anti-rabbit gammaglobulin reagent, should have been employed. The unavailability of such a reagent at the time precluded this approach. The use of these technical improvements would have undoubtedly given higher precipitate cpm. However, the aim of the experiment was to establish the presence of immunoglobulins in the cell lysates and not to quantitatively assess them.

In summary, it can be said that LPS, PWM and Insol. Con.A all induced production of significant levels of 19S proteins, which included IgM, in opossum blood lymphocyte cultures. PWM in addition induced significantly increased levels of 7S proteins, which included IgG.

## CHAPTER 7

### THE KINETICS OF ANTIBODY FORMATION

#### 7.1 Introduction

Published information on the antibody responses of T.vulpecula is limited to a single paper (Yadav, 1973b). Following administration of SRBCs, S.adelaide flagella or bacteriophage ØX174 to an opossum "by a series of injections over one year", IgM and IgG antibodies against all three antigens were detected in the serum; an additional, low molecular weight antibody of about 80,000 daltons was produced in response to the bacteriophage.

In the present study the antibody responses of T.vulpecula to a particulate and a soluble antigen are compared with those given by rabbits to the same materials. Antisera were collected at intervals following immunization and their titres measured by haemagglutination techniques. Some antisera were fractionated on Sephadex G-200 and the distribution of IgM and IgG antibodies established during the primary and secondary responses of the two species.

#### 7.2 Materials and Methods

##### a. Animals

Adult opossums and rabbits weighing between 2.5 and 3.0 kg were used. The rabbits were New Zealand Whites obtained from the Small Animal Production Unit of Massey University.

##### b. Preparation of antigens

Sheep red blood cells (SRBC)

Sheep were bled from a jugular vein into Vacutainers (Beckton-Dickinson) containing heparin. The blood was

centrifuged at 1750 g for 20 min and the buffy coat and plasma discarded. The erythrocytes were washed three times with PBS, counted in a haemocytometer and resuspended in 0.15M NaCl at concentrations of approximately  $5 \times 10^9$  or  $25 \times 10^9$  cells per ml.

#### Bovine serum albumin (BSA)

BSA (crystalised and lyophilised, Sigma) was dissolved in 0.15M NaCl at a concentration of 20 mg per ml.

#### c. Immunization

Opossums were anaesthetised with halothane (Chapter 2) when being injected with the antigens and when being bled by cardiac puncture. Rabbits were restrained by an assistant and bled from marginal ear veins. Pre-immunization serum samples were collected.

#### SRBC

One ml amounts of the SRBC suspensions were injected into the tail veins of opossums or marginal ear veins of rabbits. Two of each species received  $5 \times 10^9$  and two received  $25 \times 10^9$  SRBCs. Serum samples were collected at weekly intervals. When the haemagglutination titres of these sera (see below) showed a decline towards pre-immunization levels the animals were reinjected intravenously with the same number of SRBCs as used in the primary injections. Animals were then bled at the intervals shown in Tables 7.1 and 7.2.

#### BSA

Each animal (two animals from each species) received 1 ml of a mixture of equal volumes of 0.15M NaCl, containing 20 mg of BSA per ml, and Freund's complete adjuvant (Difco) intramuscularly. When the primary

titres showed evidence of waning the animals were injected intramuscularly with a mixture of BSA-in-saline (20 mg per ml) and Freund's incomplete adjuvant (Difco). The animals were then bled at intervals shown in Table 7.4.

#### d. Serology

##### Measurement of anti-SRBC antibody

Doubling dilutions of heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) antisera were made in PBS. 50  $\mu\text{l}$  of a 1.5 per cent suspension of SRBCs were added to each 500  $\mu\text{l}$  of serum dilution. The mixture was incubated at room temperature for 3 hr. The highest serum dilution showing haemagglutination in the form of an even mat of cells was recorded for each sample. The results given are reciprocals of these titres.

Some anti-SRBC antisera were fractionated on Sephadex G-200 (Chapter 2). The individual fractions were pooled around obvious protein peaks and concentrated by dialysis against PEG to the original volume of the serum sample fractionated. The three fractions so obtained were designated Fractions I (rich in IgM), II (rich in IgG) and III, Fraction I being the first peak eluted. Each of these three fractions was assayed for anti-SRBC activity as described above. Additionally, the presence of IgG and IgM in each fraction was determined by IEP (Chapter 2).

##### Measurement of anti-BSA antibody

A microtitre haemagglutination system was used (Marx et al., 1971) employing indicator erythrocytes sensitised by the method of Evans et al. (1974). One ml of thrice-washed, packed SRBCs was mixed with 9 ml of PBS containing 3 mg BSA. The suspension was stirred at

room temperature for 3 min. One ml of 2.5 per cent glutaraldehyde in 0.15M NaCl was added over a period of 15 min and the mixture stirred for a further 45 min. The BSA-coated SRBCs were then washed three times with 0.15M NaCl and resuspended in saline to give a 1.5 per cent cell suspension.

Doubling dilutions of heat-inactivated ( $56^{\circ}\text{C}$  for 30 min) antisera were made in 50 ul volumes of PBS in micro-titre plates (Kayline Plastics). Ten ul of a 1.5 per cent suspension of BSA-coated SRBC were added to each well. The plates were incubated at room temperature for 3 hr. The highest dilution of antiserum showing agglutination was recorded.

### 7.3 Results

#### a. Antibody responses to SRBCs

The haemagglutination titres of opossums and rabbits injected intravenously with  $5 \times 10^9$  SRBCs are given in Table 7.1. Plots of the  $\log_{10}$  values of these titres against time after injection are shown in Fig. 7.1. A marked difference in the response of the two species was seen. In the opossum the primary response was completed in about 30 days whereas in the rabbit this took nearer to 60 days. Following a booster injection the maximum titres attained by the opossums were 10,240 ( $\log_{10}=4.0$ ) while the rabbits showed titres of 81,920 ( $\log_{10}=4.9$ ) and 163,840 ( $\log_{10}=5.2$ ). The secondary responses in the opossums were appreciably shorter than those in the rabbits. Table 7.3 shows that while maximum titres were reached in opossums 40 days following an initial injection of the antigen, maximum titres were reached in 99 and 86 days in the rabbits.

This marked difference in the response of the two species was less apparent when a larger number of erythro-

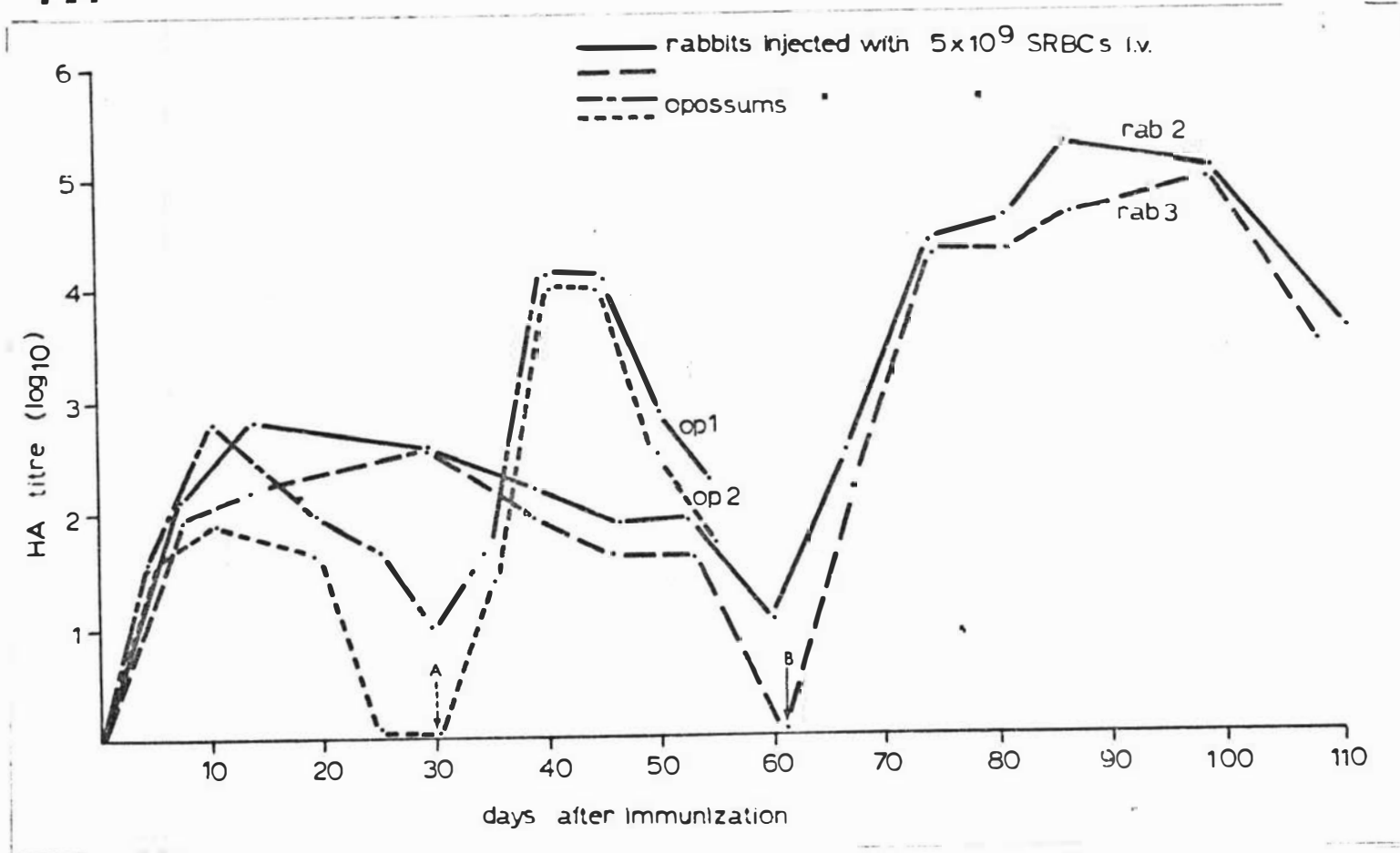
Table 7.1 The haemagglutination titres of opossums and rabbits injected intravenously with  $5 \times 10^9$  SRBCs.

Opossum			Rabbit		
Day	No.1	No.2	Day	No.1	No.2
0	0	0	0	0	0
5	40	40	7	80	160
10	80	640	14	160	640
20	40	80	29	320	320
25	0	40	39	80	160
30*	0	10	46	40	80
35	20	40	53	40	80
40	10,240	10,240	61*	0	10
45	10,240	10,240	67	160	320
50	640	320	74	20,480	20,480
55	160	40	81	20,480	40,960
			86	40,960	163,840
			99	81,920	81,920
			110	2,560	5,120
			125	640	640
			135	80	320
			146	80	160
			156	20	10

\* Booster injection of  $5 \times 10^9$  SRBCs given intravenously.

FIGURE 7.1 Immune responses of opossums and rabbits injected intravenously with  $5 \times 10^9$  SRECs. Arrows A and B indicate the days when second injections were given to opossums and rabbits, respectively.

# 7.1





cytes was injected. Table 7.2 and Fig. 7.2 show the results of injecting  $25 \times 10^9$  SRBCs intravenously. Although at this level of antigen the response curves of the opossum and rabbit are similar there is some suggestion that the primary and secondary responses of the opossums are shorter than those of the rabbits. However, the maximum titres reached are similar in the two species (Opossum,  $81,920 / \log_{10} = 4.9$ ; rabbit,  $40,960 / \log = 4.6$ ) as are the times taken to do so (50 days for opossums; 56 days for rabbits).

b. Antibody responses to BSA

In general terms the responses of opossums and rabbits to BSA injected intramuscularly with Freund's adjuvants were similar (Table 7.4 and Fig. 7.3). The maximum titres recorded for both species were similar as was the time taken to reach them (Table 7.2). The use of adjuvant made the distinction between primary and secondary responses somewhat difficult to define. However, from Fig. 7.3, there is a suggestion that the primary response in the opossums may have started to wane around day 30 or 40 whereas in rabbits this was more likely to have been around day 70. A delay of some 15 to 20 days was seen in individuals of both species in responding initially to the antigen.

c. Immunoglobulin class distribution of anti-SRBC antibodies

The haemagglutinating activities of the three Sephadex G-200-derived fractions are shown in Tables 7.5 and 7.6 and in Fig. 7.4. A comparison of the titres recorded for whole sera (Tables 7.1 and 7.2) with those of the fractions shows that fractionation always resulted in a considerable loss of antibody activity.

Table 7.2 The haemagglutination titres of opossums and rabbits injected intravenously with  $25 \times 10^9$  SRBCs.

Opossum			Rabbit		
Day	No.3	No.4	Day	No.3	No.4
0	0	0	0	0	0
5	160	20	7	20	20
10	160	1,280	14	160	80
15	640	1,280	21	160	1,280
20	20	640	28	640	10,240
31	-	640	35	160	320
40*	10	320	42	80	160
45	160	40,960	49*	40	10
50	20,480	81,920	56	40,960	10,240
60	5,120	20,480	63	5,120	5,120
67	5,120	2,560	70	2,560	5,120
74	2,560	1,280	78	2,560	5,120
80	2,560	640	84	2,560	2,560
90	1,280	640	91	2,560	2,560
97	640	640	99	2,560	1,280
102	640	40	107	1,280	640
108	40	40	112	1,280	640
			120	1,280	160
			128	1,280	160
			136	80	40

\* Booster injection of  $25 \times 10^9$  SRBCs given intravenously.

FIGURE 7.2 Immune responses of opossums and rabbits injected intravenously with  $25 \times 10^9$  SRBCs. Arrows A and B indicate the days when second injections were given to opossums and rabbits, respectively.

## 7.2

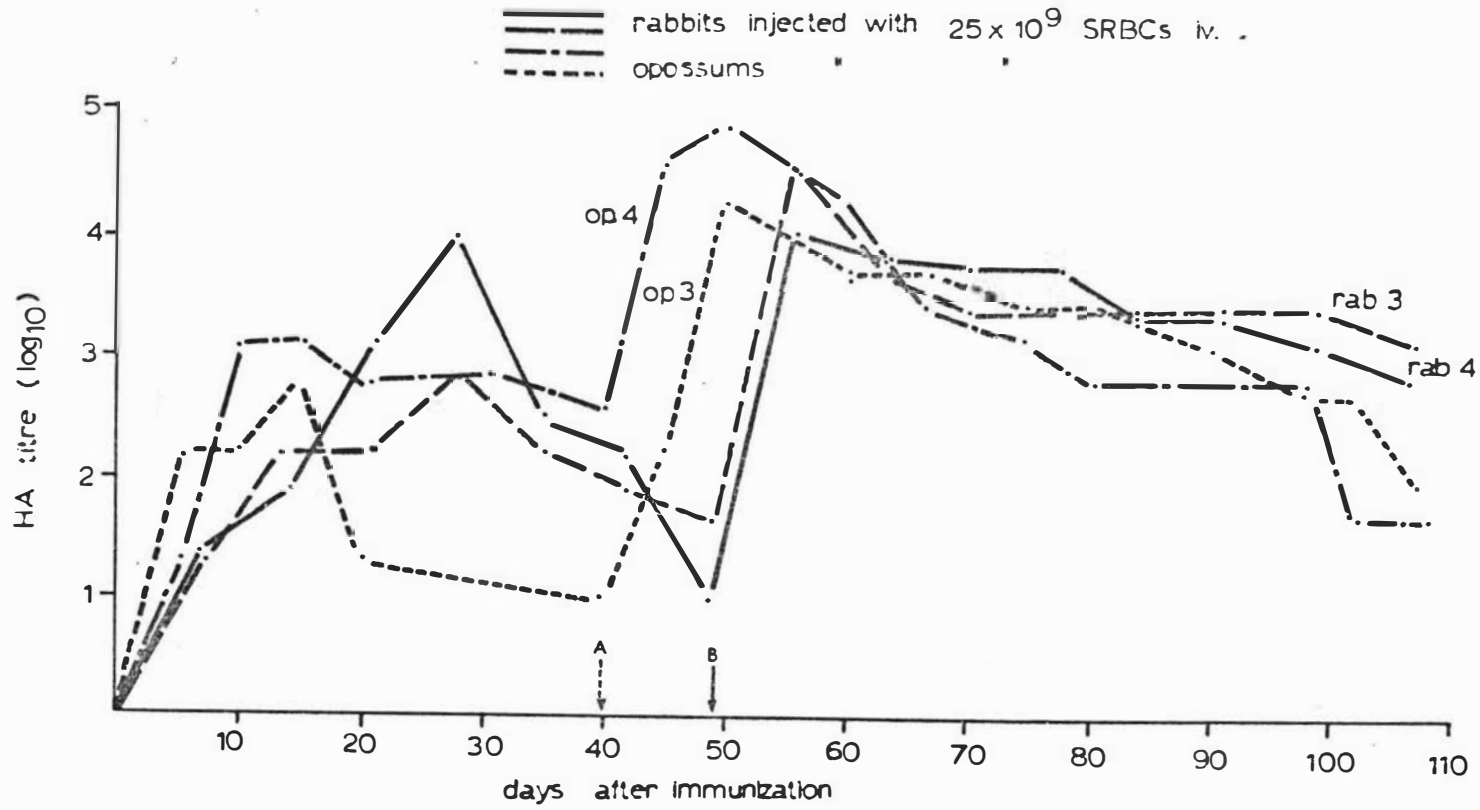


Table 7.3 The  $\log_{10}$  values of maximum haemagglutination titres recorded for opossums and rabbits injected either with SRBCs or BSA.

Antigen	Animal	Primary		Secondary		Max. days <sup>3</sup>
		Titre	Days <sup>1</sup>	Titre	Days <sup>2</sup>	
SRBCs $5 \times 10^9$	Op.1	1.9	10	4.0	10	40
	Op.2	2.8	10	4.0	10	40
	Rb.1	2.5	29	4.9	38	99
	Rb.2	2.8	14	5.2	25	86
SRBCs $25 \times 10^9$	Op.3	2.8	15	4.3	10	50
	Op.4	3.1	10	4.9	10	50
	Rb.3	2.8	28	4.6	7	56
	Rb.4	4.0	28	4.0	7	56
BSA, 20 mg with FCA <sup>4</sup>	Op.5	2.2	26	4.9	66	110
	Op.6	2.5	37	5.2	40	83
	Rb.5	4.6	66	6.1	19	106
	Rb.6	4.3	66	5.2	19	106

1) Refers to days following primary injection; 2) Refers to days following secondary injection; 3) Refers to maximum days from initial injections of antigens; 4) FCA- Freund' complete adjuvant.

Table 7.4 The haemagglutination titres of opossums and rabbits injected intramuscularly with 20 mg of BSA in Freund's adjuvants. Initial injection using Freund's complete adjuvant.

Opossum			Rabbit		
Day	No.5	No.6	Day	No.5	No.6
0	0	0	0	0	0
7	0	0	11	160	0
14	20	0	21	640	0
26	160	40	28	1,280	80
37	80	320	35	2,560	320
44 <sup>*</sup>	160	320	50	5,120	10,240
51	640	320	59	20,480	10,240
59	640	640	66	40,960	20,480
76	1,280	20,480	73	40,960	10,240
83	5,120	163,840	81	20,480	5,120
100	20,480	81,920	87 <sup>*</sup>	5,120	2,560
110	81,920	81,920	94	40,960	5,120
116	81,920	81,920	101	81,920	40,960
124	20,480	40,960	106	655,360	163,840
130	20,480	40,960	118	40,960	81,920
141	10,240	40,960	129	20,480	81,920
148	<u>Died</u>	20,480	144	20,480	10,240
159		5,120	153	10,240	5,120
169		2,560	164	5,120	2,560
184		2,560	180	160	40

\* Booster injection of 20 mg of BSA in Freund's adjuvant given intramuscularly.

FIGURE 7.3 Immune response of opossums and rabbits injected intramuscularly with 20 mg of BSA in Freund's complete adjuvant. Arrows A and B indicate the days when second injections were given to opossums and rabbits, respectively.

### 7.3

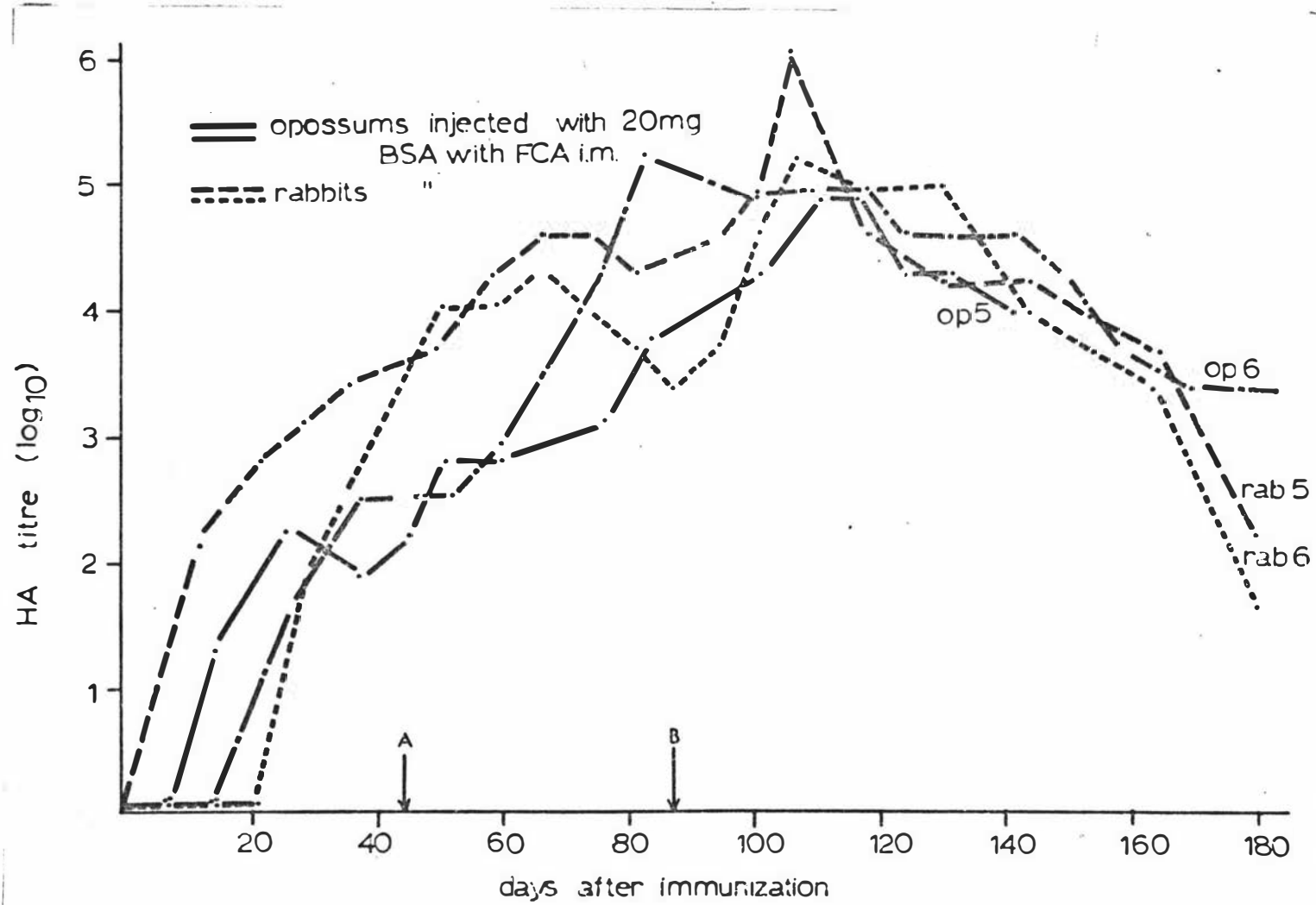




Table 7.5 Kinetics of antibody response in adult opossum and rabbit immunized with  $5 \times 10^9$  SRBCs, intravenously.

Day	Opossum 2			Day	Rabbit 2		
	F1*	F2	F3		F1	F2	F3
0	0	0	0	0	0	0	0
5	40	20	5	7	160	40	0
10	320	20	0	14	640	160	20
15	80	20	5	29	160	160	0
25	80	40	10	53	80	40	10
35**	5	0	0	61**			
40	80	640	0	74	320	10,240	0
45	80	5,120	10	99	20	5,120	0
50	80	5,120	0				
55	20	2,560	0				

\*Sephadex G-200 gel filtration fractions; \*\* Second injection given.

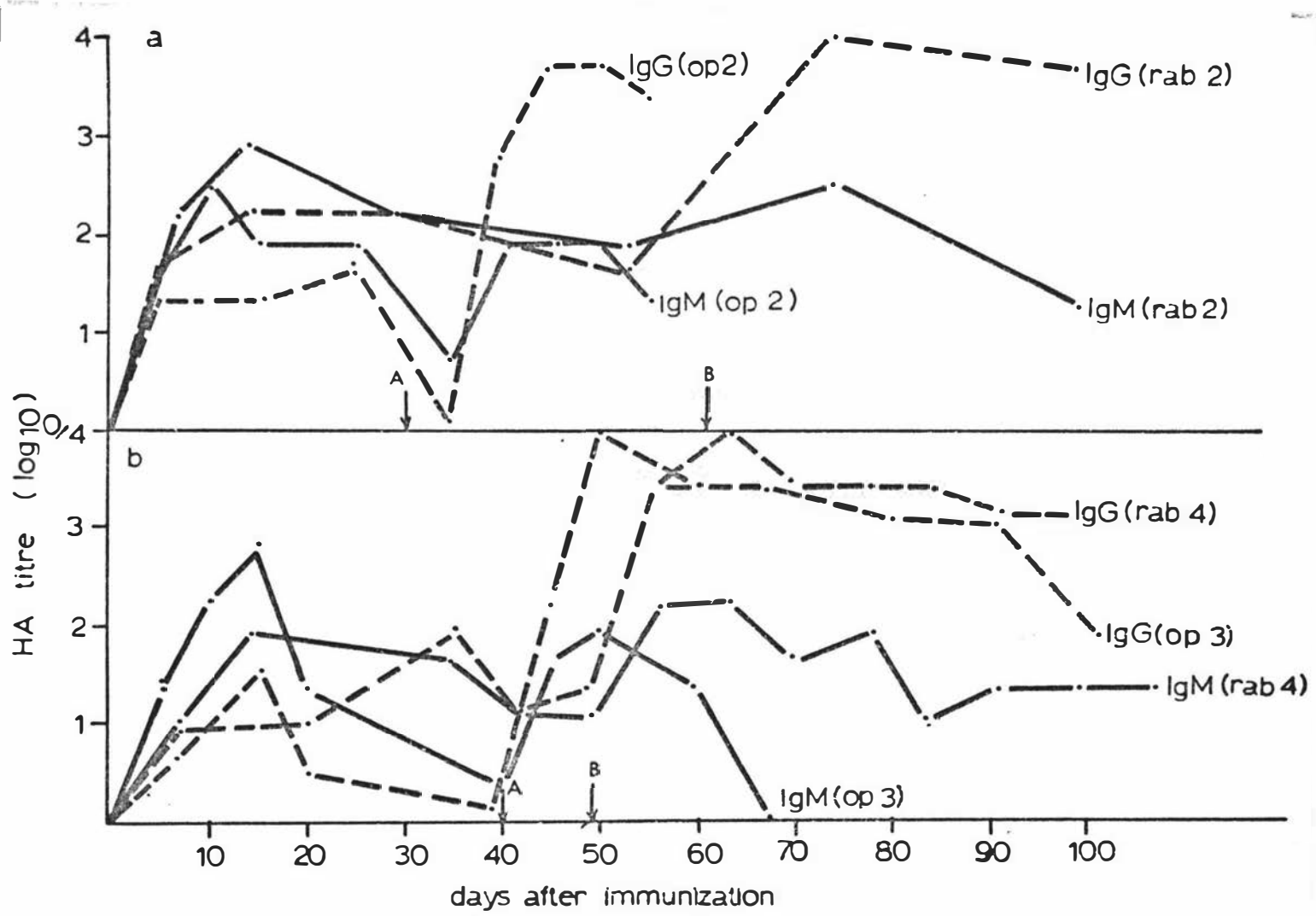
Table 7.6 Kinetics of antibody response in adult opossum and rabbit immunized with  $25 \times 10^9$  SRBCs, intravenously.

Day	Opossum 3			Day	Rabbit 4		
	F1*	F2	F3		F1	F2	F3
0	0	0	0	0	0	0	0
5	20	5	0	7	10	10	0
10	160	160	20	14	80	10	0
15	640	40	20	35	40	80	10
20	20	5	0	42	10	10	0
40**	5	0	0	49**	10	20	0
45	40	160	0	56	160	2,560	320
50	80	10,240	0	63	160	10,240	5
60	20	2,560	0	70	40	2,560	5
67	0	2,560	20	78	80	2,560	20
80	5	1,280	0	84	10	2,560	20
90	0	1,280	0	91	20	1,280	20
102	5	640	0	99	20	1,280	5
				107	20	160	10
				128	0	40	0

\* Sephadex G-200 gel filtration fractions; \*\* Second injection given.

FIGURE 7.4 The levels of antibody activity in the IgM and IgG fractions of immune sera from opossums and rabbits, given  $5 \times 10^9$  SRBCs (a) or  $25 \times 10^9$  SRBCs (b). Arrows A and B indicate the days when second injections were given in opossums and rabbits, respectively.

# 7.4



In both opossums and rabbits the majority of the antibody activity was found in the IgM fraction during the primary response. Following secondary stimulation both species showed sharp increases in their IgG levels, reaching peak titres in 10 to 15 days following secondary injection. There was also an initial increase in the IgM level during the secondary response, but this was always less than the primary response level. With higher concentrations of antigen both species showed elevated levels of IgG antibody for longer periods of time.

The data are too limited to make any definite assessment of the antibody distribution between the IgM and IgG classes. From Fig. 7.4 it does appear, however, that the distribution of haemagglutinating activity between the two immunoglobulin classes was similar in both species for both doses of antigen at any given phase of the immune response. An exception to this may be the more transient appearance of IgM in the secondary responses of opossums than occurred in the secondary responses of rabbits.

#### 7.4 Discussion

Any discussion of these results must be prefaced with an admission that they are derived from a small number of animals. The use of additional animals would have made serum fractionation impracticable and the choice was made to follow the antibody responses of a few animals at short intervals rather than monitor a greater number less frequently. Furthermore it should be pointed that this discussion is confined to a consideration of the antibody responses of metatherians to either SRBCs or BSA only.

Obvious differences were seen in the responses of the two species to the lower dose of SRBCs injected.

Firstly, the time taken to reach the peak haemagglutination titre in the opossums was about half that of the rabbits. This was less apparent when a larger dose of SRBCs was used although there still remained an indication that the opossums reacted more promptly than did rabbits. These results might indicate that a relationship clearly exists between the amount of antigen injected and the timing of the response to this antigen. This possibility could be explored by using a greater variety of erythrocyte doses for immunization.

Secondly, the haemagglutination titres of the rabbits injected with the lower dose of sheep erythrocytes were 8- to 16- times those of the opossums. When the larger dose of SRBCs was used the titres of the two species were very similar, a situation brought about by improved responses on the part of the opossums and a relative decline in those of the rabbits.

Yadav (1973b) reported that the serum of an opossum which had received, over a period of one year, an unspecified number of intra-peritoneal injections of 2 ml amounts of a 20 per cent suspension of SRBCs had a haemagglutination titre of 512, the assay system used was not specified. What this single titre means relative to the results given here cannot be imagined.

Thomas et al. (1972) immunized quokkas simultaneously with SRBCs, S. adelaide flagella and bacteriophage ØX174. Two 2 ml amounts of a 20 per cent SRBC suspension were injected intra-peritoneally over 21 days. Seven days after the second injection the single animal reported on had anti-SRBC agglutinins at a titre of 16, the serological method used was not described.

Marx et al. (1971) described maximum haemagglutination titres of 64 in D. virginiana. However, the immuniza-

tion and bleeding schedules were somewhat unorthodox and may have contributed to these low values. The authors claimed that the "cell density" of the opossum spleen was 1/10th that of a mouse spleen; furthermore, the opossum spleen response, as measured by a plaque assay, was "inferior" to "those of other species". Plaque assay systems provide a direct measure of the number of lymphocytes within a population that are capable of responding to a given antigen and are well suited to study the response to SRBCs. This approach was contemplated in the present study, but preliminary experiments with the plaqueing system showed that large numbers of animals would be needed to give significant results.

The distribution of antibody activity between the IgM and IgG immunoglobulin classes of immune sera has received some attention in the literature. This distribution is doubtless dependent on many factors including the prior antigenic experience of the individual, the animal's (immune) genetic composition, the type of antigen used and in particular its degree of thymus dependency or independency. Furthermore, it may also depend on the relative efficiencies of antibodies of different immunoglobulin classes in being able to participate in the assay system used. For these reasons it seems pointless to try to compare the present limited results with equally limited ones from other species. A fair general statement would be that the results fit a "typical" eutherian pattern in that IgM dominated the primary responses and IgG the secondary responses.

In general terms the distribution of the haemagglutinating activity between the IgM and IgG immunoglobulin classes throughout the antibody responses was similar in the two species. IgM may have had a more transient

role in the secondary responses of the opossums than in rabbits but the data are so limited that this must remain only an impression.

It was initially planned to study the carrier effect and hapten discrimination in T.vulpecula by using combinations of KLH-TNP and BSA-DNP. However, after repeated injections of the BSA-DNP conjugate alone into opossums over a 3 month period no antibody to BSA could be detected by passive haemagglutination technique. The use of an adjuvant seemed necessary if a response to a soluble protein was to be achieved in the opossum.

As judged by the anti-BSA titres reached and the times taken to do so the two species showed similar capabilities. Table 7.4 could possibly be interpreted as showing that the primary responses of the opossums were again more brief than those of rabbits and also of a lower magnitude. In retrospect the booster injections of the opossums could well have been delayed somewhat in order to give the animals more time in which to show a definite change in their antibody levels.

Twenty-one days after the injection of 100 mg of BSA in Freund's complete adjuvant a quokka had an anti-BSA titre, measured by a passive haemagglutination technique, of 1024 (Thomas et al., 1972). This species, as was also noted for T.vulpecula, did not show a vigorous secondary response. Thus twenty five days later, and after receiving a further 50 mg of BSA intra-peritoneally, the titre remained at 1024. After a further BSA injection the titre eventually rose to  $10^6$ , a titre some 4 times greater than that recorded here for T.vulpecula, but in general the responses of these two species to BSA were not dissimilar.



Following intramuscular injections of BSA into D.virginiana, Taylor and Burrell (1968) showed that one of the two opossums showed a peak haemagglutination titre of 1024 about 3 weeks after a booster injection, the other one did not show any antibodies. When the authors used KLH as the antigen, three out of four opossums showed haemagglutinating antibodies although one animal showed antibodies only after 18 weeks of primary injection. The peak titre obtained (4096) following a booster injection with KLH was higher than that attained against BSA in the other animals.

From the data available it would seem that T.vulpecula is as efficient as other modern metatherians in its ability to respond to either SRECs or BSA. Indeed, the antibody responses of this entire group seem typically eutherian in both the expected levels of antibody produced and in the immunoglobulin classes expressed.

CHAPTER 8CONCLUDING STATEMENT

This investigation has considered a number of B cell functions in T.vulpecula. It has established that this species has B cells which, in their surface markers and tissue distribution, resemble those of eutherians. It has also shown that in culture these cells respond to recognized mitogens in a manner similar to their eutherian counterparts- the morphologies of the transformed cells and their immunoglobulin products being the same in both cases. Immunoglobulins analogous to eutherian IgG, IgM and IgA have been identified in T.vulpecula. Admittedly, the study has failed to demonstrate the occurrence of sub-classes of immunoglobulins, particularly in regard to IgG, but as discussed previously this is likely to be due to the approach used. Finally, the ability of T.vulpecula to respond to antigenic stimulation was seen to be essentially the same as that of rabbits.

In brief, no evidence has been produced that suggests that T.vulpecula is in any way deficient in B cell capability. However, the co-operative role of B and T cells in most antibody responses is appreciated and studies investigating T cell mediated help and suppression in antibody production seem warranted.

Each class of vertebrate animal must be acknowledged as being immunologically competent or else it would not survive. Nevertheless the evolution of vertebrates has been accompanied by an increasing complexity of immunological capacity. By studying the immune capabilities of "lower" animals we may be able to visualize the strata on which the final eutherian form of immunity is based. It is in this respect that comparative immuno-

logy provides a fruitful and important area for investigation.

It is because of this importance that comparative immunology holds that this thesis ends with a plea. Valid comparisons of the immune capabilities of different classes of animals will be possible only when a more standard investigative approach is used. It is the author's firm hope that the use of a battery of standard antigens, employed in an agreed fashion, the responses to which are assayed by standard techniques can be adopted. It would not be difficult to extend this concept to cover fields such as mitogen responsiveness by cells in culture and the many other diverse areas which make comparative immunology so fascinating. Such an approach would not preclude the use of additional antigens either as a personal preference or for a particular purpose but it would certainly reduce the confusion that now exists.

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APPENDIX ISuppliers of products mentioned in the text

- Baker Chemical Company, Phillipsburg, New Jersey, USA.
- Baltimore Biological Ltd, Maryland, USA.
- BDH Chemicals Ltd, Poole, England.
- Beckman Instruments Inc., Fullerton, California, USA.
- Becton-Dickinson, Becton, Dickinson and Company, Oxnard, California, USA.
- Calbiochem, San Diego, California, USA.
- Cappel Laboratories Inc., Cochranville, P.A 19330, USA.
- Central Veterinary Laboratory, Weybridge, Surrey, England.
- Cooke, Dynatech Laboratories Inc., Alexandria, Virginia, USA.
- Difco Laboratories, Detroit, Michigan, USA.
- Falcon Plastics, Division of Becton, Dickinson and Co., Oxnard, California, USA.
- Fenwal Laboratories, Deerfield, Illinois, USA.
- Flow Laboratories Ltd, Irvine, Scotland.
- Fluka, Chemische Fabrik, Buchs SG, Switzerland.
- GIBCO, Grand Island Biological Co., Grand Island, New York, USA.
- ICI, Imperial Chemical Industries, Macclesfield, Cheshire, Great Britain.
- Kayline Plastics, Plympton, South Australia.
- Nyegaard Company, Torshov, Oslo 4, Norway.
- Pharmacia Fine Chemicals AB, Uppsala, Sweden.
- Radiochemical Centre, Amersham, England.
- Rohm and Haas N.Z. Ltd., Otahuhu, Auckland, New Zealand.



Sigma Chemical Company, St.Louis, USA.

Shell Chemicals N.Z. Ltd., 96, The Terrace, Wellington,  
New Zealand.

Wellcome Research Laboratories, Beckenham, Kent,  
England.

Whatman Ltd., Springfield Mill, Maidstone, Kent,  
England.

APPENDIX IIBuffers and reagentsAcetyl acetone reagent

Acetylacetone	1 ml
Sodium carbonate, 0.5N	50 ml
Freshly prepared each time.	

Amido Black stain

Amido Black	1 gm
Acetic acid, 1M	425 ml
Sodium acetate, 0.1M	425 ml
Glycerol	150 ml

Barbital buffer for immunoelectrophoresis

Sodium barbital	10.31 gm
Barbituric acid	1.84 gm
Sodium acetate	6.80 gm
Distilled water to make	1000 ml
Ionic strength	0.1M
pH	8.6

Barbital buffer for starch block electrophoresis

Sodium barbital	19.95 gm
Barbituric acid	3.41 gm
Distilled water to make	1000 ml
Ionic strength	0.1M
pH	8.6

This buffer is diluted 1:1 with distilled water to give a final molarity of 0.05M before use.

Coomassie Brilliant Blue stain

Coomassie Brilliant Blue	0.25 gm
Methanol-acetic acid-water (5:1:5)	100 ml

Cysteine reagent

Cysteine hydrochloride	3 gm
Distilled water to make	100 ml

Ehrlich's reagent

p-dimethylaminobenzaldehyde	0.8 gm
Methanol	30.0 ml
Concentrated HCl	30.0 ml

Diphenylamine reagent

Diphenylamine	1 gm
Glacial acetic acid: conc. sulphuric acid (9:1)	100 ml

Epoxy-resin mixture

Component A (Epoxy-resin, Fluka)	5.47 gm
Component B (Hardner 964, Fluka)	4.79 gm
Component C (Acceleator 964, Fluka)	0.20 ml
Component D (Di-n-butyl phthalate, BDH)	0.10 ml

Components A and B were mixed, left at 60°C for 5 min and then re-mixed thoroughly in a vortex blender. Components C and D were then added to this mixture and mixed thoroughly.

Gel buffer for SDS-PAGE

NaH <sub>2</sub> PO <sub>4</sub>	6.8 gm
Na <sub>2</sub> HPO <sub>4</sub>	20.45 gm
Sodium dodecyl sulphate	2.0 gm

Dissolve and dilute to 1000 ml with distilled water. pH should be approximately 7.0 at 25°C.

Glacial acetic acid-Sulphuric acid mixture

Glacial acetic acid	90 ml
Sulphuric acid, concentrated	100 ml

H<sub>2</sub>SO<sub>4</sub>-H<sub>2</sub>O mixture

Sulphuric acid, concentrated	6 volumes
Distilled water	1 volume

Karnovsky's fixative

Formaldehyde	2 ml
Glutaraldehyde	3 ml
Phosphate buffer, 0.1M, pH 7.2 to make	100 ml

Lead citrate stain

Lead citrate	0.025 gm
Sodium hydroxide, 10N	0.1 ml
Distilled water to make	10.0 ml

Orcinol-sulphuric acid reagent

Reagent A:	
Sulphuric acid, concentrated	60 ml
Distilled water	40 ml
Reagent B:	
Orcinol	1.6 gm
Distilled water	100 ml
Reagent A	7.5 volumes
Reagent B	1.0 volume

Phosphate buffers, pH 8.0

<u>0.03M</u>	Solution A: NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	4.68 gm/1L
	Solution B: Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	10.75 gm/1L
	Mix solution A	53 ml
	solution B	947 ml

<u>0.04M</u>	Solution A: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	6.24 gm/1L
	Solution B: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	14.33 gm/1L
	Mix solution A	53 ml
	solution B	947 ml
<u>0.05M</u>	Solution A: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	7.80 gm/1L
	Solution B: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	17.91 gm/1L
	Mix solution A	53 ml
	solution B	947 ml
<u>0.1M</u>	Solution A: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	15.60 gm/1L
	Solution B: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	35.82 gm/1L
	Mix solution A	53 ml
	solution B	947 ml

Phosphate buffer, 0.01M, pH 7.2

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.51 gm
$\text{KH}_2\text{PO}_4$	0.41 gm
Distilled water to make	100 ml

Phosphate buffer for starch block electrophoresis

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.2M	29.0 ml
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.2M	156.5 ml
Distilled water to make		1000 ml
Ionic strength	0.2M	
pH	7.5	

Phosphate buffered saline (PBS), 0.01M, pH 7.2

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.2M	16.5 ml
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.2M	33.5 ml
NaCl		8.5 gm
Distilled water to make		1000 ml

Sample buffer for SDS-PAGE

$\text{NaH}_2\text{PO}_4$	0.34 gm
$\text{Na}_2\text{HPO}_4$	1.02 gm
Sodium dodecyl sulphate	1.00 gm
2-Mercaptoethanol	1.00 gm
Bromophenol Blue	0.005gm
Urea	36.00 gm

Dissolve and dilute to 100 ml with distilled water. pH should be approximately 7.0 at 25°C.

Scintillation fluid

2,5- Diphenyl oxazole (PPO)	4 gm
1,4- bis (2 (4-methyl-5-phenyl oxazolyl) ) benzene (Dimethyl POPOP)	100 gm
Toluene	300 ml
Triton X-100	700 ml

Sodium phosphate buffer, 0.01M, pH 7.0

Solution A: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	1.56 gm/1L
Solution B: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.58 gm/1L
Mix solution A	390 ml
solution B	610 ml

Sodium phosphate buffer (for Protein A-Sepharose Cl-4B chromatography), 0.15M, pH 8.0

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.15M	53 ml
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.15M	947 ml

Tris-HCl buffer for SDS-PAGE (Separating gel),  
1.5M, pH 8.8

Trizma (Sigma)	18.15 gm
Dissolved in distilled water	50 ml
pH adjusted to 8.8 with 1N HCl	
Final volume made to	100 ml

Tris-HCl buffer for SDS-PAGE (Stacking gel),  
0.5M, pH 6.8

Trizma (Sigma)	3 gm
Dissolved in distilled water	30 ml
pH adjusted to 6.8 with 1N HCl	
Final volume made to	50

Tris-HCl-NaCl (0.1M Tris, 0.2M NaCl; pH 8.0)

Tris	24.22 gm
Sodium chloride	23.38 gm
Distilled water to make	1500 ml
Adjust the pH to 8.0 with 1N HCl	
Make up to 2000 ml with distilled water.	

Tris-Glycine buffer for SDS-PAGE

Trizma (Sigma)	6.0 gm
Glycine	28.8 gm
Sodium dodecyl sulphate	1.0 gm
Distilled water to make	1000 ml
ionic strength	0.025M Tris 0.192M Glycine
pH	8.4

Trypan Blue stain

Trypan Blue	1 gm
Sodium chloride, 0.9% to make	100 ml

Uranyl acetate stain

50 per cent ethanol with  
 saturated uranyl acetate.

Uranyl acetate was added to saturation in 50 per cent ethanol, centrifuged and the supernatant stored in brown glass bottles.

White cell diluting fluid

Methyl violet	0.05 gm
Acetic acid	0.50 gm
Distilled water to make	100 ml.



APPENDIX IIIHistology of selected lymphoid tissues of  
T.vulpeculaA4.1. Introduction

In view of the attention paid to the identification and distribution of B lymphocytes in T.vulpecula (Chapter 3), it was felt that some consideration should be given to the histology of selected lymphoid tissues from this species.

A4.2. Materials and Methods

Lymph nodes and Peyer's patches and thymic and splenic tissues were collected from an exsanguinated animal. They were embedded in paraffin, sectioned and stained with haematoxylin and eosin by standard methods.

A4.3. Results and Discussion

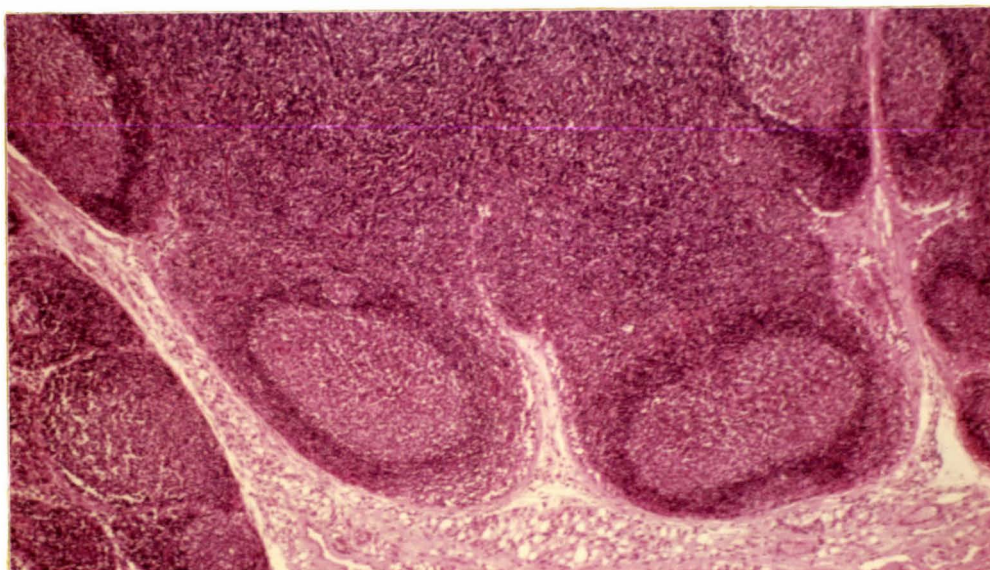
Figs. A4.1 to A4.11 show the histological appearance of the tissues examined. In all aspects the architectures and cellular compositions of the tissues resembled those of eutherian species.

FIGURE: A4.1. Lymph node. Cortical area of lymph node showing primary nodules with germinal centres. This area is encapsulated by irregularly arranged fibrous connective tissue and adipose tissue. The area above the nodule represents the zone, which in eutherians, is the thymus dependent, paracortical region. X 60

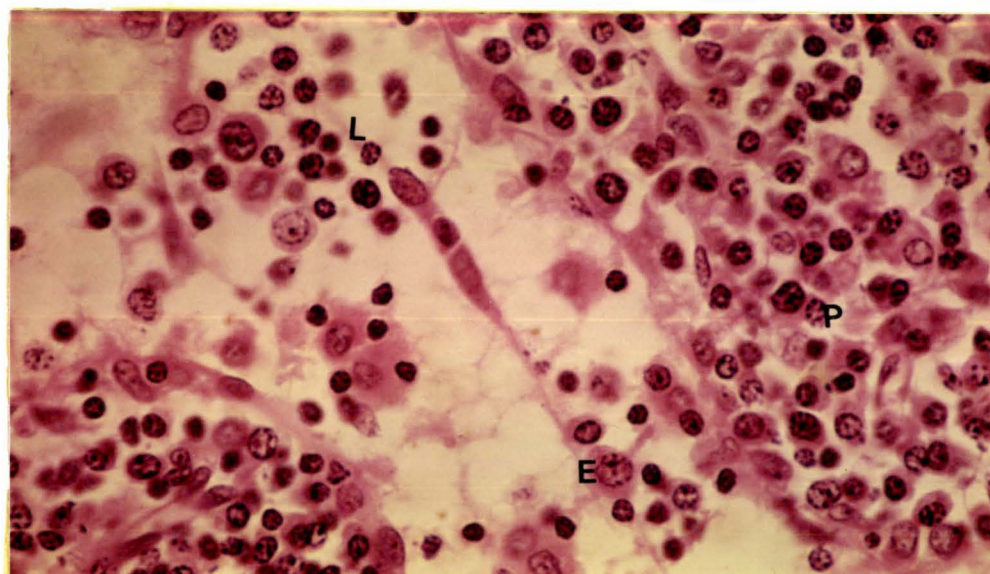
FIGURE: A4.2. Lymph node. Part of a medullary cord and a medullary sinus are shown. The medullary cord contains predominantly plasma cells (P). The few cells which contain large pale staining nuclei are reticulo-endothelial cells (E). The medullary sinus consists of a three-dimensional reticular network with free and fixed macrophages and circulating lymphocytes (L). X 600

FIGURE: A4.3. Lymph node. Three main types are recognizable within this primary nodule. Large nucleated, pale staining reticulo-endothelial cells (E), small, dark, nucleated lymphocytes (B) and intermediate cell types of the lymphoid series including lymphoblasts (Lb) or prolymphocytes. X 600

1



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3

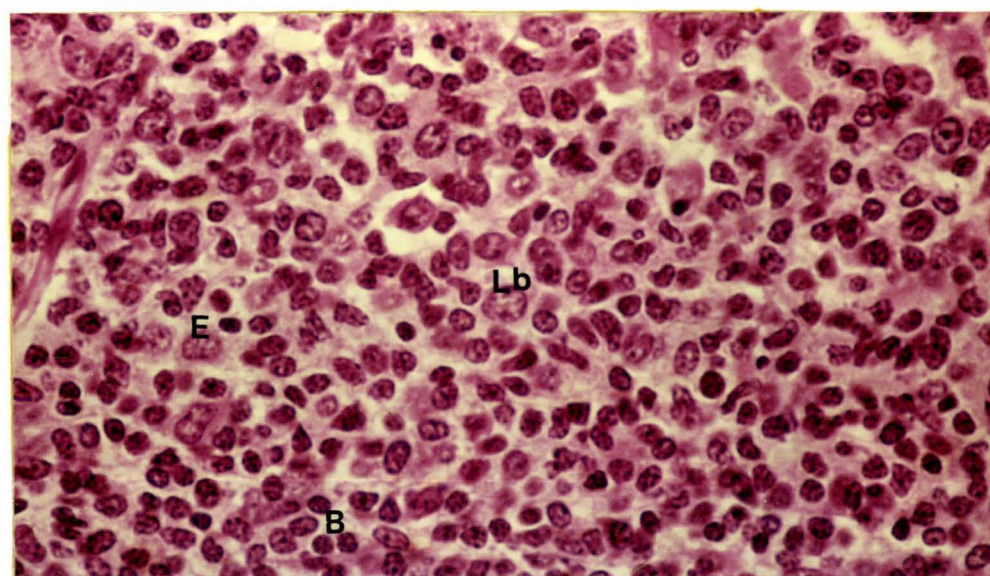


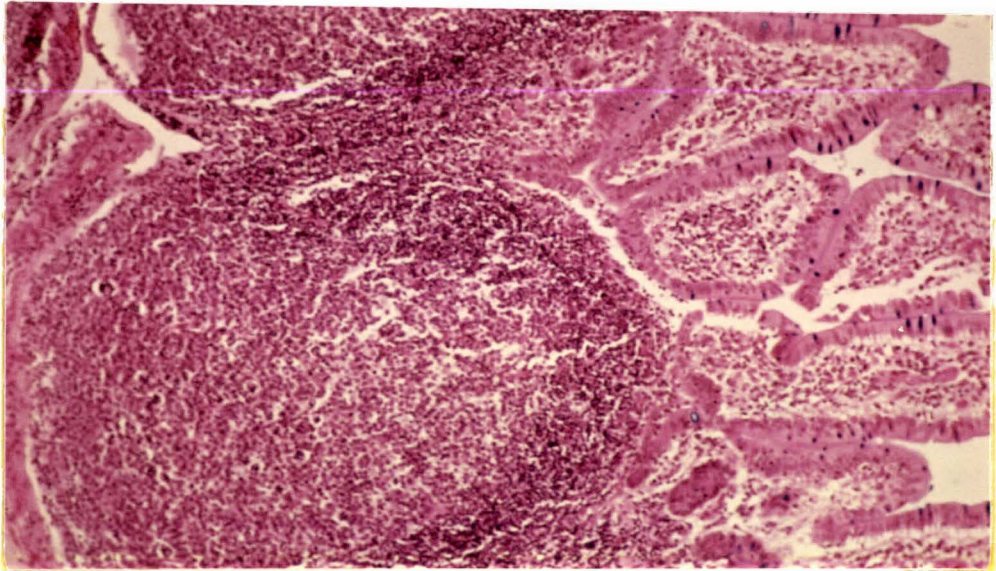
FIGURE: A4.4. Peyer's patch. Each nodule shows a dark staining corona of densely packed small lymphocytes. The germinal centre is an area of active mitotic divisions. X 100

FIGURE: A4.5. Peyer's patch. Higher magnification of Peyer's patch showing darkly staining, densely packed small lymphocytes at the periphery of the germinal centre. X 240

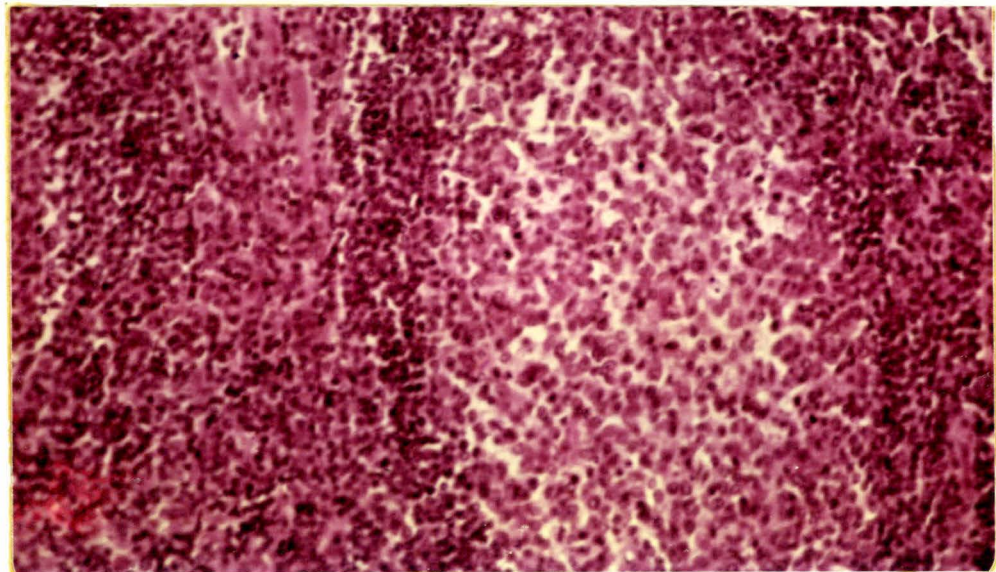
FIGURE: A4.6. Peyer's patch. Higher magnification of Peyer's patch showing densely packed, small lymphocytes (L) and reticulo-endothelial cells (E). X 600



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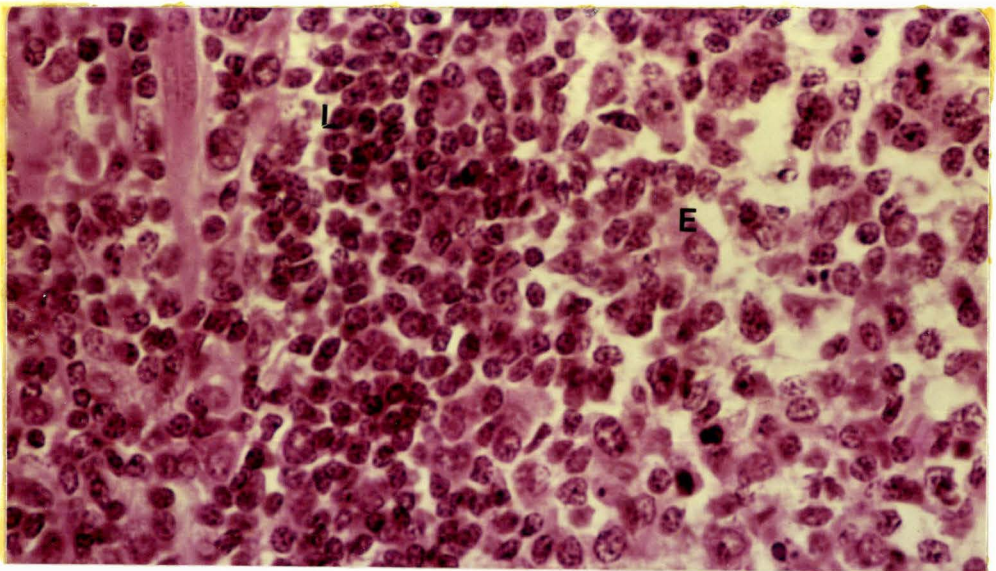
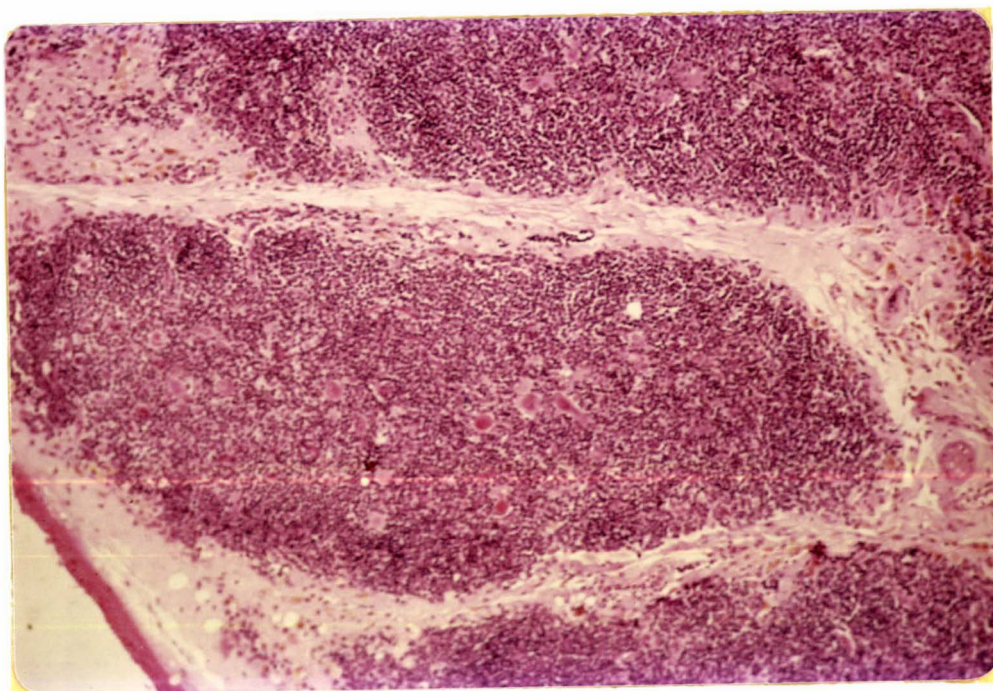


FIGURE: A 4.7. Thymus. Area of cortex showing lobules of lymphoid tissue separated by connective tissue. Haemosiderin is present in the interlobular connective tissue, probably indicating the degenerative changes associated with age involution. X 100

FIGURE: A4.8. Thymus. Medullary region showing the presence of a Hassal's corpuscle (H). Three cell types are present. Large, pale staining cells are the epithelial-reticular cells (E); small dark staining cells are mature T cells (T); mast cells (M) with blue staining cytoplasm are occasionally seen. X 600



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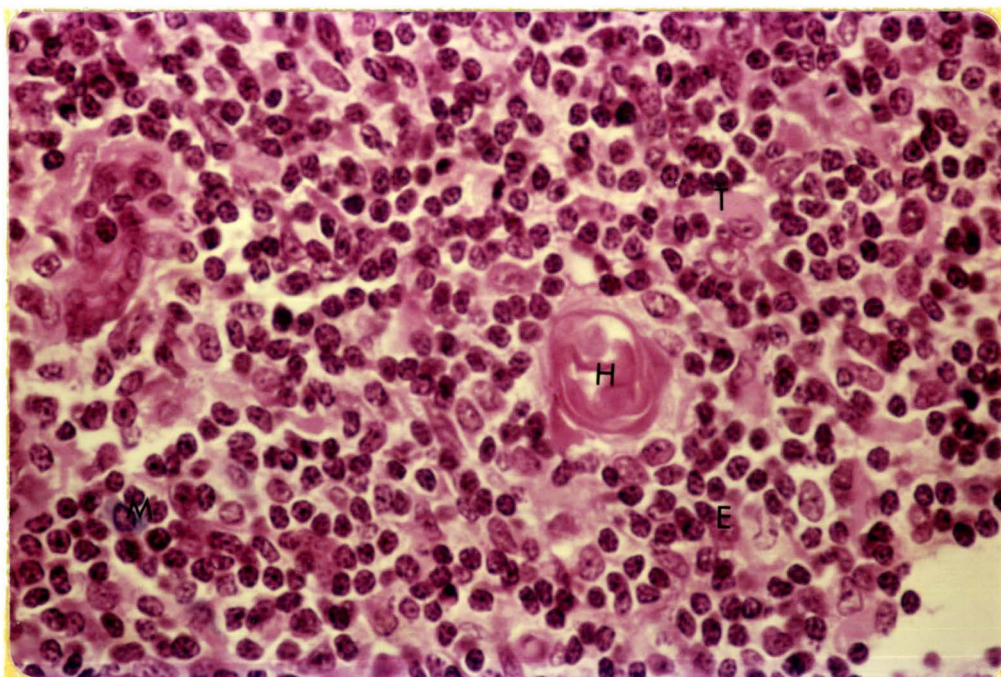


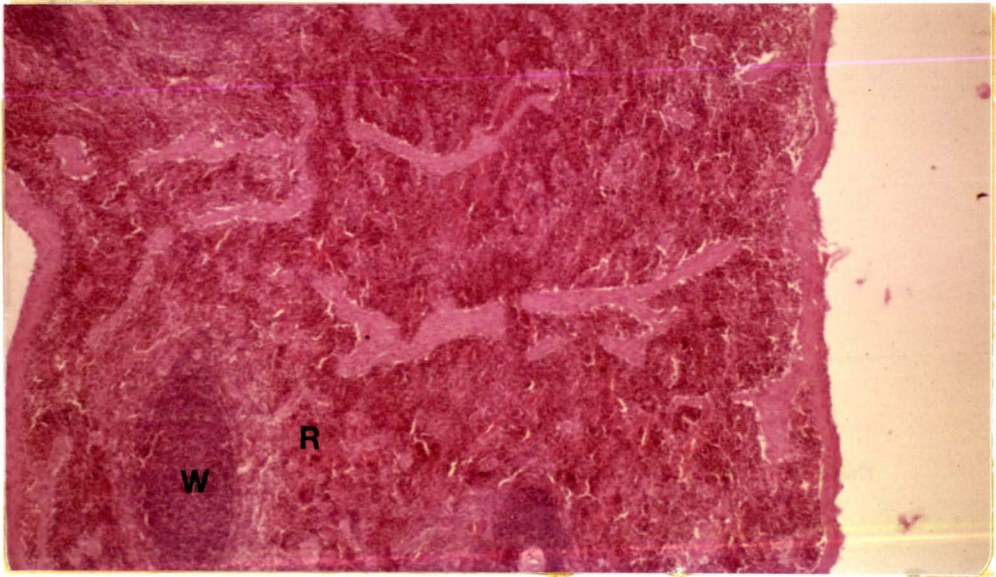
FIGURE: A4.9. Spleen. Section shows the capsule and associated trabeculae extending into the substance of the organ. Areas of red pulp (R) and white pulp (W) are visible. One arteriole surrounded by lymphoid tissue can be seen at the bottom of the photomicrograph. This is the periarteriolar lymphatic sheath populated by T lymphocytes. X 60

FIGURE: A4.10. Spleen. Higher magnification of spleen showing a periarteriolar lymphatic sheath enclosing an arteriole (A). X 240

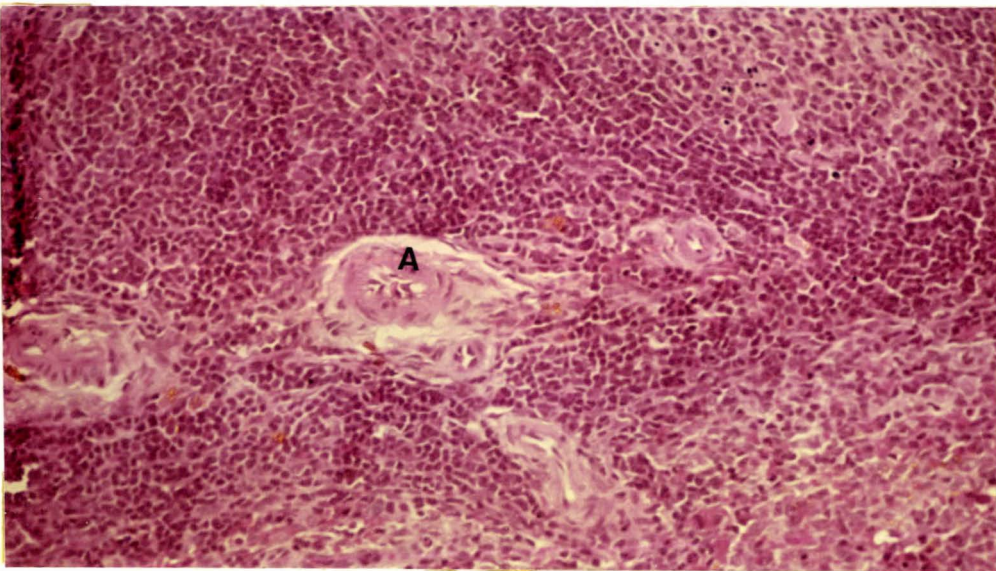
FIGURE: A4.11. Spleen. Higher magnification of the white pulp of spleen showing numerous small lymphocytes among the network formed by reticulo-endothelial cells. X 600



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11



## APPENDIX IV

### Miscellaneous techniques

#### Lymphocyte counting

The lymphocytes to be counted were suspended in 2 ml of MEM-FCS. 200 ul of this suspension was mixed for 1 min with 4 ml of white cell diluting fluid (Appendix II). A drop of this mixture was placed in a haemocytometer and examined under X10 magnification. The total number of cells in the four large corner squares was counted, and multiplied by 50 to give the number of cells per cmm; this value was multiplied by 1000 to give the number of cells per ml.

#### MacNeal's stain

Smears made from mononuclear cell suspensions were flooded with MacNeal's stain for 3 min. The added stain was then diluted with an approximately equal amount of phosphate buffer, pH 6.8, 0.01M, and left for 6 min. The stain was washed from the slide with distilled water and the smear air-dried.

#### Preparation of liver powder

Opossum livers were obtained from exsanguinated animals. They were diced and washed twice with 0.15N NaCl prior to being homogenized. The homogenate was mixed with 4 volumes of acetone and centrifuged at room temperature. The precipitate was washed twice with acetone and, filtered on a Buchner funnel and dried at 37°C overnight. The dried coarse powder was ground in a mortar and sieved. The fine powder so obtained was stored at 4°C.

### Staining thick sections of blocks (electron microscopy)

Stains consisting of 1 per cent toluidine blue or 4 per cent basic fuchsin were used to stain thick sections of embedded block. Staining was for 1 min at 80°C for each stain. The smears were then washed with distilled water and air dried.

### Staining thin sections of blocks (electron microscopy)

The grids containing thin sections were stained with uranyl acetate for 5 min. They were then washed with 50 per cent ethanol for 2 min and stained with lead acetate for 5 min. The grids were then washed with distilled water and air dried.

### Trypan blue staining for Cell viability

200 ul of lymphocyte suspension were mixed with 1.8 ml of trypan blue stain (Appendix II). After 5 min two hundred cells were examined and the number stained, presumably dead cells, was counted.