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THE NEISSER-WECHSBERG PHENOMENON

by

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ABSTRACT

This study concerns the Neisser-Wechsberg (NW) phenomenon - a prozone phenomenon that commonly occurs in the complement-mediated bactericidal reaction. It involves the deviation of complement activity at relatively high levels of associated antibody. Thus, target bacteria survive in the presence of relatively low dilutions of a hyperimmune antiserum but can be killed in the presence of higher dilutions. This study, together with other studies, indicate that the NW phenomenon is an *in vivo* phenomenon, and not just an *in vitro* artefact. The humoral arm of immune defense is significantly weakened in hyper-immunized animals and in humans with chronic infections involving Gram-negative bacteria. The phenomenon is probably not the cause of chronic infection but is an important consequence and may have a role in sustaining such an infection.

The system studied here consists of the *Salmonella typhimurium* M206 strain of bacterium, rabbit antisera raised against this bacterium, and human or guinea pig serum as the complement source. Bacteria, sensitized with a prozoning dilution of antiserum, still displayed the prozone phenomenon when washed and incubated with a complement source. Significant complement consumption was shown to occur in the prozone situation and the consumption was dependent on the presence of target bacteria. Thus, deviation of complement activity in the bactericidal reaction appears to be associated with phenomena occurring on the surface of the target bacteria, rather than with interactions

between antiserum and complement in the fluid-phase.

Two rabbits were periodically vaccinated with heat-killed bacteria over eight weeks and, unlike the bactericidal activity (1 week after initial vaccination), prozoning activity appeared later (after 2-3 weeks) in the rabbit sera and peaked after four weeks. Hyperimmune antisera were subjected to single step and stepwise affinity chromatography on protein A-Sepharose columns and the prozoning activity was found to reside exclusively with the IgG immunoglobulins. Fractions containing IgA and IgM immunoglobulins did not display prozoning activity. IgG fractions, having different reactivities for protein A, were isolated and all contained prozoning antibody and all were able to fix complement. It is concluded that complement-fixing (bactericidal) IgG antibody is the immunoglobulin species responsible for the NW phenomenon.

Adsorption studies, utilizing mutant strains of various Salmonella species, showed that the prozoning antibody is specific for antigenic determinants associated with the bacterial lipopolysaccharide (LPS) (O-somatic antigens, in this system), and no other outer membrane (OM) component. As the isolation of specific prozoning antibody by elution from whole M206 cells or insolubilized LPS proved extremely difficult, it is concluded that this antibody is very avid. A protein A binding assay, that measures cell-bound IgG, was used to establish that near antigen-saturating levels of specific IgG antibody are required to effect the prozone phenomenon.

Through the use of certain R reagents (C-142, C-EDTA, C-EGTA) and the demonstration of immune adherence with

prozoning mixtures, it was established that both complement pathways are activated to at least the C3 component in the NW phenomenon. Through the use of other R reagents (RaC6-9, C6-def RaC) and an assay to detect deviated lysis, it became apparent that the membrane attack complex (MAC) of complement is not formed or, once formed, cannot bind to an OM site where it can effect a lesion. Further, the prozone phenomenon was demonstrated with sheep red blood cells and *S. minnesota* R595 cells sensitized with the LPS of *S. typhimurium* C5; this constituted evidence for the cooperation of LPS-antibody complexes in promoting the fixation of MAC to cells, that are subsequently killed and lysed by it.

The data allows the following speculations to be made, regarding the NW phenomenon. Avid IgG antibody quickly and efficiently covers the LPS antigens of the target bacteria. This dense cover imparts a highly hydrophobic character to the surface of these cells. Either, the formation of the large, amphiphilic MAC of complement is sterically and electrostatically hindered or, once formed, is prevented from interacting with the LPS-antibody complexes. Any MAC, formed in the fluid-phase is quickly inactivated by the inhibitor, S-protein.

STATEMENT

The material in this thesis has not been previously submitted for a degree in any University, and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

(A. CAON)

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Dr. Bruce Reynolds for his guidance during the study and subsequent preparation of this manuscript. Acknowledgements also go to Garry Penney for his support and Jan Hemming for her typing. I especially thank my family for allowing me to devote time away from them in order to pursue this study.

ABBREVIATIONS

Å	angstrom unit
CM	cell membrane
cpm	counts per minute
CWP	cell wall protein
C6-def RaC	C6-deficient rabbit complement
DID	double immunodiffusion
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
FFA	free fatty acids
GpC	guinea pig complement
HA	haemagglutination
HuC	human complement
ID	immunodiffusion
IEP	immunoelectrophoresis
KDO	2-keto-3-deoxyoctonate
LPE	lysophosphatidylethanolamine
LPS	lipopolysaccharide
MAC	membrane attack complex
2ME	2-mercaptoethanol
Mg^{2+} saline	0.002M $MgSO_4$ in N-saline
MKC	minimum killing concentration
MW	molecular weight
ND	not detected
NRS	normal rabbit serum
NT	not tested
N-saline	0.9% W/V NaCl
NW	Neisser-Wechsberg
OD	optical density

PE	phosphatidylethanolamine
PMN	polymorphonuclear
RaC	rabbit complement
RaC6-9	rabbit complement, lacking C3 and C5 activity
RBC	red blood cell
SRBC	sheep red blood cell
SRID	single radial immunodiffusion
TCA	trichloroacetic acid
TESA	Tris-EDTA-saline-azide
Tris	tris-(hydroxymethyl)-aminomethane
UDPG	uridine diphosphate-galactose
VBS	veronal buffered saline
V/V	volume per volume
W/V	weight per volume

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CHAPTER 1 INTRODUCTION

CHAPTER 1.1 The complement-mediated bactericidal reaction

1.1.1 The history of complement and the bactericidal reaction

The first important theory of mammalian resistance to disease was proposed by Metschnikoff in 1887. For several years, he studied the behaviour of leucocytes in many animals, and attributed the destruction of bacteria in the body to the digestive activity of these cells. He proposed that phagocytosis was the major mammalian defense against infectious agents.

Grohmann in 1884 showed that cell-free mammalian plasma could kill bacteria and fungi. The phagocytic process was questioned as being the only important factor in the defense against infectious agents, by this and other work. Buchner (1889) showed that fresh, cell-free serum was capable of killing and lysing several different bacteria, whilst other bacteria were unaffected. Susceptible bacteria included the cholera vibrio, typhoid bacillus and bacterium coli. Buchner termed the serum component responsible "alexin". Its activity was thought to be enzymatic because it was destroyed by heat (55^{o}, 60 minutes) and was most readily demonstrated at body temperature.

A major advance in the understanding of this serum bactericidal reaction came with the work of Pfeiffer (1893). He found that cholera vibrios were destroyed in the cell-free peritoneal cavity of immunized guinea pigs,

as well as in the serum from such animals. The heated serum did not kill the vibrios but, when the heated serum plus live vibrios were injected into the peritoneal cavity of normal guinea pigs, these vibrios were destroyed. Such experiments indicated the importance of the serum bactericidal reaction in the mammalian defense against pathogenic bacteria. Some considerable time was to elapse before it was agreed that both the cellular and humoral mechanisms constituted important defense systems or that some humoral components accelerated the phagocytic process.

Bordet (1898) recognized that two components were required for the serum bactericidal reaction: (i) a heat-stable substance, whose concentration was increased in the serum of immunized animals, and (ii) the heat-labile substance alexin, present in the serum of normal and immunized animals. The terms that were eventually accepted for the two "mandatory" components in the bactericidal reaction were "antibody" and "complement", respectively (Ehrlich and Morgenroth, 1898a). "Antiserum" also replaced the term "immune serum". It was further observed that, although some minimal concentration of antibody was needed to promote complement-mediated killing, excess antibody could inhibit it (Neisser and Wechsberg, 1901). This was termed the Neisser-Wechsberg phenomenon and its investigation forms the subject of this thesis.

Bordet (1898) also discovered a phenomenon that he termed "immune haemolysis". If the blood of one species of mammal was injected into an individual of another species, the serum of the recipient developed the ability

to lyse the red blood cells (RBCs) of the donor species. He recognized the requirement of the same two components as in the serum bactericidal reaction. He showed that the haemolytic activity of a rabbit antiserum, destroyed by heating to 56°, could be restored by the addition of fresh normal rabbit serum (NRS).

Antibody, capable of lysing cells other than bacteria or RBCs eg., nerve cells, spermatozoa, leucocytes, liver cells and gastric epithelia, could also be produced by immunization (Bolton, 1906). There was a shift in emphasis from study of the complement-mediated bactericidal system to that of the immune haemolytic system. RBCs were easier to handle and standardise in the *in vitro* assays than bacteria because of their larger size and non-viable state. Thus various phenomena, previously established for the bactericidal system, were shown to hold true for the haemolytic system.

For many years, study of the immune haemolytic system revealed fundamental relationships between the target RBCs, the specific antibody and the complement. These studies also guided the less frequent investigations of the serum bactericidal system. Ehrlich and Morgenroth (1899a, 1899b, 1900) established the sequential nature of immune haemolysis. They showed that the combination of specific antibody and RBCs (sensitization) could occur at 0°, and that lysis could then occur in the presence of complement only at elevated temperatures (preferrably 37°). By the kinetic analysis of immune haemolysis, Brooks (1919) and several other workers discovered that curves of the time course of haemolysis, and those of

the complement dose and lytic response, were sigmoidal. These observations and the assumption that complement was a single substance led some workers (Alberty and Baldwin, 1951) to suggest that a target cell was lysed only after a critical number of damaged sites had accumulated on its surface.

There was a succession of workers who demonstrated the multi-component nature of complement, by physico-chemical techniques. Ferrata (1907) identified precipitating and soluble components of complement by dialysis in water. Brand (1907) showed that the precipitating "midpiece" could combine with sensitized RBCs and, after washing, these could be lysed by the soluble "endpiece". A third subunit or component was identified as combining with yeast cells and being destroyed by cobra venom factor. A fourth component was identified as being sensitive to ammonia (Gordon *et al.*, 1926). Pillemer and Ecker (1941) proposed the terminology C'1, C'2, C'3 and C'4 for the four components, identified by these techniques. Ueno (1938) established the order in which these components interacted with sensitized RBCs ie., C'1, C'4, C'2 and C'3.

In the ensuing years, the mechanism of immune haemolysis was studied with "R" reagents; these were specifically lacking in one or more components. The results were often difficult to interpret as the R reagents were not functionally homogeneous and the techniques used to measure the degree of haemolysis were not precise. The quantitative spectrophotometric procedure for the titration of haemolytic complement was eventually

adopted. The sigmoidal relationship between complement dose and haemolytic response was utilized to accurately gauge complement concentration as CH50 units per ml. This procedure ushered in an era, during which parameters influencing immune haemolysis (e.g., the nature and concentration of anti-RBC antibody, temperature, pH, electrolyte concentration, reaction volume) were studied. Mayer, in particular, was a prominent worker in this field. He reintroduced the kinetic approach, isolated reaction intermediates and formulated the "one hit theory" of immune haemolysis (Mayer, 1961). Rapp and Borsos (1970) provided a mathematical basis for this theory; their book also contains a thorough historical review of the early complement studies. Mathematical analysis by Rapp (1958) also provided strong evidence that the activity, then ascribed to C3, was not a single factor.

Müller-Eberhard, Götze, Cooper and Nelson, and others used sophisticated chromatographic techniques during the 1960's to isolate pure complement components, including five additional ones, that were recognized as participating in immune haemolysis. A thorough review of these techniques was recently published by Williams and Chase (1977). A standard nomenclature for the complement components and reaction intermediates in immune haemolysis was established by a committee of the World Health Organization (1968); it is used throughout this thesis. The complement pathway that leads to haemolysis, through the activation of components C1 to C9 on sensitized RBCs, was termed the "classical pathway".

This pathway has been reviewed by Müller-Eberhard (1975); Fig. 1.1, taken from this article, is a schematic representation of the molecular events.

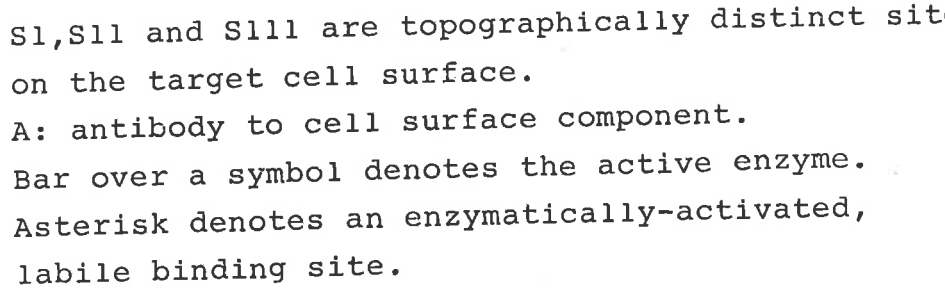
The early observations of C3 depletion from serum by cobra venom factor and yeast cells initially led to independent lines of research. They eventually merged together with the realization of a common, second pathway of complement activation, termed the "alternative pathway". This can be activated by naturally occurring polysaccharides and lipopolysaccharides (LPS), and by immunoglobulins. However, this pathway may also operate in the complete absence of specific antibody and immunoglobulins (Schreiber *et al.*, 1979). The pathway involves only components C3 to C9 of the classical pathway, and is endowed with a unique amplification mechanism. Much attention has been focused on the alternative pathway recently and up to date review articles (Götze and Müller-Eberhard, 1976; Müller-Eberhard and Schreiber, 1980) exist. Fig. 1.2, schematically representing the molecular events of this pathway, is taken from an article by Schreiber *et al.*, (1978).

Various inhibitors and modulators of the complement pathways have also been known for many years. S-protein, the most recently discovered inhibitor, combines to the membrane attack complex (MAC) (Kolb and Müller-Eberhard, 1975; Podack *et al.*, 1976) and inactivates it. The MAC consists of the five terminal complement components (Kolb and Müller-Eberhard, 1973; Podack *et al.*, 1978) and forms the subject of much current work.

Although not mentioned thus far, there have been

FIGURE 1.1

Schematic representation of the molecular events in the classical pathway of complement



First site: Activation of Recognition Unit

1. $S1A + C1q\begin{cases} r \\ s \end{cases} \longrightarrow S1A\text{-}C1q\begin{cases} \overline{r} \\ \downarrow \\ \overline{s} \end{cases}$

Second site: Assembly of Activation Unit

2. $C4 \xrightarrow{S1AC\overline{1}} C4a + C4b^*$

3. $C2 \xrightarrow{S1AC\overline{1}} C2a^* + C2b$

4. $S11 + C4b^* + C2a^* \longrightarrow S11C\overline{4b,2a}$

5. $C3 \xrightarrow{S11C\overline{4b,2a}} C3a + C3b^*$

6. $S11C\overline{4b,2a} + C3b^* \longrightarrow S11C\overline{4b,2a,3b}$

Third site: Assembly of Membrane Attack Mechanism

7. $C5 \xrightarrow{S11C\overline{4b,2a,3b}} C5a + C5b^*$

8. $S111 + C5b^* + C6 + C7 + C8 + C9 \longrightarrow S111C5b,6,7,8,9.$

(taken from Müller-Eberhard, 1975)

FIGURE 1.2

Schematic representation of the molecular events in the alternative pathway of complement

Control: the situation that exists in a non-challenged state, in the fluid phase, or upon introduction of a nonactivator particle.

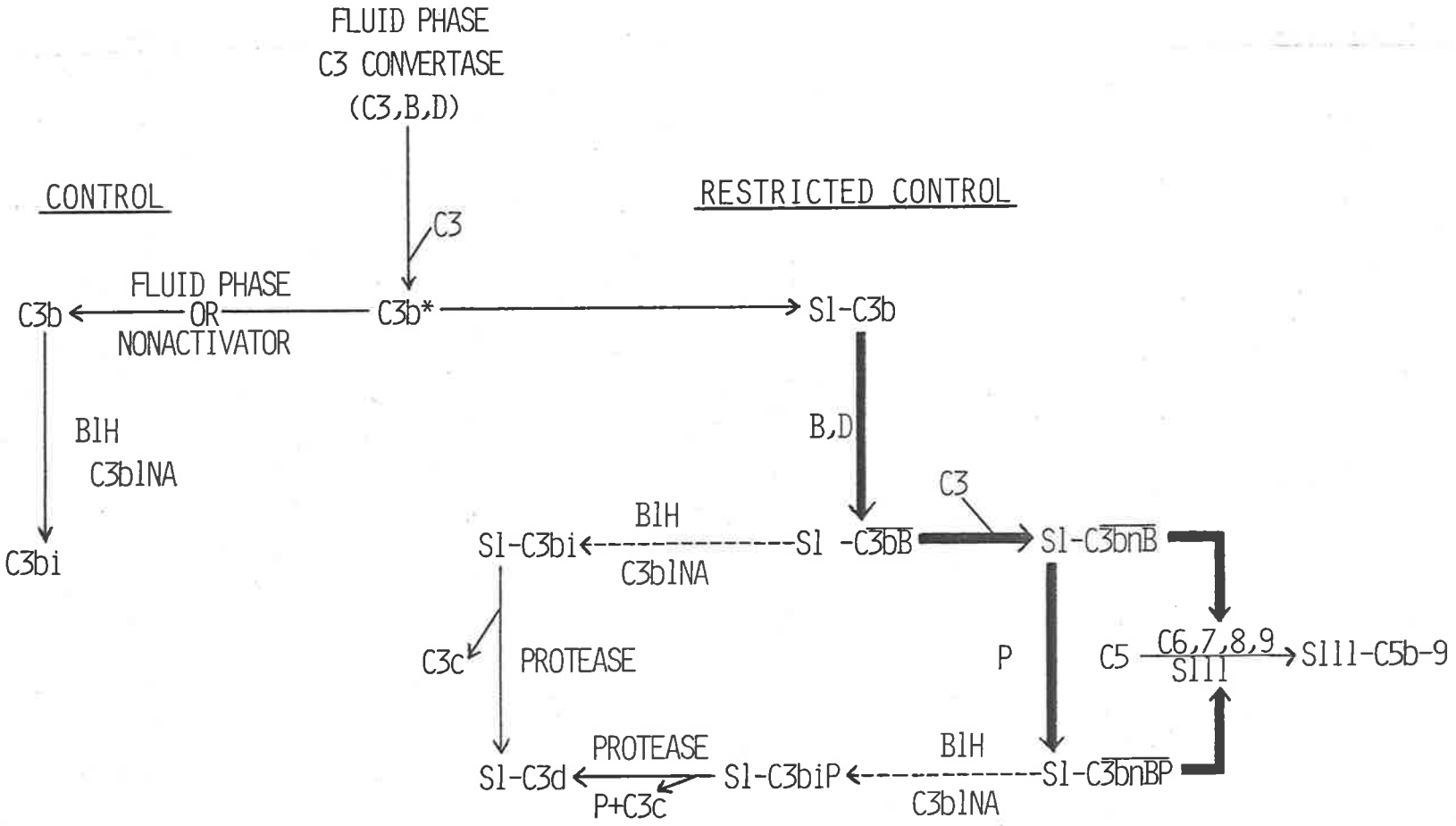
Restricted control: the situation that occurs upon introduction of a pathway activator.

C3b*: nascently formed C3b before decay of its labile binding site.

S1: the site of initial C3b deposition and its secondary interaction with structures of an activator particle.

S111: the attachment site of the membrane attack complex, C5b-9.

Control reactions are in small type; amplification reactions are in bold type. The heavy solid arrows indicate amplification; the thin-dashed arrows denote restriction of control reactions.



(taken from Schreiber et al., 1978)

extensive studies done on complement-dependent host defense mechanisms, other than the serum bactericidal system; the biosynthesis and metabolism of complement components; phylogeny of the complement system; inherited deficiencies of complement components in various mammals; complement-mediated cytotoxicity with cells other than RBCs as targets, and; complement-mediated participation in inflammation and tissue injury. However, these aspects will not be considered in detail here, as they are not relevant to the Neisser-Wechsberg (NW) phenomenon.

Studies of the serum bactericidal system have revealed basic similarities but important differences from the immune haemolytic system. The latter stem from basic differences in the two target cells ie., cell wall structure and viability. One example is the existence of serum-resistant bacteria, known for almost a century (Buchner, 1889). This topic is expanded in 1.1.2.

The requirement of antibody in the complement-mediated bactericidal reaction, postulated at the turn of the century by Bordet (1898), has been scrutinized over the past 25 years. Some workers considered the bactericidal antibodies to be non-specific or of limited specificity (Gordon and Carter, 1932). However, others (Turk, 1959; Sterzl *et al.*, 1962; Wardlaw, 1962) found that antibody-deficient serum could kill bacteria. Serum from precolostral animals or animals raised in germ-free environments was also found to kill bacteria (Franek *et al.*, 1961; Kim *et al.*, 1966; Dlabac, 1968). The presence and participation of natural antibody of low affinity, in these sera, has been difficult to disprove and several

workers (Inoue *et al.*, 1959; Muschel and Jackson, 1963; Rowley, 1968) demonstrated an antibody requirement. It is now generally agreed that the bactericidal reaction, mediated by the classical pathway of complement, requires the interaction of antibody, specific for the target bacterium.

The issue of antibody involvement was clarified when the full implications of the alternative pathway of complement were realized. In the assumed absence of antibody, bactericidal action against certain Gram-negative bacteria was first attributed to the so-called "properdin system" by Wardlaw and Pillemer (1956). Some (Osawa and Muschel, 1960; Reed and Allbright, 1974) suggested that antibody was involved in the bactericidal reaction, mediated by this pathway. The use of factor β-depleted serum (Götze and Müller-Eberhard, 1971) and C4-deficient guinea pig serum (Root *et al.*, 1972) established the involvement of alternative pathway proteins in the bactericidal reaction. Recently, Schreiber *et al.*, (1979) demonstrated bactericidal activity through the interaction of the 11 isolated plasma proteins, that comprise the cytolytic alternative pathway (Schreiber and Müller-Eberhard, 1978). Immunoglobulins were absent and so specific antibody was shown not to be necessary.

The bactericidal and bacteriolytic actions of complement were recognized as two distinct events nearly twenty years ago. Kinetic studies by Davis *et al.*, (1966) showed a succession of events: cell death, accompanied by a loss of phospholipids; conversion of

cells to spheroplasts, and; conversion to ghosts. They monitored killing with the colony count assay, and lysis with optical density (OD) measurements and nucleic acid release. Lysozyme, an enzyme normally present in mammalian serum, attacks the glycopeptide component of the cell wall of Gram-negative bacteria. Inoue *et al.*, (1959) and Glynn and Milne (1967), through kinetic studies with *Escherichia coli*, established that lysozyme is not necessary for the killing but is necessary for the lysis of bacteria. There is no such requirement for immune haemolysis.

The requirements for magnesium and calcium ions in the bactericidal reaction was found to be similar to that of immune haemolysis (Treffers, 1958). Dozois *et al.*, (1943) showed that all four known components of complement were required for immune bacteriolysis. Inoue *et al.*, (1968b) used highly purified preparations of components to show that all nine, nowadays recognized as constituting the classical pathway of complement, are required for immune bacteriolysis.

Several years after the visual demonstration of lesions on RBCs in immune haemolysis (Humphrey and Dourmashkin, 1965), similar lesions were demonstrated on whole bacterial cells (Glynn and Milne, 1967; Bladen *et al.*, 1967), on bacterial spheroplasts (Bladen *et al.*, 1966) and on bacterial LPS (Bladen *et al.*, 1967), by the action of specific antibody and complement. The cell walls of bacteria are relatively complicated structures compared to RBC membranes. For this reason, the nature of the site and the event that results in the killing of

bacteria, in the complement-mediated bactericidal reaction, are not well understood. This is discussed further in 1.1.2.

1.1.2 Sensitivity of bacteria and the mechanism

Gram-positive bacteria are not ordinarily susceptible to killing or lysis by specific antibody and complement, whereas Gram-negative bacteria are. Susceptible bacteria are termed "serum-sensitive" while those that are not susceptible are termed "serum-resistant".

Serum substances, termed "β lysins", have been known to kill various Gram-positive bacteria such as *Bacillus anthracis*, *Bacillus subtilis*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Muschel, 1960). They are thermostable and are not increased by vaccination. Little is known about the nature of these substances, although Hirsch (1960) showed that blood platelets release them during the clotting process. This means that the bactericidal activity of these substances may well be absent from circulating plasma. However, antibody and complement are still important in the body defense against Gram-positive bacteria by promoting their phagocytosis (Cohen and Morse, 1959).

Buchner (1889) showed that strains of several Gram-negative bacteria ie., *Vibrio cholerae*, *Salmonella typhi* and *Escherichia coli*, are killed by fresh cell-free serum. The list of serum-sensitive Gram-negative bacteria was extended to include species of Vibrio, Salmonella, Proteus, Shigella, Haemophilus, Brucella, Chromobacter and Escherichia (Muschel, 1965). Pfeiffer and Friedberger

(1903) first showed that not all Gram-negative bacteria are serum-sensitive. Certain strains of *Paracolobactrum ballerup, Salmonella typhimurium* and *Salmonella paratyphi C* are resistant to both normal and immune serum (Muschel, 1965). The more virulent strains of Gram-negative bacteria, often associated with systemic infections in humans (Roantree and Rantz, 1960) are usually the smooth type. The corresponding rough strains of the same species usually show greater susceptibility to killing by antibody and complement (Dlabac, 1968; Muschel, 1965; Rowley, 1968). There may even be differences between smooth strains of the same species. For example, the C5 and M206 strains of *Salmonella typhimurium* are serum-resistant and serum-sensitive, respectively (Furness and Rowley, 1956; Krishnapillai and Karthigasu, 1969). These two strains were extensively used in the thesis work.

Gram-negative bacteria have a common cell envelope structure. Electron micrographs of stained cells show three electron-dense layers, with electron-sparse areas in between, surrounding the cells (Kellenberger and Ryter, 1958; Bladen and Mergenhagen, 1964). The work of De Petris (1967) provided a chemical status for each of these complex layers. By coupling electron microscope studies with studies of the susceptibility of layers to particular solvents, he established that the cell envelope contained considerable quantities of phospholipid, glycopeptide, protein and polysaccharide. Fig. 1.3 is a diagrammatic representation of the basic findings, which hold true today (Glauert and Thornley, 1969; Costerton

FIGURE 1.3

The cell envelope of E.coli

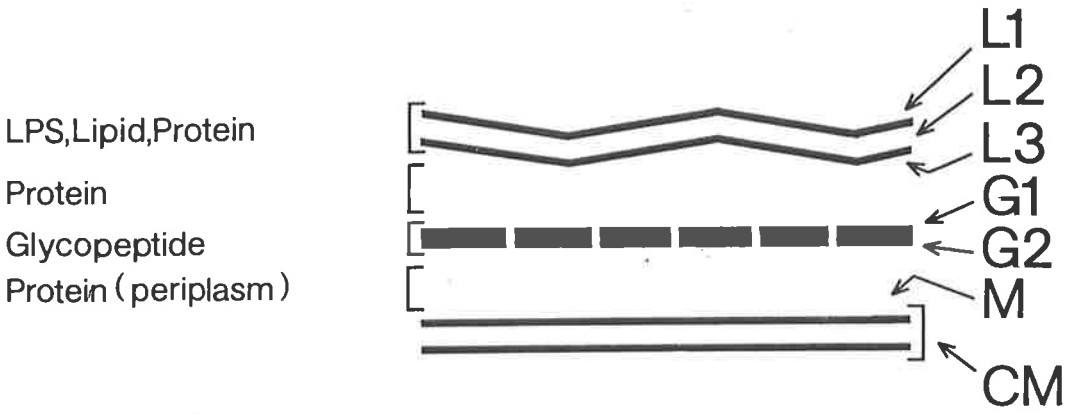


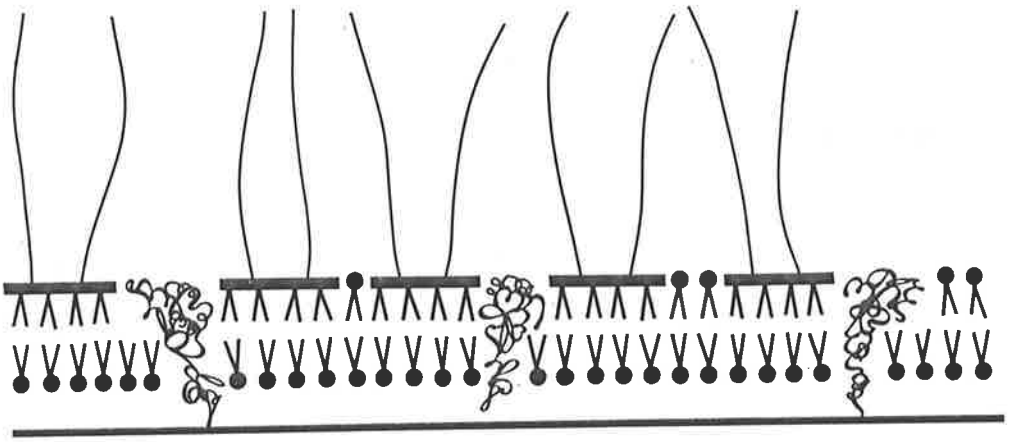
Figure and terminology adapted from De Petris (1967

et al., 1974). The inner membrane is referred to as the cell, plasma or cytoplasmic membrane (CM). It consists of a phospholipid bilayer, containing mostly phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin in *S. typhimurium* and *E. coli* strains. The inner electron-sparse area, termed the periplasm, contains protein. The middle electron dense layer (G1 and G2), termed the murien sacculus, consists of glycopeptide and may contribute between 5% to 20% of the total cell mass. This layer is common to both Gram-negative and Gram-positive bacteria, although it is thicker in the latter (Salton, 1964). The outer cell membrane (L1, L2 and L3), like the cytoplasmic membrane, is 75Å thick but the two are 100Å apart. The outer cell membrane is composed of a phospholipid bilayer, protein and LPS. Comprehensive reviews have been published on the membrane (Osborn *et al.*, 1974; Inoue, 1979) and the protein content (DiRienzo *et al.*, 1978). Fig. 1.4 depicting a proposed model for the structure of the outer cell membrane of *S. typhimurium*, is taken from an article by Smit *et al.*, (1975). The LPS, and the polysaccharide component in particular, is the outermost structure of the cell. This is not so for some species, that have a distinctly separate polysaccharide capsule.

The LPS component occupies about 45% of the surface of the outer cell membrane. It contains the O-somatic antigens and endotoxins of Gram-negative bacteria. The LPS consists of three chemically and biologically distinct regions. The branching polysaccharide

FIGURE 1.4

Proposed model for the structure of the outer membrane of S.typhimurium



(adopted from Smit et al., 1975)

(region 1), carrying the main serological specificity in the form of the O-antigens, is linked to the core polysaccharide (region 2), common to groups of gram-negative bacteria. The core polysaccharide is linked via the 2-keto-3-deoxyoctonate (KDO) trisaccharide to the lipid component, lipid A (region 3) (Luderitz *et al.*, 1971). The lipid A portion of the LPS molecule replaces part of the phospholipid in the outer layer of the lipid bilayer. The chemical structure of lipid A has been extensively analysed as well as its role as endotoxin and the major biologically active component of LPS (Luderitz *et al.*, 1973). Intensive study of the LPS structure of Gram-negative bacteria, particularly Salmonella species, in which the biosynthesis of specific LPS precursors was blocked (Nikaido *et al.*, 1964; Osborn *et al.*, 1964; Luderitz *et al.*, 1966), followed the initial report by Nikaido (1961) of an epimerase-less mutant strain of *S. typhimurium*. The study of these so-called "R mutants" led to a detailed understanding of the structure, biosynthesis and underlying genetics of the polysaccharide component of the LPS of various Salmonella species. Fig. 2.1 shows the LPS structures of the various Salmonella strains used in this thesis.

The change from a rough form to the smooth form of a gram-negative bacterium is accompanied by a change in the LPS structure, the polysaccharide component, in particular (Luderitz *et al.*, 1966). The change is also accompanied by a decrease in sensitivity to the serum bactericidal reaction (Dlabac, 1968). The partial removal or structural disorganization of the LPS was therefore

thought to be a way of increasing the sensitivity of an otherwise resistant strain. The chelating agent, ethylenediaminetetra acetic acid (EDTA), and the univalent tris-(hydroxymethyl)-aminomethane (TRIS) markedly increase the serum sensitivity of smooth strains of Gram-negative bacteria (Reynolds and Pruul, 1971a) and Gram-positive bacteria (Reynolds and Rowley, 1969), when present in reaction mixtures. This becomes more apparent in the absence of magnesium ions. Reynolds and Pruul (1971b) showed that EDTA and Tris disrupt the structure of the LPS of Gram-negative bacteria and proposed separate actions on the divalent cations (Mg^{2+}), that are structurally important components. The large distance between the site of complement activation on the O-antigens and the target cell membrane (Shands, 1965) was commonly believed to be the reason for the serum-resistance of smooth strains (Muschel, 1965; Feingold *et al.*, 1968b; Archer and Rowley, 1969; Reynolds and Rowley, 1969). However, Reynolds and Pruul (1971b) showed that the sensitization of smooth bacteria did not involve the fixation of antibody and complement closer to the target cell membrane but rather the promotion of a later event. Reynolds *et al.*, (1975) identified this later event by showing that the LPS of the smooth *S. typhimurium* C5 prevents the activated C5 complement component from binding to its membrane receptor site. The MAC appeared to be sterically hindered by the extensive LPS structure on the outer cell membrane. More recent work by Joiner *et al.*, (1982a,b) also suggests that the MAC is formed and does associate with the

bacterial surface, but that it fails to insert into hydrophobic regions of the outer membrane.

The locus of cell death in the complement-mediated bactericidal reaction is uncertain, although considerable work has been done to ascertain this. The O-polysaccharide antigens of Gram-negative bacteria have long been recognized as the principal antigens operating in the reaction (Cundiff and Morgan, 1941; Adler, 1953; Muschel, 1960). Overwhelming evidence has shown that they are the antibody binding sites and the activation sites for the classical complement pathway. Bladen *et al.*, (1967) demonstrated similar lesions on the isolated LPS, as on whole cells of *Veillonella alcalescens*, in the serum bactericidal reaction. Muschel and Jackson (1966) suggested that complement action directly damages the inner cell membrane. This suggestion raised many questions based on the known structure of the Gram-negative cell envelope. It meant that the late acting complement components ie., the MAC, would have the two cell membranes as targets. Cell death does occur in the serum bactericidal reaction without the participation of lysozyme (Glynn and Milne, 1967). The problem raised by this was how the large MAC of complement can gain access to the inner cell membrane through the dense glycopeptide layer.

Damage to Gram-negative cells in the serum bactericidal reaction has been studied by monitoring the release of intracellular and extracellular cell components. These components have been active enzymes or structural components, labelled by growing the bacteria in medium containing a radioactive tracer eg., ^{14}C-glucose

or ^{32}P-orthophosphate. Spitznagel (1966a) showed that when a smooth strain of *E. coli* is grown in medium containing ^{32}P and then incubated with guinea pig serum, ^{32}P-labelled compounds of both high and low molecular weight (MW) are released from the cells. The low MW compounds, having characteristics of phospholipids, are in the majority and indicate membrane damage. The high MW compounds have characteristics of nucleic acids and so indicate some release from the cell cytoplasm. A further report (Spitznagel, 1966b) showed that the heat-labile complement and heat-stable antibody are required for ^{32}P release from labelled *E. coli* cells in the serum bactericidal reaction.

The lethal effect of lysozyme-free serum on *E. coli* cells can be inhibited by placing these cells in a hypertonic sucrose solution (Feingold, 1968a). The inner cell membrane is protected from the secondary lethal changes that normally occur in bactericidal reaction mixtures. The *E. coli* cells, whose glycopeptide is labelled with ^{3}H-diaminopimelic acid, release the label in the serum bactericidal reaction when lysozyme is present but not when it is absent. Lysozyme, therefore, seems essential for the breakdown of the structure maintaining the cell shape - glycopeptide layer. Feingold believed that sequential damage to the two supporting structures of the cell envelope ie., the outer membrane and the glycopeptide layer, is lethal, whereas damage to one or the other is tolerated under certain conditions.

Alkaline phosphatase and β-galactosidase are two degradative enzymes that have been shown to be present in

the periplasm and the cytoplasm, respectively, of Gram-negative bacteria (Done *et al.*, 1965; Heppel, 1967; Neu and Chou, 1967). They are both released into the fluid-phase when *E. coli* cells are incubated with whole serum, whereas only alkaline phosphatase is released with lysozyme-free serum (Inoue *et al.*, 1968; Feingold, 1968b). Moreover, β-galactosides were shown to be accessible to the intracellular enzyme, where killing occurs with lysozyme-free serum (Feingold, 1968b). The two studies also showed that the target cells remain viable and morphologically similar if exposed to lysozyme-free serum in a hypertonic medium. In this situation, alkaline phosphatase is released but β-galactosides are not accessible to the intracellular β-galactosidase. Feingold suggested that damage to the inner cell membrane is essential for killing to occur and that a defective cell wall upsets the normal osmotic forces operating at the inner cell membrane.

Inoue *et al.*, (1974a) examined the radioactivity released from ^{14}C-glucose labelled *E. coli* cells, when they were incubated with specific antibody and complement, in the absence of lysozyme. Among the phospholipids released, phosphatidylethanolamine (PE) appears first and then increasing levels of the breakdown products, free fatty acids (FFA) and lysophosphatidylethanolamine (LPE), appear. No hydromyristic acid from the lipid A moiety of the LPS is detected. These workers suggested that a phospholipase is present at the periphery of the bacterial cells and that this enzyme is activated by complement-associated damage to the outer membrane. They (Inoue *et*

al., 1974) also discovered a mutant *E. coli* strain that presumably lacks this enzyme, because cells liberate PE but only trace amounts of FFA and LPE when they are incubated with specific antibody and complement. Phospholipase activity has also been demonstrated in the outer membrane of *S. typhimurium* cells (Osborn *et al.*, 1972). Inoue and co-workers postulated that the normal release of the degradation products in the parent strain occurs after the bacterial enzyme gains access to its substrate - the inner membrane phospholipid - via channels in the cell wall. These channels are formed by complement action (Inoue *et al.*, 1968a).

The question of complement-associated damage to both cell membranes of *E. coli* was taken up recently by Wright and Levine (1981a). Using similar bacterial enzyme and radioactive label release techniques, they showed that damage to the two membranes occurs simultaneously and with identical dose responses. They proposed that complement acts at a single locus, contiguous with both the outer and inner cell membranes. Bayer (1975) previously described the loci of close inner and outer membrane apposition. Wright and Levine (1981b) further proposed that the lethal event occurs by a two hit-mechanism, as two terminal complexes are required for inner membrane damage, outer membrane damage and killing. This is consistent with the concept of Biesecher *et al.*, (1979) that the MAC of complement functions as a dimer of C5b-9. These recent propositions indicate that damage to the inner cell membrane is required for the killing and lysis of Gram-negative bacteria by complement.

1.1.3 Antibody involvement

It is generally accepted that specific antibody is required for the killing of Gram-negative bacteria by the classical pathway of complement. Early experiments showed that the serum of precolostral laboratory animals and of animals raised in a germ-free environment can kill certain bacteria (Turk, 1959; Sterzl *et al.*, 1962). Antigenic stimuli, beyond the control of these investigators, probably resulted in a low serum level of natural antibody that was only detected in the very sensitive bactericidal reaction. In several well-controlled experiments, such antibodies have been produced in animals due to the introduction of dead or live bacteria in their diet (Springer, 1959; Wagner, 1959). Natural antibodies have been shown to display the same specificity as those produced by vaccination or symptomatic injection, and the natural antibodies act similarly in the bactericidal reaction (Mackie and Finkelstein, 1932; Adler, 1953; Muschel *et al.*, 1962). There is no fundamental difference between natural and immune antibodies.

Specific antibody of the different classes is not equally efficient in the complement-mediated bactericidal reaction. IgM antibody is considered to be more efficient on a molar or weight basis than IgG antibody in the killing of a variety of Gram-negative bacteria: *Neisseria meningitidis* (Griffiss, 1975; Griffiss and Bertram, 1977); *Brucella abortus* (Hall *et al.*, 1971), *Pseudomonas aeruginosa* (Bjornson and Michael, 1970); *Haemophilus influenzae* (Johnston *et al.*, 1973); *Vibrio cholerae* (Pike and Chandler, 1969), and; *Salmonella*

species (Robbins *et al.*, 1965; Daguillard and Edsall, 1968; Eddie *et al.*, 1971; Schulkind *et al.*, 1972). It is believed that the reason is similar to that for the immune haemolytic system. Here, a single IgM molecule, specific for and bound to a RBC membrane, is sufficient to trigger the classical pathway of complement, whereas two such IgG molecules are required in close proximity (Borsos and Rapp, 1965; Ishizaka *et al.*, 1966; Borsos *et al.*, 1968). IgM molecules are decavalent and more than two cell-binding sites can be filled by them. However, non-complement-fixing IgM antibodies with diverse specificities have been described in various species (Cowan, 1973; Rosse, 1968), and rabbit antibodies of the IgG class have been described as more efficient in causing haemolysis once bound to RBCs (Frank and Gaither, 1970).

Adinolfi *et al.*, (1966) suggested that colostral IgA antibody is bactericidal in the presence of complement and lysozyme. Ishizaka *et al.*, (1965) showed that IgA antibody, specific for human group A RBCs, could not lyse these cells in the presence of complement. IgA antibody can, in fact, inhibit the haemolytic activity of IgG and IgM antibody (Russell-Jones *et al.*, 1980). Other workers have shown that colostral IgA antibody cannot kill bacteria in the presence of complement alone (Knop *et al.*, 1971; Heddle *et al.*, 1975), or with lysozyme (Eddie *et al.*, 1971; Heddle *et al.*, 1975). The latter workers doubted the validity of the work by Adinolfi *et al.*, due to the fractionation and assay techniques used by this group. The weight of evidence therefore indicates that

IgA antibody is not bactericidal, nor per se can it trigger the alternative pathway.

In recent years, IgG subclasses of various mammalian species have been investigated for complement-fixing and bactericidal activity. Human IgG_1, IgG_2 and IgG_3 antibodies fix complement in the immune haemolytic system whereas IgG_4 antibody does not (Ishizaka *et al.*, 1970; Spiegelberg, 1974). Their ability to fix complement in the complement-mediated bactericidal reaction probably follows this. Siroťák *et al.*, (1976) showed that IgG_2 antibody from guinea pigs killed bacteria by the classical pathway of complement, whereas IgG_1 antibody killed by the alternative pathway. Mouse IgG_{2a} and IgG_{2b} antibodies have been shown to fix complement (Miller and Nussenzweig, 1974) whilst there is evidence for two isotypes of IgG_1 antibody - one that fixes and another incapable of fixing complement (Klaus *et al.*, 1979; Ey *et al.*, 1979; Ey *et al.*, 1980). This thesis deals with rabbit immunoglobulins. No recognized subclasses of rabbit IgG, or of IgA or IgM, have been described, although Schlamowitz *et al.*, (1975) showed the fractions of IgG, derived from column chromatography techniques, to have basic differences.

Following the immunization of laboratory animals with Salmonellae, an increase in bactericidal activity per unit mass of IgG and IgM antibodies has been noted with time (Schulkind *et al.*, 1972). This paralleled the increase in avidity also noted with time. This increase in avidity is also apparent with other immunogens viz., *Vibrio cholerae* (Holmgren and Svennerholm, 1972),

E. coli LPS (Ahlstedt *et al.*, 1973) and haptens (Eisen and Siskind, 1964; Goidl *et al.*, 1968; Werblin *et al.*, 1973a and b). In any particular serum, the avidity of IgM antibody is lower than that of IgG antibody, specific for the same Gram-negative bacterium (Robbins *et al.*, 1965; Holmgren and Svennerholm, 1972).

Early work by Pillemer *et al.*, (1954) showed that properdin-depleted serum was deficient in bactericidal activity towards certain bacteria and in other complement-mediated biological activities. Properdin, a hydrazine-sensitive component (factor A), and a heat-labile component (factor B) could together activate C3 to generate serum bactericidal activity (Wardlaw and Pillemer, 1956). Through the use of factor B-depleted serum (Götze and Müller-Eberhard, 1971) and C4-deficient guinea pig serum (Root *et al.*, 1972), the alternative pathway of complement was established to have an important role in bactericidal reactions with certain Gram-negative bacteria. Schreiber *et al.*, (1979) demonstrated bactericidal activity with only the 11 proteins of the alternative pathway and so conclusively proved that this pathway can be bactericidal in the complete absence of specific antibody.

Bacterial LPS is believed to directly activate complement via the classical pathway (Loos *et al.*, 1974) and the alternative pathway (Gewurz *et al.*, 1968; Marcus *et al.*, 1971; Götze and Müller-Eberhard, 1971; Dierich *et al.*, 1973). Evidence suggests that the polysaccharide moiety of LPS activates the alternative pathway while the lipid A activates the classical pathway (Lachmann and

Nicol, 1973; Loos *et al.*, 1974; Morrison and Kline, 1977). The direct activation of either pathway, in the absence of specific antibody, does not seem to result in killing in assays where a diluted component source is used.

1.1.4 Bactericidal assays

The standard method for determining the number of bacteria surviving serum action is the plate count assay. It was used by Neisser and Wechsberg (1901) in the first description of the NW phenomenon. It is still used today although instruments, media, etc. have changed since the turn of the century. The assay involves a mixture of diluent, antibody source, complement source and log-phase bacteria being incubated at 37^{o} for a specific period. At the beginning and end of this period, samples of the mixture are plated out on nutrient agar plates, and the numbers of colonies are counted after overnight incubation of these plates. There are some disadvantages inherent in this assay: (i) bacterial numbers initially increase due to cell division, (ii) mixtures, containing greater than 10^{4} bacteria per ml, require to be diluted before samples are taken and this increases the statistical error, and (iii) bacteria may agglutinate in the presence of specific antibody and so colony counts can be falsely low, especially if the numbers added are high ($>10^{6}$ per ml).

The rapid photometric growth assay was developed by Muschel and Treffers (1956) to counter some of these objections. The assay, like the plate count assay,

includes an initial period when bacteria, antibody and complement are allowed to interact; the bacteria can still divide in this period. The subsequent addition of nutrient broth halts the bactericidal reaction and allows the surviving cells to increase exponentially. The mixture is incubated for several hours after which the relative growth rates of the bacteria in test and control situations are estimated by photometric means. Muschel and Treffers found the assay to be accurate and more time-saving than the plate count assay. The principal disadvantage is the excessive use of reagents, especially complement. Inoue *et al.*, (1974) modified the photometric growth assay to remedy this. Their microplate count assay begins with the incubation of a small volume (75 µl) of bactericidal reaction mixture in a micro-titration plate. Concentrated nutrient broth (25 µl) is then added and the plate is incubated at 37^{o} overnight. The viability of bacteria at the end of the bactericidal reaction is gauged by the turbidity of the mixture or the number of colonies on the bottom of the well. This assay allows a larger number of reaction mixtures to be tested, because of the small volumes used and the speed of pipetting and diluting. However, it is just as time consuming as the plate count assay as overnight incubation of the plate is required.

Another assay that uses small volumes of reagents is the radial diffusion agar plaque assay (Holmgren *et al.*, 1971). Microdrops of antibody source and complement source are placed on the surface of a nutrient agar plate, that incorporates live bacteria. Incubation of the plate

at 37^{o} for several hours results in plaques forming at the killing sites. The radial diffusion of antibody source, prior to the addition of the complement source, results in plaques, whose area is proportional to the bactericidal antibody concentration. Because IgM antibody diffuses poorly and avid antibody binds to bacteria in the drop rather than diffusing out, this modification has its limitations.

The multiplication of target bacteria is a problem inherent in all the above bactericidal assays. Recently, a mutant strain of *E. coli* has been described that does not multiply at the normal bactericidal assay temperature of 37^{o}, but does so at 25^{o} (Hooke *et al.*, 1978). This bacterium appears to be an ideal target organism for a bactericidal assay as it cannot multiply during the assay but does multiply to form colonies on agar plates, if the latter are incubated at 25^{o}.

A form of the original plate count assay is used in this thesis. In the majority of experiments, the initial bacterial cell concentration is 5X10^{3} per ml. Such a low number results in very close agreement between the initial colony counts of different mixtures, in any experiment. Agglutination of the target bacteria is also prevented. The important advantage of this assay is that target cells can be centrifuged down and studied further after a bactericidal assay has been performed.

A number of assays have been developed that measure that later action of specific antibody and complement on Gram-negative bacteria - lysis. The release of nucleic acid from the cytoplasm (Amano *et al.*, 1958), the release

of ^{32}P (Spitznagel, 1966a) or ^{14}C (Kasper and Wyle, 1962) from labelled target cells and β-galactosidase activity of these cells (Feingold, 1968b) are examples. The NW phenomenon is an abrogation of the bactericidal reaction and so a detailed discussion of these assays is not included in this thesis.

1.1.5 A comparison with complement-mediated lysis of animal cells

Complement has been shown to interact with and kill protozoa (Anziano *et al.*, 1972), nucleated mammalian cells (Green *et al.*, 1959; Green and Goldberg, 1960) and viruses (Almeida *et al.*, 1972). The fundamental mechanisms of complement-mediated haemolytic, bactericidal and cytolytic reactions appear to be similar.

The close historical and mechanistic ties that exist between the serum bactericidal and immune haemolytic reactions have already been described. Both require all nine components of the classical pathway of complement (Inoue and Nelson, 1966; Inoue *et al.*, 1968b) and both result in similar "doughnut" type lesions on the cell surface (Humphrey and Dourmashkin, 1965; Glynn and Milne, 1967; Bladen *et al.*, 1967). However, there are basic differences also. These differences stem from the obvious differences in cell structure and viability. Whereas previous evidence has indicated that, like immune haemolysis (Mayer, 1961), the bactericidal reaction is a one-hit process (Inoue *et al.*, 1976), recent evidence (Wright and Levine, 1981b) suggests that it is a two-hit process. Target RBCs are not viable; target bacteria are

viable and their killing precedes spheroplast formation and lysis (Davis *et al.*, 1966). RBCs of all mammalian species are susceptible to immune haemolysis, whereas ordinarily, no Gram-positive bacteria and only some Gram-negative bacteria appear to be susceptible to the serum bactericidal reaction. The glycolipid cell membrane is the site of complement activation and damage in immune haemolysis. The polysaccharide O-somatic antigens of the outer membrane of Gram-negative bacteria are the important sites of complement activation in the serum bactericidal reaction. The phospholipid bilayer of this outer membrane is certainly a site of complement damage, but the inner cell membrane apparently needs to be damaged before cell death and lysis occurs.

Like bacteria, nucleated mammalian cells display great variability in their susceptibility to killing by complement (Ohanian *et al.*, 1973; Ferrone *et al.*, 1975; Young-Roderichuk and Gyenes, 1975). However, this variability cannot be solely ascribed to physico-chemical properties of the cell membrane. The metabolic properties of nucleated cells may also be important. These cells may repair or re-seal damage caused by complement, they may shed or degrade bound complement components, or they may modify other physical properties of the cell membrane (Ohanian and Borsos, 1977). The various properties and behaviour of nucleated cells, that may influence the result of complement action, have been listed (Ohanian *et al.*, 1978) but the relative importance of each is relatively unknown.

Bacteria are more susceptible to the serum bactericidal reaction in the logarithmic phase of growth. Nucleated cells, however, are most susceptible to complement-mediated cytolysis during the G1 phase and least susceptible during the logarithmic phase (Lerner *et al.*, 1971; Pellegrino *et al.*, 1974; Cooper *et al.*, 1974). Unlike bacteria, nucleated cells differ in their susceptibility to complement from different species (Ohanian *et al.*, 1973; Pellegrino *et al.*, 1974). Inefficient killing, despite hundreds of thousands of bound complement molecules, is also recognized (Lerner *et al.*, 1971; Ohanian and Borsos, 1975). Morphological changes have been observed at the surface of nucleated cells following exposure to antibody and complement. In particular, multiple protrusions have been demonstrated using scanning electron microscopy (Ohanian *et al.*, 1978). The surfaces are "moth-eaten" in appearance and point to complement acting at discrete sites on the cell membrane. The protrusions are similar to those demonstrated on bacterial cells in similar circumstances (Schreiber *et al.*, 1978).

The classical pathway of complement appears to be more important than the alternative pathway in the complement-mediated killing of nucleated cells. This is so for the serum bactericidal reaction. However, there are several reports (see Müller-Eberhard and Schreiber, 1980) of nucleated cells activating the alternative pathway and being subsequently killed. Like bacteria (Schreiber *et al.*, 1979), at least one type of nucleated cell has been shown to be killed and lysed only in the

presence of the eleven proteins of the cytolytic alternative pathway (Schreiber *et al.*, 1980).

CHAPTER 1.2 Aberrent phenomena associated with the complement-mediated bactericidal reaction

1.2.1 The Neisser-Wechsberg phenomenon

The phenomenon was first described by two German workers, Neisser and Wechsberg, in 1901. They reported that low dilutions of some antisera are unable to kill the corresponding serum-sensitive bacteria in a plate count bactericidal assay, whereas higher dilutions can kill until specific antibody is no longer present. The lack of killing with the low antiserum dilutions, ie., with high levels of specific antibody, constituted the NW phenomenon.

Neisser and Wechsberg demonstrated this prozoning activity in antisera raised in rabbits, goats and dogs against two species of Vibrio, and *Salmonella typhi*. They further showed (i) that the bactericidal activity of a normal serum could be diminished by the addition of heat-treated antiserum with this prozoning activity, and (ii) that the prozoning activity of an antiserum could be reduced by the addition of normal serum. These last two observations are very important to the understanding of the mechanism of the NW phenomenon, although this was not realized at the time. Their explanation was consistent with the current theory of immune haemolysis, although they could not demonstrate such a prozone phenomenon in the haemolytic system. According to Neisser and Wechsberg,

specific antibody (amboceptor) needs to combine with complement, before attaching to all the antigenic sites (receptors) of the target bacterial cells, if cell death is to occur. When excess antibody ie., low dilutions of antiserum, is present in a bactericidal assay mixture, a fraction does not combine with complement. If the avidity of the uncombined antibody is higher or the same as that of the combined antibody, the former preferentially binds to available antigenic sites. The target cells are not killed as uncombined antibody is bound to some or all of the antigenic sites.

Current theory on the mechanism of the serum bactericidal reaction does not support this first explanation for the NW phenomenon. Many other explanations have been forwarded since 1901, and the purpose of this thesis is to provide an explanation based on a study of the various parameters of the bactericidal reaction.

Fifty years after the first description, a reference was made tothe phenomenon in one of the standard Microbiology textbooks of the day (Wilson and Miles, 1957). The authors claimed that such a prozone phenomenon is demonstrable in other simpler immune systems and vaguely paralleled the NW phenomenon with the zone phenomena seen in agglutination and precipitation systems.

Various hypotheses were later aired by several noted immunologists in a workshop setting (Muschel, 1965). Muschel believed that the NW phenomenon is due to some steric hindrance to complement components and that the precise mechanism is more difficult to explain than for the inhibition of agglutination by excess antibody. Coombs

suggested that soluble bacterial antigen combines with antibody in the fluid-phase and that this soluble complex deviates complement away from the target bacteria. Boyden suggested that only one Fab combining site per antibody molecule combines to antigenic sites on the bacteria, and so complement activation does not occur. Very little experimental evidence was presented for these hypotheses during this discussion.

The agglutinating and bactericidal properties of IgM and IgG antibodies, specific for somatic antigens 9 and 12 of *Salmonella typhi*, were studied by Daguillard and Edsall (1968). They pooled rabbit antisera and first separated antibody, specific for the single antigens and both antigens, by adsorption to and subsequent elution from acetone-dried bacteria. They then separated the specific IgM and IgG antibody by gel filtration on Sephadex G-200 and by subsequent anion-exchange chromatography of the crude IgG fraction on DEAE-cellulose. Both specific antibody preparations showed the NW phenomenon and so Daguillard and Edsall claimed that specific IgG and IgM antibody possess this prozoning activity.

Certain experimental aspects of this work seem unsatisfactory. The vaccination schedule was short in comparison with other studies, in which prozoning activity was demonstrated (see 1.4.1). Specific IgA antibody could have been present in the various fractions, as an otherwise unidentified β-globulin appeared in sera collected late in the vaccination schedule. The antisera used to test the various fractions were not well characterized. Elution of specific antibody from acetone-dried *S. typhi* cells by

acetate buffer (pH 3.5) followed a previously published method (Robbins *et al.*, 1965). Incubation of the mixture at 37^{o} for 90 minutes, before centrifugation at 37^{o}, must have destroyed some complement-fixing activity of the specific IgM antibody (Stollar *et al.*, 1976). This may well explain why the IgM antibody displayed prozoning activity.

In a detailed study of the NW phenomenon, displayed by hyperimmune rabbit antiserum and *S. typhi* bacteria, Muschel *et al.*, (1969) dispelled some of the early beliefs regarding the phenomenon. One of the important findings was that target bacteria, sensitized with a prozoning dilution of antiserum, could be washed and still display the prozone phenomenon on addition of complement. This seemed to indicate that the phenomenon occurs on the cells. These workers also showed that haemolytic complement is consumed in the prozoning situation and that the prozoning activity is dependent on the complement concentration. Serum fraction studies indicated that both IgM and IgG antibody exhibit prozoning at high concentrations, however, characterization of the fractions obtained was poor and there was no regard taken of IgA antibody. Muschel *et al.*, concluded that excess antibody plus non-specific anticomplementary factors found in heat-treated antiserum are responsible for the NW phenomenon.

At about the same time, Edebo and Normann (1969) published the first of a series of articles on the phenomenon. The bactericidal activity of normal human serum on rough *Salmonella typhimurium* mutants was shown to be inhibited by specific rabbit antiserum. The prozoning

activity of the antiserum appeared to be specific for the immunizing mutant or for mutants belonging to the same chemotype. This suggested that antibody specific for the outer membrane LPS antagonizes the bactericidal action of normal serum. In a second article, Edebo and Normann (1970) reported that the killing of these mutants by normal cattle serum can also be inhibited by rabbit antiserum.

Normann (1972) provided evidence to suggest that a lengthy immunization schedule is required to produce prozoning activity in the serum of immunized rabbits. Such a lengthy schedule resulted in serum with comparatively high prozoning activity but with low bactericidal and haemagglutinating activities. By fractionation of the antiserum on DEAE-cellulose and identifying the fractions using immunoelectrophoresis, Normann revealed that the prozoning activity resides with only the IgG fraction ie., with the specific IgG antibody, presumably. He asserted that IgM and IgA antibodies are unimportant.

In the most important paper of this series, Normann *et al.*, (1972) analysed some basic parameters of the prozone phenomenon, displayed by hyperimmune rabbit antiserum. They showed that *S. typhimurium* cells can be sensitized and washed, but still remain in the prozoning situation on incubation with complement. They also showed that when the antiserum is absorbed with the isolated LPS, the prozoning activity is lost. Other results suggested that the prozoning activity remains stable over a large bacterial concentration range and that it is abrogated by the pepsin digestion of the antiserum. These workers

postulated that in the prozoning situation, complement activity is scattered or deviated so that it does not effect a lethal lesion in the bacterial envelope. Unfortunately, the above-mentioned parameters of the NW phenomenon were not studied with the isolated IgG fraction of hyperimmune rabbit antiserum. This could have afforded very strong evidence for the involvement of IgG antibody in this phenomenon.

So far, the series of articles dealt with the effects of hyperimmune rabbit antiserum on the bactericidal reaction involving a rough strain (395 MRO) of *S. typhimurium*. Normann *et al.*, (1973) also showed that IgG antibody could also abrogate the killing of a smooth strain of *S. enteritidis*. They postulated that IgG antibody can abrogate the killing of Gram-negative bacteria, whether their LPS is "complete" or not.

One other study (Eddie *et al.*, 1971) was important because it focussed on the possible role of IgA antibody. These workers first vaccinated rabbits with live *S. typhimurium* bacteria. They then isolated specific secretory IgA antibody from these animals, by adsorption and chromatography techniques. As little as 10 µg of secretory IgA antibody was found to inhibit the bactericidal activity of specific IgM or IgG antibody. These results suggest that specific IgA antibody may have an important role in the NW phenomenon.

1.2.2 Clinical Brucellosis

Brucella abortus is a Gram-negative bacterium that commonly infects cattle, sheep, horses, and humans who by

profession have contact with these domestic animals. Bacteraemia, manifested by spiking body temperature, can proceed to serious complications such as abortion and the malfunction of several body organs.

Acute and chronic phases exist in this disease and they can be differentiated serologically with tube agglutination, complement fixation, bactericidal and modified Coombs tests (Buchanan *et al.*, 1974). A prozone phenomenon is commonly observed in the agglutination test with heat-killed *Br. abortus* organisms and low dilutions of serum from chronically-ill humans. The prozoning activity was found to be associated with the serum β_{2A} (γ_{1A})-globulin, viz, the IgA fraction (see 1.3.1).

Mammalian sera also display a similar prozone phenomenon in the complement-mediated bactericidal reaction with this bacterium. Initially, Hall (1950) noted that low dilutions of serum from chronically-ill humans are unable to kill. The phenomenon has been demonstrated in whole blood (Joos and Hall, 1968) and serum (Hall *et al.*, 1971) from hyperimmunized rabbits.

The first kinetic study of the bactericidal activity of fresh, whole blood closely resembled the *in vivo* situation (Joos and Hall, 1968). Normal rabbits and rabbits, immunized with heat-killed *Br. abortus* organisms, were assayed for blood bactericidal activity. Whole blood and serum, from all normal and most immunized rabbits, efficiently killed the target *Br. abortus* cells in a very short time. However, some specimens from immunized rabbits and all from hyperimmunized rabbits were unable to kill.

Weekly immunizations over eight weeks constituted hyper-immunization, and the appearance of this prozoning activity in the blood coincided with the appearance of prozoning activity in the tube agglutination test. Joos and Hall concluded that a complement deficiency and a blocking antibody are the causes of the activity shown by immune and hyperimmune blood, respectively.

Hall *et al.*, (1971) attempted to identify the serum factor(s), responsible for this prozone phenomenon, by fractionating hyperimmune rabbit antiserum. Surprisingly, the whole serum showed only slight prozoning activity, in bactericidal assays with target *Br. abortus* cells. The β-globulin fraction, resulting from starch block electrophoresis, showed the most prozoning activity and bactericidal activity. The IgM and IgG fractions, isolated from the β- and γ-globulins, showed only bactericidal activity. The IgA fraction, isolated from the β-globulins, showed both activities. These workers postulated that this manifestation of the NW phenomenon is due to some property of the specific IgA antibody. However, they admitted that the IgA fraction was significantly contaminated with IgG immunoglobulin and, more recently, Griffiss (1975) has added transferrin, albumin and IgG degeneration products as probable contaminants. The anticomplementary activity of the latter may explain the observed prozoning activity. The observed bactericidal activity is not consistent with the established properties of IgA antibody (Eddie *et al.*, 1971; Knop *et al.*, 1971; Heddle *et al.*, 1975) and was probably due to the contaminating IgG antibody. For these reasons, this study was inadequate to define what serum factor(s) are

responsible for this prozoning phenomenon.

1.2.3 Clinical Meningococcal infection

Neisseria meningitidis is a Gram-negative bacterium that may cause septicaemia and meningitis in humans. The immunological mechanisms, which enable the infected host to kill meningococci, have evaded elucidation since the earliest studies with this bacterium. Matsunami and Kolmer (1918) first reported that specific antiserum may not be bactericidal for meningococci at low dilutions. They also demonstrated an increase in the bactericidal activity of whole blood from convalescing patients and immunized animals. Citrated blood was used in the bactericidal assays. As citrate ions are known to antagonize complement activities, the bactericidal activity of citrated blood was probably not complement-mediated.

As part of a general investigation of meningococcal infection, Thomas *et al.*, (1943), and Thomas and Dingle (1943) established that bactericidal activity frequently occurs in the serum of normal humans and that there exists an extraordinary variation in the sensitivity of various meningococcal strains. Sera from convalescing patients and immunized rabbits often showed no killing in the bactericidal assay, but on dilution and addition of complement, they did show killing. These sera also abrogated the bactericidal activity of normal sera, with no demonstrable consumption of haemolytic complement. A clear explanation of these observations was not given.

Susceptibility of humans to systemic meningococcal

disease is directly related to a deficiency of specific antibody (Goldschneider *et al.*, 1969a). These workers found that sera from 51/54 prospective cases of meningococcal infection were deficient in specific antibody by bactericidal and fluorescent antibody assays. A further study by these workers (Goldschneider *et al.*, 1969) reported sharp rises in serum bactericidal activity and specific IgA, IgM and IgG antibody, following carriage of a particular meningococcal serotype in the nasopharyngeal area, or following infection by that serotype.

The serum bactericidal antibody profile of four patients convalescing from recent meningococcal infection was further studied by Griffiss (1975). Sera were fractionated in a series of chromatography steps. The resulting IgG, IgM and IgA fractions were shown to be free of contaminants by immunoelectrophoresis and quantitative radial immunodiffusion. The whole sera were bactericidal but, for three patients, exhibited prozoning activity also. IgM and IgG fractions were bactericidal but IgA fractions were not bactericidal. This is consistent with the reported inability of IgA antibody to participate in complement-mediated cytolytic reactions (Ishizaka *et al.*, 1965; Eddie *et al.*, 1971; Knop *et al.*, 1971; Colten and Bienenstock, 1974; Heddle *et al.*, 1975). Griffiss also found that the IgA fractions could specifically inhibit the bactericidal activity of both IgM and IgG fractions. The extent of inhibition was directly proportional to the ratio of the IgA immunoglobulin and the bactericidal immunoglobulins. This study agrees with others on the

ability of IgA antibody to inhibit both bactericidal (Eddie *et al.*, 1971; Hall *et al.*, 1971) and haemolytic (Russell-Jones *et al.*, 1980) reactions, mediated by IgG and IgM antibody.

Griffiss concluded that specific but non-complement-fixing IgA antibody can compete with IgG and IgM antibody for antigenic sites on the target bacterial cells. He noted that significant inhibition could only be demonstrated when quantities of IgA immunoglobulin, far in excess of that in whole serum, were added to the bactericidal assay mixtures. He concluded that if IgA is to account for the prozoning activity of these convalescent sera then it does so by an as yet undefined mechanism. Thus, another manifestation of the NW phenomenon remains unexplained.

In a further study, Griffiss and Bertram (1977) postulated that the sera of individuals, susceptible to meningococcal infection, contain low levels of specific IgA antibody, that inhibits the complement-mediated killing of *N. meningitidis*. These sera are already known not to have specific bactericidal activity (Goldschneider *et al.*, 1969a). Acute phase sera contained little bactericidal activity in the later study; however, when IgA had been removed by affinity chromatography, this activity was significantly raised.

1.2.4 Less defined examples

The previous three sections deal with the three commonly demonstrated and most thoroughly investigated examples of the prozoning (NW) phenomenon in the serum

bactericidal reaction. In particular, they deal with the lack of bactericidal activity in low dilutions of hyperimmune antisera and of convalescent sera, with *S. typhimurium*, *S. typhi*, *Br. abortus* and *N. meningitidis* as the immunogens or pathogens. This section deals with some less defined examples of this type of phenomenon in clinical situations. It emphasizes the variety of such situations that show similar features as those with hyperimmunized laboratory animals.

Waisbren and Brown (1966) studied the serum bactericidal activity of patients, chronically infected with Gram-negative bacteria. Infected body organs included the lungs, peritoneum, heart and kidneys. The infecting organisms included Pseudomonas, Proteus, Salmonella and *E. coli* strains. These two workers found that the sera themselves failed to kill the infecting bacterium in the serum bactericidal reaction, as well as inhibiting the bactericidal activity of normal serum in this reaction. The inhibitory activity was heat-stable and specific for the infecting bacterium. Waisbren and Brown stated that there was no correlation between the serum inhibitory activity and haemagglutination or agglutination titres, but the data presented was too scanty for this conclusion to be scrutinized. The specific antibody activities of only four sera were calculated; the haemagglutination activity of three seemed to correlate with the inhibitory activity. They concluded that specific antibody is responsible for this inhibitory activity. Although the study did not endeavour to establish whether bactericidal activity appeared on diluting the

patient sera, all the cases cited were possibly examples of the NW phenomenon.

Bacteria that cause systemic infections in humans are nearly always serum-resistant (Roantree and Rantz, 1960). *E. coli* strains, causing upper urinary tract infection, are mostly serum-resistant and smooth (Kimball *et al.*, 1964). Gower *et al.*, (1972) discovered that, in this clinical situation, the infecting *E. coli* strain was serum-resistant, or was serum-sensitive with no bactericidal activity being present in the patient's serum. They even cited one patient as developing an upper urinary tract infection, although initially having normal serum bactericidal activity. Specific haemagglutination titres rose but the serum bactericidal activity disappeared. In follow-up studies, the haemagglutination titres declined and normal serum bactericidal activity returned. These workers inferred that the lack of specific bactericidal activity could be responsible for the continuation rather than the establishment of chronic infection.

The sera from two such patients, infected with *Proteus mirabilis*, were investigated by Taylor (1972). They both lacked specific bactericidal activity and inhibited the bactericidal activity of normal serum on this bacterium. They were fractionated by column chromatography methods and the resulting IgG fractions appeared to contain the inhibitory activity. IgM and IgA immunoglobulins were shown to be absent from these fractions by immunoelectrophoresis with class-specific antisera. Taylor thus stated that specific IgG antibody is responsible for the lack of specific bactericidal activity and the inhibitory

activity in the serum of such patients. He also inferred that, in such aberrations of the complement-mediated bactericidal reaction, high concentrations of specific IgG antibody effectively compete with the bactericidal IgM antibody for antigenic sites on the target bacteria. Human IgG antibody is bactericidal, although the sub-classes widely differ in complement-fixing activity (Spiegelberg, 1974). It seems likely that the studies of Gower *et al.*, and Taylor both refer to examples of the NW phenomenon in the clinical setting. The exact mechanism of this phenomenon is again unknown.

More recently, the serum bactericidal activity of patients suffering from chronic rhinoscleroma was investigated (Krasilnikov *et al.*, 1974). This disease is invariably caused by a Klebsiella species, and a capsular form of this bacterium was used as the target organism. Serum bactericidal activity was found to be low for sufferers but high for normal persons. The serum of sufferers could also inhibit the bactericidal activity of normal human serum and normal guinea pig serum. This again appears to be an example of the NW phenomenon.

The serum bactericidal activity of typhoid carriers may relate to the NW phenomenon. Specific activity against *S. typhi* is significantly lower in these individuals than in non-carriers with or without any typhoid history (Karolček *et al.*, 1962; Karolček *et al.*, 1975). Cižnár *et al.*, (1975) claimed that there are increased levels of specific IgA antibody and reduced levels of specific IgM antibody in the serum of carriers. The evidence was the result of absorbing these sera with H, O and Vi

antigens of *S. typhi* and then measuring the level of absorbed antibody of each class. It was an indirect and clumsy method to use. Karolček *et al.*,(1975) showed that serum from carriers assumed specific bactericidal activity after absorption with Vi antigen. They also postulated that specific but non-complement-fixing IgA antibody in the sera of carriers compete with IgM and IgG antibody, for antigenic sites on the target cell wall; due to its higher avidity, the IgA antibody effectively competes and so little complement fixation occurs on the bacteria. Unfortunately, insufficient experimental data were provided to support the proposed mechanism.

CHAPTER 1.3 Blocking phenomena associated with other immune reactions

1.3.1 Introduction

There are a number of other instances in which an immune reaction may be blocked by particular sera and by particular serum components, or may display prozoning at low serum dilutions. The best known example of the latter occurs in precipitation and agglutination reactions with soluble and particulate antigens, respectively. Over forty years ago, studies were undertaken with both protein (Heidelberger and Kendall, 1935) and polysaccharide (Heidelberger and Kendall, 1937) antigens. They established that precipitates, from reaction mixtures of constant antibody concentration but increasing antigen concentrations, progressively appeared, peaked and lastly

disappeared. The first of these three phases constitutes a prozone-like phase, due to excess antibody. The antigen-antibody complexes are small and so are still soluble.

The examples of blocking phenomena to follow are more complex. Participation of particular classes of antibody or other serum components, rather than of excess antibody, is implicated. Their relationships to the NW phenomenon are discussed.

1.3.2 Agglutinins in clinical Brucellosis

The *in vitro* tube agglutination test has been a serological aid in the diagnosis of human Brucellosis for many years. A prozone phenomenon is frequently noted with sera collected in the late acute and chronic phases of this disease. With constant bacterial concentration and increasing serum dilutions, a zone of no agglutination (the prozone phenomenon), then one of agglutination and lastly one of no agglutination (the antigen excess zone) are frequently observed. The first zone often completely masks the zone of agglutination, and this constitutes part of the evidence for rejecting antibody excess as the explanation for this prozone phenomenon.

The antiglobulin or modified Coombs test was found to be a valuable aid in detecting and studying the phenomenon (Hall and Manion, 1953). A normal tube agglutination test is performed and those tubes with no agglutination are centrifuged. The bacteria are washed several times before being resuspended in and incubated with a dilution of anti-human immunoglobulin antiserum.

The antibody detected by this test is called blocking or non-agglutinating antibody. Zinneman *et al.*, (1959) established that it appears late in the acute illness and throughout the chronic illness. By using starch block electrophoresis to fractionate serum, these workers found the blocking antibodies to migrate with the β-globulins. They postulated that this prozone phenomenon is due to the coating of *Br. abortus* cells by such blocking antibodies. Glenchur *et al.*, (1961) found that these antibodies appeared in rabbit sera only after prolonged and intensive antigenic stimulation. This seemed to parallel their appearance in human disease.

There is still conjecture over the class of antibody that this blocking antibody belongs to. Heremans *et al.*, (1963) reported the presence of agglutinating antibody in the IgG, IgM and IgA fractions of sera from two Brucellosis patients. Zinneman *et al.*, (1964) characterized the blocking antibodies as $\beta_{2A}(\gamma_{1A})$ glycoproteins, with a MW of 180,000 and with similar carbohydrate content as 7S γ_2 globulins. They were thus identified as non-agglutinating antibodies of the IgA class. The work of Dana-Arie and Serre (1974) supports this. However, Wilkinson (1966) and Kerr *et al.*, (1968) reported the presence of such antibodies in both the IgG and IgA fractions of convalescent-phase sera, but not in the IgM fraction. These differences probably relate to the different fractionation procedures adopted, however, there is a general consensus that specific IgA antibody can function as blocking antibody.

The appearance of blocking antibody in the serum of

Brucellosis patients, or animals hyperimmunized with this bacterium, parallels the appearance of the NW phenomenon with this (see 1.2.2) and other Gram-negative bacteria (see 1.2.1 and 1.2.3). Blocking IgA antibody, incapable of fixing complement, may be responsible for the NW phenomenon with *Br. abortus* and with other bacteria. However, unlike the NW phenomenon, the prozone phenomenon in the agglutination test can completely mask the active zone of agglutination. This indicates a mechanistic difference.

1.3.3 Phagocytosis by polymorphonuclear cells

In 1975, Lawrence *et al.*, discovered that human polymorphonuclear (PMN) cells contained surface receptor sites for unaggregated IgA myeloma protein. These sites were distinct from those for unaggregated IgG myeloma protein. Sera from patients with IgA myeloma were found to suppress chemotaxis between PMN cells and a number of chemotactic attractants (Van Epps and Williams, 1976). Little or no suppression of chemotaxis was observed with sera from patients having IgG myeloma or Waldenstrom's macroglobulinaemia (IgM myeloma).

Actual inhibition of phagocytosis by IgA was shown by Wilton (1978). In this study, IgA from normal human serum, a myeloma IgA and colostral IgA were all found to inhibit the IgG-dependent phagocytosis of a pathogenic yeast by PMN cells. Involvement of the Fc portion of the IgA molecules was found to be as necessary as the binding of these molecules to the cells, for inhibition to occur. Van Epps *et al.*, (1978) showed that IgA myeloma protein

could also inhibit the phagocytosis of the bacteria, *E. coli* and *Staphylococcus aureus*, both sensitized with specific IgG antibody. The inhibition was dependent on the IgA concentration and only occurred when the IgA was in a polymeric or aggregated form. These two observations suggest that, in this type of inhibition, steric hindrance has a major role; Fc receptors for IgG on the cell membrane are possibly blocked by bound IgA molecules.

A similar explanation may apply to the NW phenomenon. Specific IgA antibody may bind to the target bacterial cells and may then prevent the complement-fixing antibody (IgG and IgM) from binding to antigenic sites.

1.3.4 Blocking of ragweed antigen-induced histamine release

Ragweed antigen is one of many allergens able to trigger the release of histamine from the leukocytes of allergic individuals. This is referred to as immediate type hypersensitivity, and specific IgE antibody is known to be the mediator of the phenomenon. Ishizaka (1970) established that this antibody binds to the basophils and mast cells of allergic individuals and then reacts with the allergen. This action ultimately results in the release of vasoactive substances from the cells. Loveless, in 1940, first noted a blocking phenomenon in the serum of patients, who had received vaccinations of the pollen antigens to which they were sensitive. This blocking activity was demonstrated in an *in vitro* assay by Melam *et al.*, (1971). They observed no histamine

release when an antigen, previously incubated with the serum of a vaccinated individual, was then reacted with the leukocytes of an allergic individual.

This blocking activity has been explained by the ability of blocking antibody from the immune sera to bind to the particular allergen, and thus prevent the latter from binding to the IgE-sensitized leukocytes. Lichienstein *et al.*, (1968) used column chromatography techniques to separate IgG, IgM and IgA fractions from the pooled serum of 22 vaccinated individuals. Only the IgG fraction showed blocking activity. These workers proposed that specific IgG antibody is responsible for the blocking phenomenon, although an increase in specific serum IgA antibody has been reported to occur during the above-mentioned immunotherapy (Deuschl *et al.*, 1977).

The blocking of ragweed antigen-induced histamine release is an example of specific IgG antibody inhibiting an immune reaction. In this case, specific IgG antibody inhibits the binding of an allergen to IgE-sensitized leukocytes. The NW phenomenon may operate in a similar way. Specific IgG antibody may saturate the target bacterial cells and so sterically hinder complement components from binding to their receptor sites on the cells.

1.3.5 Immune haemolysis

Both blocking and prozone phenomena have been described for the immune haemolytic system, with sheep red blood cells (SRBCs) as target cells, but with antisera and complement from a number of mammalian species. There

are basic similarities between these phenomena and the NW phenomenon; this is expected as all are aberrations of the cytolytic activity of complement.

Serum haemolytic complement is measured clinically by a variety of methods, including the agarose plate diffusion technique. In this, SRBCs are first sensitized with rabbit haemolysin and then set in agarose gel. Test sera are pipetted into cut wells and the agarose plate is incubated at 37^{o}. The diameter of the zones of haemolysis around the wells reflect the levels of haemolytic complement in the sera. Thompson and Rowe (1967) reported that certain sera could cause an inner zone of lysis, an intermediate zone of no lysis and then an outer zone of lysis. Such sera showed agglutination in the antiglobulin test with SRBCs and anti-human IgG antiserum, but not with anti-IgA, anti-IgM or anti-IgD antisera. Thompson and Rowe fractionated these sera and found that IgG antibody caused the intermediate zone of no lysis to occur. They therefore concluded that such human sera contain natural anti-SRBC antibody of the IgG class, that can block the immune haemolytic reaction. This appears to be another example of IgG antibody blocking an immune reaction - in this case, a complement-mediated cytolytic reaction.

Thompson and Rowe suggested three possible factors that could be operating in the blocking phenomenon in the plate lysis assay. They are: (i) displacement of the cell-bound rabbit antibody by human IgG antibody; (ii) a prevention of the attachment of complement components to the red cells, and; (iii) a competition for available

complement between the rabbit antibody and the human IgG antibody. They implied that the human IgG antibody is "non-lytic". Aho *et al.*, (1964) described naturally-occurring human IgG antibody, specific for SRBCs but unable to sensitize them for lysis due to an inability to fix complement. Human antibody of the entire IgG_4 subclass is unable to fix complement (Ishizaka *et al.*, 1970; Spiegelberg, 1974). It is possible that non-complement-fixing IgG antibody similarly competes with "lytic" antibody in the NW phenomenon.

Bordet (1904) was the first to discover an inhibition or blocking phenomenon in the immune haemolytic reaction. He found that guinea pig antiserum, specific for rabbit serum, could inhibit the lysis of SRBCs, sensitized with rabbit haemolysin. Romeyn and Onysko (1964) found that the relative concentrations of complement and guinea pig antiserum determines the degree of inhibition, and that there is no complement fixation. They concluded that the inhibition is due to a competition between complement and guinea pig antibody for the same or closely situated receptor sites on the rabbit haemolysin. A similar competition between complement components and specific antibody, for receptor sites on the target bacterial cells, may exist in the NW phenomenon.

It is recognized that immune haemolysis can be inhibited if high concentrations of some sensitizing haemolysins are incorporated (Mayer, 1961). On dilution of such haemolysins, there is a haemolytic phase

and eventually a non-haemolytic phase. Whilst investigating various facets of the NW phenomenon, Muschel *et al.*, (1969) used the seemingly identical prozone phenomenon in immune haemolysis to see which, if any, complement components are fixed. SRBCs, sensitized with a prozoning level of haemolysin did display immune adherence. This indicated that C3 is fixed and is activated on the RBC membrane. When prozoning SRBCs were washed and then reacted with C-EDTA, these cells lysed. This indicated that C1, C4 and C2 are also activated in the prozoning situation and that excess of the remaining components of the classical pathway can abrogate the prozone phenomenon. It is possible that saturating levels of haemolysin may sterically hinder the short-lived MAC, incorporating components C5 to C9, from attaching to receptor sites on the RBC membrane. By increasing the concentrations of these components, the steric hindrance can be overcome. Such observations and conclusions may be true for the NW phenomenon but one must remember that the immunogens and target cells in the two systems are chemically and biologically very different.

Russell-Jones *et al.*, (1980) have recently shown that specific IgA antibody can inhibit the IgG antibody-dependent lysis of trinitrophenyl-SRBCs by complement. Perhaps this IgA antibody has an important role in the prozone phenomena of both systems.

CHAPTER 1.4 Previous investigations of the major parameters of the Neisser-Wechsberg phenomenon

1.4.1 Vaccination schedule

The nature of the vaccination schedule with Gram-negative bacteria is important in the development of this prozoning activity in rabbit sera. In particular, the dose and frequency of vaccinations, plus the time span of the schedule, are important.

Neisser and Wechsberg (1901) first stated that a lengthy vaccination schedule is required before an anti-serum shows good prozoning activity in the bactericidal reaction. More recently, Joos and Hall (1968) showed that whole blood from normal rabbits, and rabbits vaccinated intravenously, with one dose of heat-killed *Br. abortus* bacteria, were bactericidal for this bacterium. However, whole blood from rabbits, hyperimmunized with such a dose (once a week for 8 weeks), was not bactericidal.

Daguillard and Edsall (1968) refuted the need for a lengthy and intensive vaccination schedule. They injected rabbits with various doses (0.5 to 3.0mg) of *S. typhi* 0901 bacteria. The injections were three days apart and numbered between three and six. The rabbits were then bled after 7 and 60 days, and 9 days after re-injection with the highest dose. Specific IgG and IgM antibody pools from all three bleeding dates showed prozoning activity. Titres were not stated for the three pools and flaws in the experimental design reduce the validity of the conclusions reached by these workers (see 1.2.1 also). Their results contradict all other published results on

this subject. For example, Normann (1972) vaccinated rabbits with *S. typhimurium* 395 MRO bacteria in a long schedule (0.2mg injected thrice weekly for 9 weeks) and a short schedule (0.15 to 0.75mg injected thrice over 15 days). Antisera from the long schedule consistently showed lower haemagglutination titres but higher prozoning titres in the bactericidal assay, than those from the short schedule.

The need for a lengthy vaccination to produce good prozoning activity in rabbit antiserum correlates well with the clinical situation in humans. A number of workers (Waisbren and Brown, 1966; Gower *et al.*, 1972) have shown that serum from patients, with a chronic Gram-negative bacterial infection, often lack specific bactericidal activity. During such chronic infections, the immune system is frequently being challenged with bacterial antigens over an extended period.

The development of the prozoning activity in relation to other immunological parameters (eg., bactericidal titre, haemagglutination titre), during and after the vaccination period, has not been investigated thoroughly. There have been many studies of the concentration and avidity of antibody, following single and repeated vaccinations of rabbits with haptens, or whole Gram-negative bacteria or their LPS.

Studies of primary responses with haptens, such as dinitrophenols, have established that there is an increase in the affinity constant of specific antibody with time. This increase is most obvious when small doses are used (Eisen and Siskind, 1964; Goidl *et al.*, 1968). Makela *et*

al., (1967) showed that specific serum IgM and colostral IgA antibodies can appear 4 days after a single vaccination and that serum IgG antibody appears 7 to 11 days after the vaccination. He also found that the early IgM and IgA antibodies had low affinities for the hapten but the IgG antibody had high affinity. The affinities of all three antibody classes rose with time. Other studies revealed that there is a gradual shift, from a normal distribution of affinity about a low mean value, to a distribution skewed towards higher affinity, with time (Werblin *et al.*, 1973a; Werblin *et al.*, 1973b).

There have been similar studies of antibody avidity during vaccination schedules with Salmonella bacteria. Robbins *et al.*, (1965) showed that specific IgM antibody is more active in secondary antigen-antibody interactions (eg., agglutination, opsonization and complement-mediated killing) but is less avid than IgG antibody, during the primary response of rabbits to *S. typhimurium*. Gupta and Reed (1971) showed that the primary antibody response to vaccination with *S. enteritidis* peaks early (day 10), with IgM antibody accounting for over half of the antigen binding capacity. The latter rises significantly on further vaccination, with the contribution by IgM antibody declining with time. Schulkind *et al.*, (1972) noted a progressive increase in the avidity of both IgM and IgG antibodies, following primary and secondary vaccinations of rabbits with *S. typhimurium*. They also showed that specific IgM antibody is more active in complement-mediated killing and agglutination, and that these specific activities increase

with time for both IgM and IgG antibodies.

Holmgren and Svennerholm (1972) studied the immune response of rabbits to challenge by *Vibrio cholerae*. They showed that antibody of the primary response has lower avidity than antibody of the secondary response, and that the avidity is inversely proportional to the booster antigen dose. Ahlstedt *et al.*, (1973) confirmed these findings by studying the immune response of mice to challenge by *E. coli* LPS. Here, the increase in avidity paralleled the rise in specific IgG antibody; IgM antibody peaked at day 4 and significant IgG antibody appeared by day 30.

It seems fair to say that, if specific antibody is a major factor in the NW phenomenon, it would be an avid antibody as prozoning activity appears late in rigorous vaccination schedules. This antibody could well be of the IgG or IgA class, as both peak at this time.

1.4.2 The role of bacteria

It appears that specific antibody of a prozoning antiserum must bind to the corresponding Gram-negative bacteria, for the NW phenomenon to be evident. The addition of complement to sensitized, then washed *S. typhi* cells (Daguillard and Edsall, 1968; Muschel *et al.*, 1969) and *S. typhimurium* cells (Norman *et al.*, 1972) has no effect on the prozoning titres of the sensitizing antisera. However, few studies have been done on the actual antigenic sites involved on the target bacteria; the effect of varying bacterial numbers on the prozoning activity of antiserum, and; the effect of incorporating bacterial

strains of the same species, but with different cell wall structures, on the prozoning activity.

The antigenic sites of Gram-negative bacteria, that are involved in the complement-mediated bactericidal reaction, have been well characterized. The LPS component of the outer cell membrane and, in particular, the polysaccharide or O-somatic antigens are the initial sites with which bactericidal antibody combines (Cundiff and Morgan, 1941; Adler, 1952; Muschel, 1960). There is some evidence to suggest that this is also true for the prozone situation. Thus, the prozoning activity of hyperimmune rabbit antiserum was found to be specific for the immunizing R-mutant of *S. typhimurium* or for mutants of the same chemotype (Edebo and Normann, 1969). There has been no further study on this subject.

Work by Normann *et al.*, (1972) indicated that the NW phenomenon is insensitive to bacterial numbers. A hyperimmune rabbit antiserum showed the same prozoning activity when it was incorporated into bactericidal assays with *S. typhimurium* cells, numbering between 2×10^3 per ml and 2×10^8 per ml. This insensitivity to bacterial numbers indicates that perhaps steric hindrance, due to saturating levels of specific antibody, does not satisfactorily explain the NW phenomenon.

Hyperimmune rabbit antiserum, specific for a smooth, virulent strain of *S. enteritidis*, showed good prozoning activity with this bacterium. This is a clear example of a Gram-negative bacterium, with a full or complete LPS structure, displaying the NW phenomenon. Any correlation of the prozoning activity, with strains having incomplete

LPS structures (R-strains), was not undertaken. This area seems important because, as mentioned before, a saturation of the bacterial cell by specific antibody is one possible mechanism for the NW phenomenon (Muschel, 1965; Muschel *et al.*, 1969). There may well be a quantitative difference in the prozoning activity of an antiserum when it is incubated with a R-strain, having less antigenic sites.

1.4.3 The role of complement

An early observation, made about the NW phenomenon, was that there was no haemolytic complement consumption (Thomas and Dingle, 1943). However, Muschel *et al.*, (1969) later demonstrated considerable haemolytic complement consumption, in the prozone situation with *S. typhi* cells and hyperimmune rabbit antiserum. These contradictory reports may reflect basic differences in the nature of the two prozoning situations, as Thomas and Dingle used *N. meningitidis* bacteria in their study. It should also be remembered that specific IgG antibody has been implicated in the prozoning situation with *Salmonellae* (Normann *et al.*, 1972); whilst specific but non-complement-fixing IgA antibody, in the serum of patients convalescing from meningococcal infection, has been assigned inhibitory activity (Griffiss, 1975).

Muschel *et al.*, (1969) assumed that the mechanisms of the prozone phenomena in the immune haemolytic and serum bactericidal reactions were similar. This group demonstrated immune adherence with prozoning SRBCs, and so inferred that there is a corresponding activation of the

first four complement components (C1, C4, C2, C3) in prozoning mixtures in the bactericidal reaction. The addition of C-EDTA to prozoning SRBCs resulted in the total lysis of these cells. This provided further evidence for the activation of the first three components in the apparently similar situation of the bactericidal reaction. This is the only study that has investigated which complement components are activated in the NW phenomenon.

The prozone phenomenon can be abrogated by the addition of a sufficiently high concentration of whole complement. There have been no reports to the contrary. This result has been demonstrated with *N. meningitidis* (Thomas and Dingle, 1943; Thomas *et al.*, 1943; *Br. abortus* (Joos and Hall, 1968) and *S. typhi* (Muschel *et al.*, 1969). The last report provided evidence suggesting that the prozoning activity of a hyperimmune antiserum is inversely proportional to the complement concentration.

In summary, although it is certain that excess complement can abrogate the NW phenomenon, the questions of which complement components, if any, are activated in this prozoning situation and why the cytolytic pathway fails, remain unanswered.

1.4.4 Serum fractionation studies

Various workers (Waisbren and Brown, 1966; Muschel *et al.*, 1969; Normann *et al.*, 1972) have suggested that antibody specific for the LPS of bacterial outer membrane is important in the NW phenomenon. This was realized by observing that (i) a prozoning situation remained after sensitized bacteria were washed and then incubated with

complement, and (ii) prozoning activity was removed from an antiserum by absorbing it with the LPS of the corresponding bacterium.

Another approach in the study of the role of antibody in the phenomenon has involved the fractionation of prozoning antiserum by physicochemical means, and the subsequent characterization of the fractions. This characterization has included the concentration of particular immunoglobulin classes, the prozoning activity, the bactericidal activity, etc.

Daguillard and Edsall (1968) undertook the first such fractionation study using rabbit antiserum specific for *S. typhi*. Antibody, specific for the O-somatic antigens were adsorbed to and eluted from Salmonella bacteria with a common O-somatic antigen. The specific antibody was then separated into IgM and IgG fractions by gel filtration and anion-exchange chromatography. Prozoning activity was noted with low dilutions of both fractions, in the bactericidal reaction with *S. typhi*. The step, separating specific antibody, has already been criticized for the probable inactivation of complement-fixing sites on the IgM antibody, so creating potential "blocking antibody" (see 1.2.1). This is supported by the observation that no prozoning activity could be demonstrated in the IgM fraction of the whole antiserum. This study was also misleading because no mention was made of IgA antibody. The unidentified β-globulin component, appearing only late in the vaccination schedule, may have been the increasing level of specific IgA antibody (Heremans *et al.*, 1963).

Muschel *et al.*, (1969) also fractionated antiserum specific for *S. typhi* by gel filtration and ultracentrifugation. The description of their procedures was scanty but they appeared to be relatively crude. Most fractions with bactericidal activity also showed prozoning activity. The heaviest fraction from the ultracentrifugation method contained the highest prozoning activity. This fraction presumably contained IgM antibody; however, no detailed immunochemical analysis of the immunoglobulin classes, contained in each fraction, was included.

Hall *et al.*, (1971) applied more exacting fractionation procedures to hyperimmune rabbit antisera, specific for *Br. abortus* and with prozoning activity. They first separated β- and γ-globulins by starch block electrophoresis. They then used gel filtration (Sephadex G-200) to separate the β- and γ-globulins into IgM and IgG/IgA fractions. The latter two immunoglobulins were separated by anion-exchange chromatography on DEAE-Sephadex. Only the resulting IgA fraction exhibited prozoning activity in the bactericidal reaction; it also inhibited the bactericidal activity of the IgM and IgG fractions. However, the IgA fraction did show killing at higher dilutions. IgA antibody is not bactericidal (Knop *et al.*, 1971; Eddie *et al.*, 1971; Heddle *et al.*, 1975), and the observed killing was probably due to the admitted 15 to 20% contamination by IgG. Anticomplementary breakdown products of immunoglobulins eg., IgG Fc fragments (Plaut *et al.*, 1972), may also have contributed to the prozoning and inhibitory activities, as suggested by Griffiss (1975).

Griffiss improved the previous fractionation procedure, by using immunoadsorbent columns specific for particular immunoglobulin classes, in an investigation of the reduced serum bactericidal activity of patients convalescing from meningococcal infection. Tests for the purity of individual immunoglobulin class preparations were more exacting and concentrations, in mg per ml, were calculated. Again, the IgA fraction was found to inhibit the bactericidal activity of both IgM and IgG fractions. However, as with the study by Hall *et al.*, (1971), this inhibition only occurred by adding IgA far in excess of the serum concentration.

In a clinical study, Taylor (1972) examined the prozoning sera of two patients, convalescing from *Proteus mirabilis* infections. The sera were subjected to gel filtration on Sephadex G-200 and the resulting 7S fraction was subjected to anion-exchange chromatography on DEAE-Sephadex. Prozoning and inhibitory activity in the bactericidal reaction only appeared in the IgG fraction. This fraction identified with IgG immunoglobulin in an immunoelectrophoresis test and was shown to be free of other serum proteins by immunodiffusion and polyacrylamide gel electrophoresis tests. Normann (1972) also demonstrated prozoning activity in the IgG fractions of hyperimmune rabbit antisera, specific for *S. typhimurium*. These fractions, twice chromatographed on DEAE-cellulose, showed only one precipitin line, identifying with IgG immunoglobulin, in immunoelectrophoresis tests. They also showed a sedimentation coefficient consistent with IgG by analytical ultracentrifugation.

In summary, particular or all immunoglobulin classes have been implicated as participating in the NW phenomenon, by various workers. Serum fractionation methods have often been incomplete and tests for the purity of resulting fractions have been lacking. Investigations, using the more rigorous techniques, have indicated that IgG antibody can mediate the NW phenomenon and that IgA antibody can, at least, inhibit the bactericidal activity of IgM and IgG antibodies.

CHAPTER 1.5 Speculative mechanisms of the Neisser-Wechsberg phenomenon

The main aim of this thesis is to provide an explanation for the mechanism of the NW phenomenon, based on experimental evidence gained from one laboratory example. This involves the serum-sensitive *S. typhimurium* M206 strain and rabbits immunized against it. There have been many hypotheses reported for the same and similar prozone phenomena in the serum bactericidal reaction. All the past and presently possible explanations for the NW phenomenon are listed below. It is possible that several of the latter contribute to the phenomenon.

1. Neisser and Wechsberg (1901), the discoverers of the phenomenon, believed that excess free antibody, present in the lower antiserum dilutions, fix complement and so deviate it away from the target bacteria. This explanation was offered at a time when the activation of complement by specific antibody was poorly understood. The necessity of antibody to be structurally altered, by union with its

antigen, before being able to fix complement (Pecht *et al.*, 1977) was not recognized. Because free antibody is incapable of fixing complement, this original explanation is most unlikely.

2. Wilson and Miles (1957) provided an explanation that was popular before 1960. They stated that the NW phenomenon is similar to the prozone phenomenon seen in the antibody excess zones of precipitation and agglutination curves (Heidelberger and Kendall, 1935; Heidelberger and Kendall, 1937). Agglutination of target bacteria was regarded as an initial step in the serum bactericidal reaction and it could not occur with excess antibody. The current understanding of the bactericidal reaction does not involve this agglutination as a necessary step; thus, this explanation is very unlikely.

3. Muschel (1965) suggested that the surface of prozoning bacterial cells is saturated with specific antibody molecules (particularly of the IgG class) from low dilutions of hyperimmune antiserum. Initial complement attachment and activation, via these bound antibody molecules, or attachment and activation of later-acting complement components is therefore sterically hindered. Either explanation is consistent with current understanding of the serum bactericidal reaction. Initial complement attachment may not be successful due to only one Fab site per antibody molecule binding to antigenic sites on the bacteria, as suggested by Boyden (Muschel, 1965). Perhaps the terminal components of complement, assembled together as the relatively large MAC, are sterically hindered from

their binding sites on the bacteria. The inhibitor, S-protein (Podack *et al.*, 1976) may then rapidly inactivate the MAC.

4. Coombs (Muschel, 1965) suggested that soluble bacterial antigen, in particular LPS, combines with the excess antibody in the bactericidal reaction mixture. Complement is then activated in the fluid-phase and is deviated away from the target cells. A consequence of this is that sensitized, then washed, bacteria would not display the prozone phenomenon, if incubated with complement.

5. Benacceraf (Muschel, 1965) suggested that there may be differences in the lytic capability of different antigenic sites on the target bacteria. Hyperimmune antiserum may contain a high proportion of antibody specific for those of low lytic capability. With low dilutions of the antiserum, complement components may therefore be deviated away from the sites of high lytic capability.

6. Muschel *et al.*, (1969) suggested that anticomplementary factors in hyperimmune antisera, especially when heat-treated, play an important role in the NW phenomenon. Sera with elevated γ-globulin levels eg., hyperimmune antisera, have been known to have anticomplementary activity for many years (Davis *et al.*, 1944). Plaut *et al.*, (1972) showed that Fc fragments of IgM can fix complement. Both heat-labile and heat-stable anticomplementary activities have been shown to appear on heating sera at 56^{o} (Soltis *et al.*, 1979). Complement components may be removed in the fluid-phase of a bactericidal assay mixture by such

factors, existing in the low dilutions of hyperimmune antiserum. Sufficient complement to kill the target bacteria, may then not be present.

7. A popular explanation is that "lytic" and "non-lytic" antibodies compete for available antigenic sites on the target bacteria. The "non-lytic" antibody has been described as not fixing complement or as deviating complement components. Such antibody effectively competes for antigenic sites and so inhibits killing, when present in high concentration ie., at low dilutions of antiserum.

Non-complement-fixing IgA antibody is an effective inhibitor of the bactericidal reaction, mediated by both IgM and IgG antibodies, in both laboratory and clinical situations (Joos and Hall, 1968; Hall *et al.*, 1971; Griffiss, 1975; Griffiss and Bertram, 1977). However, the concentration of IgA antibody required for this inhibition, is far in excess of what exists in the whole antiserum.

Several workers (Taylor, 1972; Normann, 1972) have proposed that IgG antibody is responsible for the NW phenomenon. Subclasses of IgG, from a number of mammalian species are known to lack complement-fixing activity. The species include humans (Spiegelberg, 1974), guinea pigs (Siroťák *et al.*, 1976), mice (Ey *et al.*, 1980), and rabbits (Rodney *et al.*, 1979). Perhaps, non-complement-fixing IgG antibody effectively competes for the antigenic sites in the prozoning situation; or, as Normann *et al.*, (1972) suggested, cell-bound IgG antibody fixes complement but deviates it to a non-lytic pathway.

Another possible explanation is that there exists

in prozoning antisera antibody, specific for some bacterial component (eg., cell wall protein), but unable to fix complement. This antibody may combine to target bacterial cells in the prozoning situation and so sterically hinder the binding of complement-fixing antibody, specific for "lytic" antigenic sites or sterically hinder complement components.

8. The hydrophilic nature of the outer cell membrane of Gram-negative bacteria is altered by the binding of specific antibody of the IgG class (Mudd, 1934; Stendahl *et al.*, 1977); it becomes more hydrophobic. Perhaps, the surface of bacteria in the prozoning situation is so neutralized, by the high level - but not necessarily saturating level - of specific IgG antibody, that it repels complement components. The latter may well be the amphiphilic (Bhakdi *et al.*, 1978) MAC of complement, ie., the C5b-9 dimer (Biesecker *et al.*, 1979).

CHAPTER 1.6 Aims of the thesis

Preceding sections of this chapter have revealed that there is much diversity of thought concerning the nature of the prozone phenomenon in the complement-mediated bactericidal reaction. There has often been disagreement in experimental data when workers have studied the same facet of this phenomenon. Either, a number of mechanisms may operate, depending on the type of bacterium involved; or, there is one mechanism, but some of the past investigations have been defective in experimental design or execution. The aim of the work, reported in this thesis,

has been to revise and extend the experimental data on the NW phenomenon and so present an explanation of the mechanism(s) that are operating.

Two important issues needed to be resolved first. Namely, (i) does the phenomenon operate in the fluid phase of the bactericidal reaction mixture, on the target cells, or both, and (ii) does complement activation actually occur and, if so, does it occur in the fluid-phase or on the cells.

Firm evidence was obtained that the NW phenomenon operates only in conjunction with the bacterial cell surface and so it was then important to establish the bacterial structure(s) involved. Damage to both the outer and inner cell membranes is believed to occur in the serum bactericidal reaction, and the O-somatic antigens of the outer membrane are important sites for fixing at least the first component of complement. Firm evidence was also found that complement activation occurs solely on the target cells and not in the fluid-phase. It was then important to determine at which step the cytolytic pathway was aborted or deviated, and why.

Antibody, specific for the target bacteria, appears to be important in the NW phenomenon. There are investigations of the bacterial structure(s) that this antibody is specific for, the class or subclass that this antibody belongs to and the complement-fixing activity of this antibody. A study of the extent to which this antibody saturates target cells in a prozoning situation has important consequences to the understanding of the phenomenon.

The NW phenomenon is often demonstrated with the

blood and serum of mammals exposed to Gram-negative bacteria, whether by infection or by immunization. It appears to be an *in vivo* phenomenon, not just one observed in the *in vitro* bactericidal reaction. An understanding of its mechanism must help in the understanding of the immune response of the host and of the serum bactericidal reaction. The phenomenon may not be an important factor involved in the persistence of such infections to the chronic stage, but it must be an important consequence.

CHAPTER 2 MATERIALS AND METHODS

CHAPTER 2.1 Bacteria

2.1.1 Stock bacterial strains

Citrobacter 396 (O:4,5)

S. derby (O:1,4,12)

S. reading (O:4,12)

S. minnesota R595

A rough Re mutant strain derived from the smooth 218s strain. The LPS of the outer membrane consists only of the core components lipid A, KDO and ethanolamine (Luderitz *et al.*, 1966).

S. minnesota R5

A rough Rc mutant strain derived from the smooth 218s strain. The LPS of the outer membrane consists only of the core components lipid A, KDO, ethanolamine, heptose and glucose (Luderitz *et al.*, 1966).

S. typhimurium C5 (O:1,4,5,12)

A smooth strain that is virulent for mice (Furness and Rowley, 1956).

S. typhimurium M206 (O:1,4,5,12)

A smooth strain that is avirulent for mice, is serum-sensitive and was originally described by Jensen (1929). More recently, its antigenic structure has been compared to that of *S. typhimurium* C5 (Archer and Rowley, 1969).

S. typhimurium G30

A rough Rc mutant strain that is deficient in uridine

diphosphate-4-galactose epimerase (UDPG-epimerase). The LPS consists of the core components Lipid A, KDO, ethanolamine, heptose and glucose when cells are grown in the absence of galactose (Osborn *et al.*, 1964).

S. typhimurium TV119

A rough Ra mutant strain, derived from the smooth LT2 strain. It lacks only the O-somatic antigens from the LPS of the outer membrane (Nikaido *et al.*, 1964).

Figure 2.1 shows the general LPS structure of the *S. minnesota* and *S. typhimurium* strains listed above.

All above strains were stored in the freeze-dried state. When in use, they were maintained on nutrient agar slopes.

2.1.2 Cell components

(a) Acetone-dried cells of *S. typhimurium* M206, *S. typhimurium* TV119 and *S. minnesota* R595 were prepared as described by Sutherland (Weir, 1973).

(b) Lipopolysaccharide fractions were prepared from *S. typhimurium* C5 and M206, and *S. derby* by the phenol-water extraction method (Westphal *et al.*, 1952). Precipitation in alcohol, enzymatic treatments (to remove nucleic acids and protein) and washing by ultracentrifugation further purified these LPS preparations.

(c) Outer cell wall protein was prepared from *S. minnesota* R595 cells by the Triton X-100 extraction method of Schnaitman (1974).

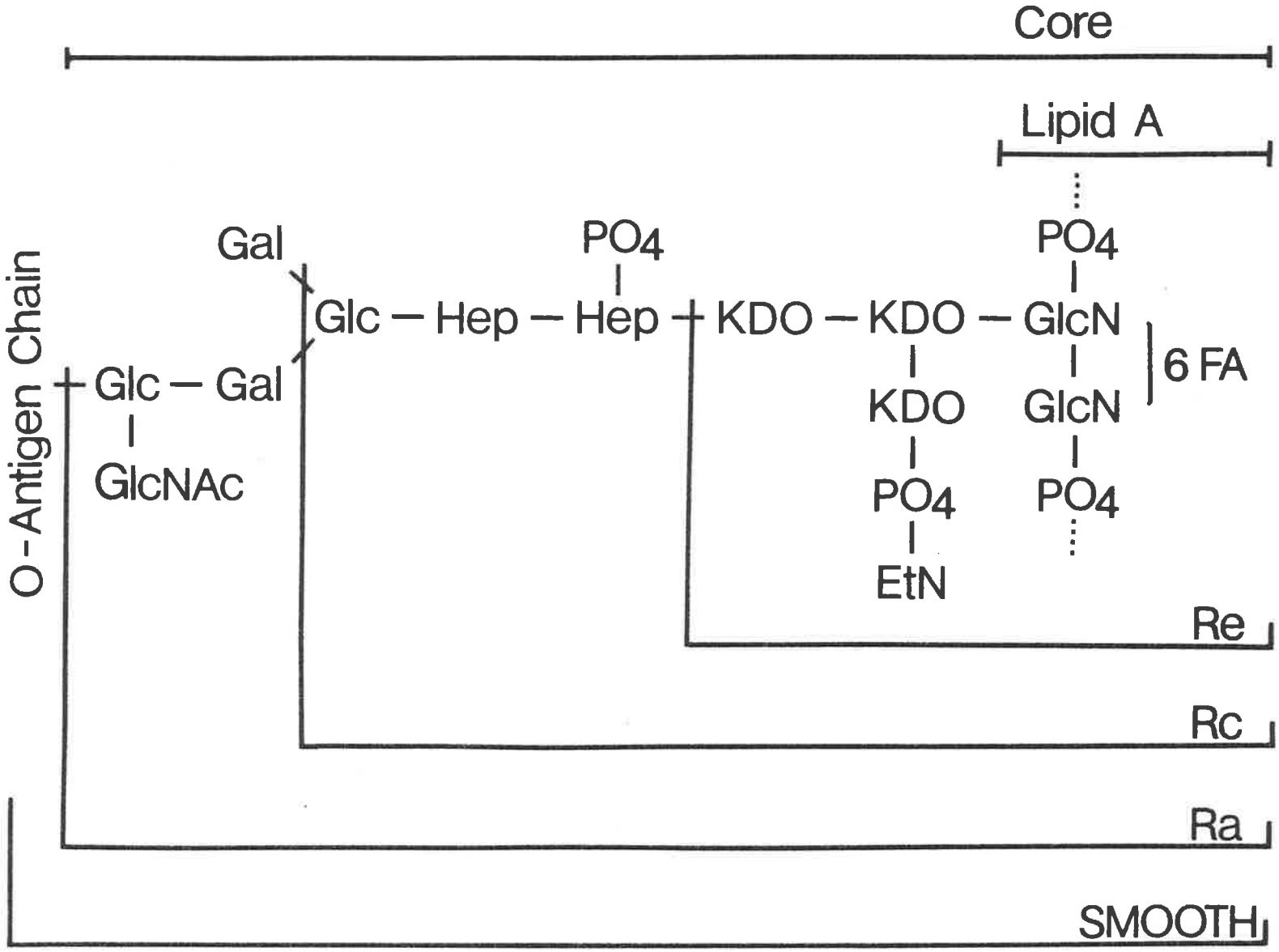
FIGURE 2.1

Lipopolysaccharide structures of the *S. typhimurium* and *S. minnesota* strains used in this study

S. typhimurium LT2, C5 and M206 strains and *S. minnesota* 218S are all smooth strains; *S. typhimurium* TV119 is an Ra mutant strain; *S. typhimurium* G30 and *S. minnesota* R5 are Rc mutant strains; and, *S. minnesota* R595 is an Rc mutant strain.

Abbreviations: FA, fatty acid; GlN, D-glucosamine; PO_4, phosphate; KDO, 3-deoxy-D-manno-octulosonate; EtN, ethanolamine; Hep, L-glycero-D-manno-heptose; Glc, glucose; Gal, galactose; GlCNAc, N-acetyl-glucosamine.

The figure shows a "monomer unit"; a molecule of LPS is thought to contain three such monomer units linked through pyrophosphate groups (dotted line).



(adopted from fig 1, Smit et al, 1975)

2.1.3 Sensitization with Lipopolysaccharide

S. minnesota R595 cells were sensitized with the LPS fraction of *S. typhimurium* C5, by incubating a suspension of the log-phase cells in Mg^{2+} saline (5×10^8 cells per ml) with C5 LPS (1.0 mg per ml) at 37^{o} for 30 minutes. The cells were then washed twice and resuspended in Mg^{2+} saline, prior to their use as target cells in a bactericidal assay.

2.1.4 Identification of the *S. typhimurium* G30 strain

(a) 4-0 agglutination test to distinguish the rough and smooth forms

A thick suspension of cells was made in N-saline on a glass slide. A drop of antiserum, specific for the Salmonella O antigen 4 (Wellcome Research Labs. Beckenham, England), was added and mixed. The slide was rotated for about 20 seconds and it was observed for macroscopic clumping of the bacteria, compared to a saline suspension control.

Smooth strains of *S. typhimurium* possess the O-somatic antigens 1,4,5,12 (Kauffmann, 1975). Their cells should therefore agglutinate in the presence of the 4-0 antiserum. Cells of the G30 strain are normally rough and so should not agglutinate. Only when grown in the presence of galactose, do they change to the smooth form (Osborn *et al.*, 1964) and so be expected to agglutinate in this test.

(b) Acriflavine agglutination test to distinguish the rough and smooth forms

A thick suspension of cells was made in N-saline on a glass slide. A drop of 1:1000 (W/V) acriflavine solution in N-saline was added and mixed. The slide was rotated for

about 20 seconds and it was observed for macroscopic clumping of the bacteria, compared to a control suspension of the smooth form.

Agglutination indicates that the cells are rough and possess an incomplete LPS (Wilson and Miles, 1957). This means that G30 cells should show no agglutination only when grown in the presence of galactose.

(c) Galactose fermentation test to identify the strain

A few drops of log-phase cells in nutrient broth (see 2.5.1) were added to duplicate bottles of phenol red-peptone water, with and without 1% galactose (see 2.2.5). The lids were left a little loose and the bottles were incubated overnight at 37^{o}.

The ability of bacteria to ferment galactose, and so effect a colour change from red to yellow, depends on the presence of the enzyme UDPG-epimerase (Nikaido, 1961; Osborn *et al.*, 1964). The G30 strain does not possess this enzyme and so should show no colour change.

CHAPTER 2.2 Media, solutions and buffers

2.2.1 Nutrient agar slopes

Oxoid Blood Agar Base No 2 mixture was dissolved in distilled water and dispensed into narrow neck glass tubes. After autoclaving, the bottles were tilted and left to cool. The stock bacterial strains were maintained on these slopes.

2.2.2 Nutrient broth

Oxoid Nutrient Broth was prepared, and suitable volumes were dispnsed into screw-top glass bottles and autoclaved.

2.2.3 Nutrient agar plates

20 ml aliquots of sterile Oxoid Diagnostic Sensitivity Agar mixture were dispensed into sterile plastic petri-dishes. The agar was allowed to set and the surface was dried. These plates were used to subculture individual bacterial colonies from the stock growth on slopes and for the bactericidal assays.

2.2.4 Brain heart infusion broth

Suitable aliquots of Oxoid Brain Heart Infusion Broth were dispensed into screw-top glass bottles and then autoclaved.

2.2.5 Phenol red-peptone water

Oxoid Peptone Water was prepared, with phenol red added as an acid-base indicator. Galactose (1% W/V) was added to some. 3.0 ml aliquots were dispensed into screw-top bottles and then autoclaved.

2.2.6 0.002 M Mg^{2+} saline (Mg^{2+} saline)

0.2 ml of a stock 1.0 M $MgSO_4$ solution in 0.9% NaCl (N-saline) was mixed with 1.0 litre of N-saline, the pH was adjusted to 7.2 and 100 ml aliquots in screw-top glass bottles were autoclaved.

2.2.7 Veronal-buffered saline (VBS)

500 ml aliquots were prepared in screw-top glass bottles using five Oxoid Complement Fixation Test Diluent tablets of the following formula:

Barbitone	0.0575 g
Sodium chloride	0.85 g
Magnesium chloride	0.0158 g
Calcium chloride	0.0028 g
Sodium barbitone	0.0185 g

The pH was adjusted to 7.2, when necessary. This was the diluent used in immune haemolytic assays.

2.2.8 Tris-HCl buffer, 0.1 M

This was prepared by mixing 450 ml of 0.2 M hydrochloric acid, 500 ml of 0.2 M TRIS and distilled water to a final volume of 1.0 litre. The pH was adjusted to 7.2 and 100 ml aliquots in screw-top glass bottles were autoclaved.

2.2.9 Phosphate buffer, 0.02 M, pH 8.0

Potassium dihydrogen phosphate	13.609 g
Sodium chloride	58.44 g
Sodium azide	5.00 g

Distilled water was added to 5.0 litres and the pH was adjusted to 8.0, when necessary. This buffer was used in serum fractionation procedures.

2.2.10 Phosphate buffer, pH 7.2

Disodium hydrogen phosphate	0.724 g
Potassium dihydrogen phosphate	0.21 g
Sodium chloride	7.65 g

Distilled water was added to 1.0 litre and the pH was adjusted to 7.2, when necessary. This buffer was used in the separation of specific antibodies from whole antiserum raised against *S. typhimurium* M206.

2.2.11 Citrate buffers, 0.05 M

Buffers of various pH values were prepared from stock 0.15 M solutions of citric acid (A) and sodium citrate (B):

pH 7.0	1.0 ml A + 99.5 ml B
pH 6.0	15.0 ml A + 125.0 ml B
pH 5.0	25.0 ml A + 90.0 ml B
pH 4.0	45.0 ml A + 90.0 ml B
pH 3.0	82.0 ml A + 48.0 ml B

One volume of the above mixtures was added to two volumes of 0.2 M sodium chloride to arrive at a 0.05 M concentration. These buffers were used in serum fractionation procedures.

2.2.12 Barbitone buffer, 0.05 M

This was prepared by mixing 250 ml of 0.2 M sodium barbitone, 63.5 ml of 0.2 M hydrochloric acid and distilled water to a final volume of 1.0 litre. The pH was adjusted to 8.2, when necessary. This buffer was used in immunodiffusion (ID) and immunoelectrophoresis (IEP) procedures.

2.2.13 Borate buffer

Boric acid	6.184 g
Sodium tetraborate	9.536 g
Sodium chloride	4.384 g

Distilled water was added to 1.0 litre and the pH was adjusted to 7.4, when necessary. This buffer was used in binding studies with radiolabelled protein A.

2.2.14 Tris-EDTA-Saline-Azide (TESA) buffer

Trisma base	3.028 g
Disodium ethylenediaminetetraacetate	0.038 g
Sodium chloride	7.743 g
Sodium azide	0.5 g

Distilled water was added to 1.0 litre and the pH was adjusted to 8.0, when necessary. This buffer was used in the preparation of IgG antibody specific for *S. typhimurium* M206.

CHAPTER 2.3 Antisera

2.3.1 Bacterial vaccines

S. typhimurium M206 cells were grown up overnight at 37^{o} in nutrient broth. On the following day, 4×10^{8} or 4×10^{9} cells (estimated by OD_{650nm} values) were centrifuged at 2,000 g for 10 minutes. They were washed once in N-saline, centrifuged again and resuspended in 2.0 ml of N-saline. The suspensions (2×10^{8} or 2×10^{9} cells per ml) were finally heated to 56^{o} for 60 minutes to kill the cells.

2.3.2 Immunization schedules

The schedules closely followed that of Muschel *et al.*, (1969). Rabbits P,S,A,C and G were injected with bacterial vaccines each week and they were also bled each week. On the same day, blood was collected from the vein of the left ear and then vaccinations were given in the vein of the right ear. 2 ml aliquots of the sera were stored at -73^{o}. Details for individual rabbits follow.

(a) Rabbits P and S were injected intravenously with 10^8 heat-killed M206 cells, once a week for six weeks. After a lapse of three weeks, they were then injected intravenously with 10^9 heat-killed cells over two weeks. Large samples of blood were collected several days after the last three injections (see table 2.1).

(b) Rabbits A and C were generously bled then injected intravenously with 10^8 heat-killed M206 cells once a week for seven weeks. Both rabbits were also bled two weeks after the last vaccination. Further details on this, the most rigorous, schedule follow in 4.2.1.

(c) Rabbits G (two) were injected intravenously with 10^8 live M206 cells once each week. The vaccination was continued and the rabbits were not bled before ten weeks. A bleeding occurred whenever a large sample of hyperimmune antiserum was required.

2.3.3 Monospecific antiserum preparation

By this procedure, an antiserum specific for only the Salmonella O-antigen 5 was prepared from a hyperimmune antiserum specific for *S. typhimurium* M206 (0:1,4,5,12). The recommended procedure of Kauffmann (1975) was followed.

S. derby (0:1,4,12) bacteria were grown up overnight in nutrient broth at 37°, from a single colony. About 10^{12} cells in 500 ml of this broth were centrifuged at 2,000 g for 10 minutes. The cells were suspended in 10 ml of N-saline and boiled for two hours. They were then washed in N-saline three times and finally resuspended in 10 ml of hyperimmune antiserum (rabbit G) specific for *S. typhimurium* M206. The mixture was incubated at 37° for two hours with

TABLE 2.1

Immunization schedule for rabbits P and S

Day	Activity
0	vaccination 1 (10^8 cells)
interim vaccinations 2,3,4,5 and bleeds 1,2,3,4	
32	vaccination 6 (10^8 cells)
38	bleed 5
44	bleed 6
53	vaccination 7 (10^9 cells)
57	bleed 7
60	vaccination 8 (10^9 cells)
65	bleed 8

occasional shaking. The bacteria were then removed by centrifugation at 10,000 g for 10 minutes and finally by filtration through a Millipore filter (0.45 μ pore size). 2 ml aliquots of the absorbed antiserum were stored at -73^{o}.

The absorbed antiserum was first tested in a haem-agglutination (HA) assay (see 2.7.1) with SRBCs sensitized with LPS from *S. derby*. The antiserum did not display agglutination and so appeared free of antibody specific for the absorbing species.

2.3.4 Removal of complement activity

All antisera were heated to 56^{o} for 30 minutes, if complement activity was required to be removed. Such antisera will be subsequently referred to as "heat-treated" antisera.

CHAPTER 2.4 Complement

2.4.1 Total complement source

Pooled human serum was the main source of complement (HuC) in this study. Blood samples were allowed to clot at room temperature for one to two hours. The sera were then centrifuged free from cells and were pooled. An absorption step, to remove antibody specific for *S. typhimurium* from the pooled serum, followed.

The first 10 preparations (HuC-1 to HuC-10) were absorbed with washed acetone-dried cells of *S. typhimurium* M206 (10 mg per ml of pooled serum). This absorption procedure proved to be unsatisfactory in certain assays because there was considerable complement consumption, when

the absorbed complement was incubated with various dilutions of an anti-M206 antiserum. As shown in table 2.2, a 50% loss of haemolytic complement occurred with the 1:1000 antiserum dilution. Presumably, antigens of the acetone-dried cells had not been completely removed from the absorbed complement. The control tube, lacking antiserum, showed a smaller loss (23%) of haemolytic complement. This loss was probably due to degradation that occurred on storage (at -73^{o}), thawing and subsequent incubation at 37^{o} in the assay.

The next 7 preparations (HuC-11 to HuC-17) were absorbed with live M206 cells (10^{10} cells per ml of pooled serum). The bacteria were grown overnight in nutrient broth, washed and suspended in the pooled serum. This mixture was incubated at 4^{o} for 60 minutes. The cells were then removed by centrifugation at 4^{o} (10,000 g for 10 minutes) and finally by filtration through a Millipore filter (0.45 μ pore size). Table 2.2 shows that a low level of haemolytic complement was lost on incubating such an absorbed serum pool with various dilutions of an anti-M206 antiserum. This loss was insignificant when one compared it with the small loss due to the physical conditions of such an assay (the control tube lacking antiserum).

Absorption of human complement with live M206 cells therefore proved to be the better method of removing natural antibodies, specific for this bacterium. Note that, in some bactericidal assays, partial and even complete killing still occurred in the control tube lacking antiserum. However, in these assays the presence of a low level of cross-reacting antibody in the complement source did not affect the significance of the results; the prozoning situation occurs in

TABLE 2.2

Loss of haemolytic complement on incubation of anti-M206 antiserum with absorbed complement sources

Complement source		Final antiserum dilution	Haemolytic complement loss	
Batch	Absorption method		CH50 units	% loss (a)
HuC-7	Acetone-dried M206 cells	1:100	8.0	100
		1:1000	3.8	48
		1;10,000	2.0	25
		Nil	1.8	23
HuC-12	Live M206 cells	1:200	1.4	35
		1:800	1.3	33
		1:3200	1.1	28
		Nil	0.7	18

(a) Comparisons between the two types of complement source are meaningless due to the different haemolytic levels initially added.

Various dilutions of heat-treated P-5 antiserum were incubated with 8.0 CH50 units of HuC-7 and 4.0 CH50 units of HuC-12 at 37^{o} for 60 minutes. The haemolytic activity, remaining in the mixtures, was determined by a haemolytic titration (see 2.4.7).

the presence of excess antibody anyway. Repeated absorptions would have decreased the level of haemolytic complement (up to 10% of activity was lost by one absorption step) and increased the level of bacterial antigens present). One preparation of human complement (HuC-18) was not absorbed.

Guinea pig serum, both unabsorbed and absorbed with live M206 cells, was used as a complement source (GpC) in some sections of the work. Unabsorbed normal rabbit serum (NRS) was also used (RaC).

All sera, employed as complement sources, were stored at 0.5 ml and 1.0 ml aliquots at -73°. The aliquots were used immediately when thawed out.

2.4.2 C6-deficient rabbit serum (C6-def RaC)

Fresh serum, from rabbits with a heritable deficiency in component C6, was kindly donated to this study by Dr. S. Neoh (Clinical Immunology Department, Flinders Medical Centre, Adelaide). 1.0 ml aliquots were stored at -73°.

2.4.3 C-EDTA

This reagent was prepared by diluting HuC or GpC to the required concentration in an assay, in 0.005 M EDTA-N-saline, pH 7.2. The mixture was incubated for 15 minutes at 37° immediately before use.

C-EDTA was a source of the last six components of the classical pathway. EDTA chelates calcium and magnesium ions and so prevents activation of components C1 and C2 of the classical pathway (Mayer, 1961) and of the C3 pro-activator of the alternative pathway (Götze and Müller-Eberhard, 1976).

2.4.4 C-EGTA

This reagent was prepared by diluting HuC or GpC to the required concentration in an assay, in 0.01 M ethyleneglycol tetraacetic acid (EGTA)-N-saline, pH 7.2. This was done immediately before use.

EGTA chelates calcium ions but not magnesium ions, and so prevents the activation of C1 of the classical pathway but allows the alternative pathway to proceed (Fine *et al.*, 1972).

2.4.5 C-142

A suspension of zymosan in N-saline (10 mg per ml) was heated in a boiling water bath for 60 minutes. After three washes in N-saline, the zymosan was resuspended in fresh guinea pig serum to a final concentration of 10 mg per ml. The mixture was incubated at 37^{o} for 60 minutes. The zymosan was centrifuged down and the absorbed guinea pig serum was stored at -73^{o} (Mayer, 1961).

The zymosan treatment activates the alternative pathway and removes at least C3 and C5 activity from the guinea pig serum, according to Lachmann *et al.*, (Weir, 1973). Components C1, C4 and C2 of the classical pathway are thus available.

2.4.6 RaC6-9

This reagent was prepared from RaC, according to the method of Cooper and Müller-Eberhard (1970). 4.0 ml of the complement source was incubated with 4.0 ml of 1 M sodium isothiocyanate for 18 hours at 4^{o}. Hydrazine hydrate was added to a final concentration of 1.5×10^{-2} M and the

mixture was incubated at 37° for 45 minutes. It was then dialysed in four changes of VBS over 24 hours. 0.5 ml aliquots were stored at -73°.

2.4.7 Haemolytic complement titration

The haemolytic complement activity of complement sources and of reaction mixtures in various experiments was measured as CH50 units per ml. The method of Rapp and Borsos (1970) was used and is briefly described below.

(a) Sheep red blood cell sensitization

SRBCs in Alsever's solution (Commonwealth Serum Labs, Melbourne) were washed twice and finally resuspended, as 1×10^9 cells per ml, in VBS. Rabbit haemolysin (Commonwealth Serum Labs, Melbourne) was diluted to a stock 1:20 dilution in VBS, with 0.5% phenol as preservative. The optimum sensitizing dose of haemolysin, previously determined for each batch used, was then made up in VBS.

Equal volumes of the SRBC suspension and the optimum sensitizing dose of haemolysin were mixed and incubated at 37° for 20 minutes.

(b) The titration

Dilutions of the complement source or reaction mixture were made up in VBS to 3.25 ml. 0.5 ml of the sensitized SRBC suspension was added, and the mixtures were incubated at 37° for 60 minutes. They were then centrifuged at 4° (1000 g for 10 minutes). OD_{541nm} values of the supernatant fluids were read on a Perkin-Elmer (model 124) spectrophotometer.

The fraction of red cells lysed (Y), for each dilution (X) of the complement source, was calculated by referring to a control tube containing distilled water instead of complement dilutions. A linear plot of log X vs log $(^{Y}/1-Y)$ was made. The intercept on the X-axis (log K in the von Krogh equation) then permitted the number of CH50 units, in 1.0 ml of the complement source or in the total volume of a reaction mixture, to be calculated. The slope of the linear plot $(^{1}/n)$ was calculated and checked with the ideal value of 0.2.

CHAPTER 2.5 Bactericidal reactions

2.5.1 Complement-mediated bactericidal reaction

Salmonella bacteria were inoculated from a single colony on a nutrient agar plate into either nutrient broth or brain heart infusion broth. After incubation at 37^{o} for two to three hours, the concentration of the log-phase cells was determined using photometric (OD_{650nm}) comparisons. These target cells were either washed in Mg^{2+} saline and re-suspended to 1×10^{9} cells per ml, or, they were diluted to 5×10^{4} cells per ml in Mg^{2+} saline.

0.1 ml of the target cell suspension was added to tubes containing 0.9 ml of antiserum dilutions and a complement source in a salt solution, usually Mg^{2+} saline. For 10 minutes prior to this addition, both the cell suspension and the tube mixtures were kept at 37^{o}. Control tubes lacking antiserum, active complement or both antiserum and complement were included.

The reaction mixtures were incubated at 37^{o}. 0.04 ml volumes were always assayed at 0 and 60 minutes, when

5×10^3 target cells were initially added. The 0.04 ml volumes were dropped onto nutrient agar plates, they were spread, and the plates were incubated overnight at 37°. When a larger number of target cells were added to reaction mixtures, suitable dilutions were plated to give a count of about 200 colonies at 0 minutes.

Percentage survival values for each tube were calculated from replicate colony counts at 0 and 60 minutes. The average number of target cells, added per tube, was calculated from the mean of the colony counts at 0 minutes.

The prozoning titre of the antiserum was assessed as the highest dilution to show <50% killing, when further dilutions showed killing with eventual total survival. The bactericidal titre was assessed as the highest dilution of antiserum to show >90% killing.

2.5.2 Polymyxin-mediated bactericidal reaction

A vial of Polymyxin B sulphate (500,000 iu, Wellcome Research Labs, Beckenham, England) was diluted in N-saline to a stock solution of 10 mg per ml and stored at 4°. The stock solution was used within a week. The bactericidal reaction was conducted in the same manner as the complement-mediated bactericidal reaction. 5×10^3 target cells were added to the reaction tubes, which contained polymyxin diluted from the stock solution rather than complement.

CHAPTER 2.6 Other complement-mediated reactions

2.6.1 The assay of antiserum by passive haemolysis

Two types of passive haemolytic assay were performed. The first type (described in (a)) was used to titre antisera

and their fractions for complement-fixing antibody, specific for *S. typhimurium*. The second type, described in (b), measured the fraction of sensitized SRBCs lysed and was the more sensitive.

(a) The semi-quantitative assay

SRBCs (Commonwealth Serum Labs, Melbourne) were washed twice in N-saline. A 2.5% suspension in N-saline was incubated with the LPS from *S. typhimurium* C5 (100 µg per ml) at 37°. The mixture was gently rolled throughout the 90 minute incubation period. The sensitized SRBCs were then washed twice in VBS and resuspended to 1.0%. A suspension of non-sensitized cells was prepared in a similar manner, with the omission of C5 LPS.

Doubling dilutions (25 µl) of the antiserum (or its fraction) were made up in VBS in a U-welled microtitre plate (Sterilin, Middlesex, England). VBS (25 µl), a 1:10 dilution of GpC (25 µl) and sensitized SRBCs (25 µl) were added. The initial dilution of antiserum (or its fraction) was also tested with the non-sensitized SRBCs. The plate was shaken and then incubated at 37° for 60 minutes, with shaking every 20 minutes. The plate was centrifuged at 4° (1000 g for 10 minutes).

The degree of haemolysis for each mixture was assessed by eye - ≥50% haemolysis was considered to be a positive result. The highest dilution of antiserum (or its fraction) showing a positive result was taken to be its haemolytic titre.

(b) The quantitative assay

The sensitization of SRBCs was carried out as in (a).

The sensitized cells were finally suspended in a volume of VBS, so that when 0.2 ml was lysed in 3.8 ml of distilled water the OD_{541nm} was 1.0.

10 ml doubling dilutions of antiserum in VBS were prepared in glass tubes. 0.2 ml of the sensitized SRBC suspension and 2.7 ml of VBS were added. A control tube, lacking antiserum, was included. The tubes were incubated at 37° for 20 minutes. 1.0 CH50 unit of GpC, contained in 0.1 ml, was added and the tubes were incubated for a further 60 minutes. OD_{541nm} values of the supernatants were measured after the tubes had been centrifuged at 4°. The fractions of cells lysed were determined by comparing these OD values with that of a further control tube, containing distilled water and sensitized SRBCs.

2.6.2 Immune Adherence

This assay, designed to demonstrate immune adherence by bacteria from bactericidal reaction mixtures, was similar to that used by Reynolds *et al.*, (1975).

A complement-mediated bactericidal assay was first performed in Tris-HCl buffer pH 7.2 with several antiserum dilutions and *S. typhimurium* C5 cells (1×10^8 cells per ml). Control tubes, lacking antiserum, HuC or bacteria, were included. At 60 minutes, the mixtures were centrifuged at 4° (10,000 g for 10 minutes) and the cell pellets were washed once in cold VBS. They were then resuspended in 0.5 ml of VBS and 50 µl doubling dilutions were made up in a U-welled microtitre plate. 50 µl of a 1% suspension of human RBCs was added. The plate was shaken and then incubated at 37° for 30 minutes. All wells were read for

haemagglutination after maintaining the plate at room temperature for 20 minutes. The last dilution of cell suspension, from each bactericidal reaction tube, to display definite haemagglutination was recorded.

2.6.3 Deviated lysis

This assay was designed to demonstrate deviated lysis by bacteria, from bactericidal reaction mixtures. The method was similar to that used in the first demonstration of the phenomenon (Rother *et al.*, 1974).

2.0 ml bactericidal assay mixtures, incorporating several antiserum dilutions, HuC and 2×10^8 log-phase cells of *S. typhimurium* M206 were first set up. Control tubes, lacking either antiserum, HuC or bacteria, were also included.

A standard bactericidal assay was performed over 60 minutes, with 0.5 ml aliquots from these mixtures.

The remaining 1.5 ml volumes were incubated at 32° for 20 minutes. A tube containing 5 mg of inulin, HuC and Mg^{2+} saline was included with this incubation. All tubes were then centrifuged at $4^{\circ}C$ (10,000 g for 10 minutes). 0.1 M EDTA-N-saline was added to the supernatants so that the final EDTA concentration was 0.01 M. A suspension of chicken RBCs was prepared by mixing 0.2 ml of washed and packed cells in 5.0 ml of 0.01 M EDTA-N-saline. 0.2 ml of the chilled chicken RBC suspension was then added to both the deposits and supernatants. All tubes were incubated at 37° for 60 minutes, shaking every 5 minutes. A control tube, containing water, chicken RBCs and a few drops of toluene, was included with the final incubation.

All tubes were placed on ice. 2.2 ml and 3.8 ml of

0.01 M EDTA-N-saline was added to the tubes containing the supernatants and deposits, respectively. All were centrifuged at 4^{o} (1000 g for 10 minutes) and the OD_{541nm} of the final supernatants were recorded.

CHAPTER 2.7 Haemagglutination and haemagglutination inhibition assays

2.7.1 Haemagglutination assay

This assay was used to measure specific antibody in anti-M206 antiserum and its fractions. When 2-mercaptoethanol (2ME) was incorporated, specific IgM and IgG antibody activities were distinguished.

The sensitization of SRBCs with LPS from *S. typhimurium* M206 was carried out as in 2.6.1(a).

An aliquot of the antiserum or it's fraction was heat-treated. It was then diluted 1:10 by either of three ways: (i) dilution in N-saline; (ii) 0.1 ml was incubated with 0.9 ml of 1% SRBCs in N-saline and the cells removed by centrifugation; (iii) 0.1 ml was incubated first with 0.1 ml of 0.2 M 2ME and then with 1% SRBCs. Incubation with SRBCs removed natural antibody specific for them. Incubation with 2ME destroyed the activity of IgM class antibody.

Doubling dilutions (25 µl) of the antiserum or it's fraction were made up in N-saline in a U-welled microtitre plate. 50 µl of N-saline and 25 µl of sensitized SRBCs were added. The initial dilution of antiserum or it's fraction was also tested with the non-sensitized SRBCs. The plate was shaken and left at room temperature for three hours.

The wells were then read for haemagglutination. The last

dilution to display this was taken to be the titre and it represented one HA unit of antiserum.

2.7.2 Haemagglutination inhibition assay

The assay, based on a competition for available antibody by the fluid-phase LPS and RBC-bound LPS, was used to determine the level of LPS remaining in the fluid-phase of reaction mixtures.

Doubling dilutions (25 µl) of the reaction mixtures and two suspensions of M206 LPS of known concentrations (1.0 mg per ml and 100 µg per ml) were made up in a U-welled microtitre plate. The diluent used throughout was N-saline. 25 µl, containing two HA units of anti-M206 antiserum (predetermined in a HA assay), and 25 µl of N-saline were added. The plate was shaken and incubated at 37^{o} for 30 minutes. 25 µl of sensitized SRBC suspension (see 2.6.1(a) for its preparation) was then added. Several controls were included: (i) initial dilutions of reaction mixtures and LPS preparations, and (ii) doubling dilutions of the anti-M206 antiserum (from 2 to $^{1}/8$ supposed HA units) were tested with the sensitized SRBCs. The plate was shaken and incubated at room temperature for three hours.

The plate was read for haemagglutination. The last dilution of reaction mixture to inhibit haemagglutination was compared with that of the standard LPS suspensions, to determine the level of M206 LPS in that reaction mixture.

CHAPTER 2.8 Protein A binding assay

This assay was used to quantitate the specific IgG antibody bound to *S. typhimurium* M206 bacteria in reaction

mixtures. It followed the method described by Mathews and Minden (1979), but there were several modifications incorporated.

2.8.1 Radioiodination of protein A

The method of Bolton and Hunter (1973) was followed and is briefly described below. The ability of the protein A molecule to bind to IgG is believed to be dependent on four of its tyrosine groups (Sjoholm *et al.*, 1973). Longone *et al.*, (1977) stated that the Bolton-Hunter method of radio-iodination is preferrable to the Chloramine-T method because it labels lysine rather than tyrosine groups.

0.4 mCi of Bolton-Hunter reagent was evaporated under a gentle stream of dry nitrogen. A 0.5 mg per ml solution of protein A (Pharmacia Fine Chemicals, Uppsula, Sweden) was made up in phosphate buffer, pH 8.0. 0.2 ml was added to the Bolton-Hunter reagent. This mixture was left at 20^{o} for 15 minutes. 0.2 ml of an amino acid mixture was added. The mixture was left at 20^{o} for a further 30 minutes. 1.0 ml of borate buffer, pH 7.4, was added.

The final mixture was subjected to molecular-sieve chromatography on an 18 cm x 1 cm column of Sephadex G-25 (Pharmacia Fine Chemicals). Borate buffer was used to rinse the column through and 1.5 ml fractions were collected. 0.1 ml samples were assayed for radioactivity (cpm) in a Searle (model 1285) gamma-counter. Those fractions, constituting the first peak of radioactivity to emerge from the column, were pooled as the ^{125}I-protein A component.

2.8.2 The binding assay

This assay was performed in parallel with a complement-mediated bactericidal assay. They contained the same number of M206 cells and the same level of specific antibody.

Mg^{2+} saline was the diluent used for the first incubation mixtures. 0.3 ml of several dilutions of anti-M206 antiserum (or its fraction) and 2.4 ml of Mg^{2+} saline were added to glass tubes, on ice. 0.3 ml of a suspension of washed M206 cells (10^8 or 10^9 cells per ml) was then added. A control tube, containing only diluent and M206 cells, was included. All tubes were incubated at 37^{o} for 30 minutes.

The tubes were centrifuged at 4^{o} (1800 g for 15 minutes). They were carefully aspirated, using a pasteur pipette with a 90^{o} bend at the tip. 3.0 ml of borate buffer, pH 7.4, the diluent used in the remaining steps, was added to the deposits. The mixtures were centrifuged, washed and centrifuged again. All cell deposits were then suspended in 0.15 ml of buffer.

3 x 50 µl aliquots of the washed cell suspensions were added to 75 mm x 10 mm plastic tubes. 0.1 ml of suitably diluted ^{125}I-protein A was added to these tubes. Two further controls were included: (i) 0.1 ml of 20% w/v trichloroacetic acid (TCA), and (ii) 50 µl of buffer were tested with ^{125}I-protein A. All tubes were incubated at 37^{o} for 60 minutes. 0.5 ml of buffer was added to all bar the above control tubes: 0.5 ml of 20% TCA was added to the TCA control tube and the protein A control tube was merely placed on ice. The tubes were centrifuged at 4^{o}

(1800 g for 15 minutes) and the washing procedure was repeated twice more. After the final aspiration, the radioactivity of the cell deposits and of the control tubes was measured and recorded as cpm.

Specific binding and % specific binding figures, for each antiserum (or fraction) dilution, were as defined by Mathews and Minden (1979):

$$\text{specific binding} = \text{cpm EXPERIMENTAL tube} - \text{cpm M206 CONTROL tube}$$

$$\%\ \text{specific binding} = \frac{\text{cpm EXPERIMENTAL tube} - \text{cpm M206 CONTROL tube}}{\text{cpm TCA CONTROL tube} - \text{cpm M206 CONTROL tube}} \times 100$$

CHAPTER 2.9 Serum fractionations

2.9.1 Protein A affinity chromatography

The IgG fraction of NRS or rabbit antiserum (raised against *S. typhimurium* M206) was eluted in one step from a protein A - Sepharose 4B column. The method was similar to that of Goding (1976). The IgG fraction of the NRS then functioned as a standard IgG preparation. Stepwise elution of the IgG fraction of antiserum, as described by Ey *et al.*, (1978), was also performed. The column work was entirely done in a cold room (4^{o}).

(a) Gel preparation

Staphylococcal protein A, covalently bound to Sepharose CL-4B (protein A-Sepharose) was purchased from Pharmacia Fine Chemicals. 1.5 g was swollen in phosphate buffer, pH 8.0, overnight. The gel was packed into a 30 cm x 1 cm chromatography column. The bed volume was about 6 ml and the protein A content was 2 mg per ml of wet gel. Before use, the column was washed with citrate

buffer, pH 3.0, and then equilibrated with phosphate buffer, pH 8.0.

(b) One step IgG elution

2.0 or 3.0 ml of rabbit serum was diluted in phosphate buffer, pH 8.0, and was passed through the column. Phosphate buffer was used to wash the antiserum through and 2 or 3 ml fractions were collected. The washing was stopped when the protein level in the fractions (gauged by OD_{280nm} values) became insignificant. The fractions containing significant levels of protein were pooled as the pH 8 eluant.

Citrate buffer, pH 3.0, was then used to wash the column through. 2 or 3 ml fractions were collected in the same volume of Tris-HCl buffer, pH 8.0. The fractions containing significant levels of protein were pooled as the pH 3 eluant. This was expected to contain IgG immunoglobulin as the only protein. The pH was quickly raised to 7.0 with IN sodium hydroxide. The column was equilibrated with phosphate buffer, pH 8.0, for future use.

Both pH 3 and pH 8 eluants were dialysed against N-saline for at least 24 hours at 4^{o}. They were then stored at -73^{o} or concentrated to a known fraction of the whole serum volume, before storage. A PM30 ultrafiltration membrane (Amicon Corporation, Mass., USA) was used for the concentration.

(c) Stepwise IgG elution

2.0 ml of rabbit antiserum was diluted in 2.0 ml of phosphate buffer, pH 8.0, and was washed through the column

with this buffer. 2.5 ml fractions were collected and those with significant levels of protein were pooled as the pH 7 eluant.

The fractionation procedure was repeated with citrate buffers of pH 6.0, pH 5.0, pH 4.0 and pH 3.0. The pooled eluants from each wash were quickly neutralized with IN sodium hydroxide. The column was equilibrated with phosphate buffer, pH 8.0, for future use.

All pooled eluants were quickly neutralized with IN sodium hydroxide. They were then dialysed against N-saline (24 hours at 4^{o}). The dialysed eluants were stored at -73^{o}.

(d) IgG concentration in the standard preparation

The IgG level of the pH 3 eluant of NRS (from one step elution on the protein A-Sepharose column) was determined in two ways.

(i) Extinction coefficient method

The absorbance at 280 nm was measured in a standard cuvette (1.0 cm light path) and in a Perkin Elmer (model 124) spectrophotometer. The IgG concentration was then calculated, knowing that a 1% w/v suspension of rabbit IgG has an absorbance value of 13.6 ± 0.01, under the same conditions (Small and Lamn, 1966).

(ii) Sulphosalicylic acid method

This determines the concentration of total protein (Hoffman, 1964). 5% sulphosalicylic acid was mixed with a sample of the standard IgG preparation to precipitate the protein present. The OD_{640nm} value of the mixture was then compared to a standard plot of OD_{640nm} vs protein concentration.

2.9.2 IgM immunoglobulin fractionation

A two-step procedure was used to purify IgM immunoglobulins from a rabbit antiserum, collected early in a vaccination schedule. The first step involved molecular sieve chromatography and was performed at room temperature. The second step involved affinity chromatography to remove IgG and was performed at 4^{o}. It utilized the protein A-Sepharose column described in 2.9.1.

(a) Sephadex G-200 gel preparation

7 g of Sephadex G-200 gel (Pharmacia Fine Chemicals) was swollen in phosphate buffer, pH 8.0, for four days. The gel was then packed into a 40 cm x 2.5 cm chromatography column (Pharmacia) and phosphate buffer was used to equilibrate it. The even passage of a sample of 0.3% blue dextran (Pharmacia Fine Chemicals) through the gel was a test for even packing.

(b) The fractionation

2.0 ml of the rabbit antiserum was passed through the G-200 column. Phosphate buffer, pH 8.0, was used to wash the antiserum through and 40 x 5 ml fractions were collected. The protein level in these fractions were gauged by their OD_{280nm} values. Fractions, representing individual peaks in protein level, were pooled and stored at -20^{o}.

The pool, considered to contain the majority of IgM, was then passed through the protein A-Sepharose 4B column with phosphate buffer. 2 ml fractions were collected and the remainder of the procedure was as described in 2.9.1(b). After dialysis, the pH 3 and pH 8 eluants were concentrated

to 1 and 2 ml, respectively, using membrane filtration (B15 Minicon cell, Amicon Corporation). Both concentrated eluants were stored at -73°.

2.9.3 Immunochemical tests of purity

The various eluants, resulting from serum fractionation procedures, were tested for specific protein content. All procedures were modifications of micro-techniques described by Ouchterlony and Nilsson (Weir, 1973). Only the single radial immunodiffusion (SRID) test was quantitative.

All tests were performed on 75 mm x 50 mm glass slides. The slides were cleaned and placed on a horizontal platform. 7 ml aliquots of 1% agarose (A grade, Sigma Chemical Company, St. Louis, USA) in barbitone buffer, pH 8.2, were poured onto the slides. The gel was allowed to set.

Antisera used in the tests were as follows:

goat anti-rabbit γ-chain, (Gar/IgG(Fc), Nordic Immunological Labs, Tilburg, The Netherlands)

sheep anti-rabbit μ-chain (Sh AR/IgM, Nordic Immunological Labs)

goat anti-rabbit α-chain, (Gar/IgA(Fc), Nordic Immunological Labs)

swine anti-rabbit serum protein, (Dako-immunoglobulins, Copenhagen, Denmark).

After the tests had been completed, the precipitation patterns were developed with Coomassie brilliant blue, as described by Weeke (Axelsen *et al.*, 1973). Briefly, the slides were washed in 0.1 M saline for 3 hours. They were dried between filter paper and then in a 37° incubator for

60 minutes. The slides were then immersed in 0.5% Coomassie brilliant blue for one minute and destained in a mixture of acetic acid, ethanol and water. They were dried between filter paper, washed in 0.1 M saline for 15 minutes and again dried.

(a) Comparative double immunodiffusion (DID)

Peripheral wells (2 mm or 4 mm diameter) were punched in a hexagonal arrangement about a central well. The distance from the central well was usually 5 mm, but sometimes 10 mm. Samples of the antigen (whole rabbit serum or its fraction) and antisera specific for particular serum proteins were added to the wells. The antigen was usually added to the central well. The slide was then incubated at 37^{o} for 48 hours in a humid chamber.

(b) Immunoelectrophoresis

2 x 2 mm diameter wells were punched in an agarose slide. 3 x 2 mm wide troughs were also cut along the length of the slide so that each well was 5 mm distant from a trough. 5µl of antigen (whole rabbit serum or its fraction) was added to the wells. Filter paper was used to connect the slide to the electrode vessels of an electrophoresis tray (Helena Labs, Texas, USA). Barbitone buffer, pH 8.2, was poured into the vessels and ice was poured into the central compartment. A constant 30 volts of electricity was applied between the electrodes for 90 minutes.

The troughs were then cleared of gel. 100 µl of suitably diluted antisera were then added to each. The slide was incubated at 37^{o} for 48 hours in a humid chamber.

(c) Single radial immunodiffusion

15 µl or 20 µl of anti-rabbit γ antiserum was mixed with the melted agarose before it was poured onto the glass slide. Two rows of 2 mm diameter wells were punched in the agarose slide. 5 µl aliquots of various dilutions of the standard IgG preparation (see 2.9.1(b) and 2.9.1(d)) and of whole rabbit antiserum (or its fraction) were added to the wells. The slide was incubated at 37°C for 48 hours in a humid chamber.

The slide was stained and the diameter of the halo (precipitated antigen-antibody complex) about each well was recorded. A linear plot of IgG concentration (mg per ml) vs diameter^2 was made, using the values for the standard IgG preparation. The IgG concentration of the tested antiserum (or fraction) was then read off this plot.

CHAPTER 2.10 Purification of specific antibody

Three methods were employed to isolate specific antibody from antiserum raised against *S. typhimurium* M206. The first method used whole rabbit antiserum as the starting material. The last two used the IgG fraction (see 2.9.1(b)).

2.10.1 Adsorption to glutaraldehyde-fixed lipopolysaccharide

LPS from *S. typhimurium* C5 was fixed with glutaraldehyde, as described by Eskenazy *et al.*, (1975). The glutaraldehyde was added to a 1% suspension of the LPS in phosphate buffer, pH 7.2, as a final 2% v/v concentration. The mixture was incubated at room temperature for 60 minutes. The fixed LPS was then repeatedly washed in distilled water.

Specific antibody was obtained from whole antiserum

in the following way. 1.0 ml of antiserum was added to the suspension of glutaraldehyde-fixed LPS and the mixture was incubated at room temperature for 30 minutes. The LPS adsorbate was then washed in phosphate buffer three times. The supernatants with significant protein levels (gauged by OD_{280nm} values) were pooled. The LPS adsorbate was resuspended in 2.0 ml of cold citrate buffer at pH 3.0. After 5 minutes at 4^{o}, the suspension was centrifuged in the cold. The supernatant was quickly neutralized with 1N sodium hydroxide. Both this pH 3 eluant and the pH 7.2 eluant were dialysed against N-saline for 24 hours. They were then stored at -20^{o}.

2.10.2 Adsorption to glutaraldehyde-fixed C5 cells

Glutaraldehyde-fixed *S. typhimurium* C5 cells were prepared as follows. A 2 litre overnight broth culture of the bacterium was centrifuged at 2000 g for 10 minutes. The cells were washed twice in N-saline and finally resuspended in a 1% v/v aqueous solution of glutaraldehyde. The mixture was incubated at 37^{o} for two hours and then at 4^{o}, overnight. The fixed C5 cells were then exhaustively washed in phosphate buffer, pH 8.0, and stored at a concentration of 5×10^{11} cells per ml at 4^{o}.

These cells were used for the isolation of specific antibody from IgG fractions, as follows. 1.0 ml of packed cells were washed once with 1M propionic acid, pH 3.0, and then three times in TESA buffer, pH 8.2. The packed cells were then suspended in 2.0 ml of the IgG fraction of an anti-M206 antiserum, and incubated at 4^{o} for 60 minutes. Centrifugation (2000 g for 10 minutes), a wash in TESA

buffer and another centrifugation followed. The supernatants were pooled.

The pelleted adsorbate was resuspended in 2.0 ml of 1M propionic acid, pH 6.0, and left at 4^{o} or 37^{o} for 30 minutes. The suspension was then centrifuged and the supernatant was neutralized with 1N sodium hydroxide. The procedure was repeated using 1M propionic acid at pH 5.0 and at pH 3.0.

Each of the resulting supernatants were dialysed against N-saline at 4^{o} for 24 hours. They were then concentrated to 2 ml with a PM 30 ultrafiltration membrane (Amicon Corporation) and stored at -20^{o}.

2.10.3 Adsorption to LPS-sensitized sheep red blood cell stromata

200 mg of SRBC stromata (provided by Dr. Reynolds of the Department of Microbiology and Immunology, University of Adelaide) were washed three times in N-saline (centrifugations were at 2000 g for 10 minutes). The pellet was finally suspended in 10 ml of N-saline. 5 ml of a C5 LPS suspension in N-saline (4 mg per ml) was added. The mixture was slowly rotated at 37^{o} for 90 minutes. The sensitized stromata were then washed three times in TESA buffer, pH 8.0. They were mixed with 5 ml of glass beads of average diameter 2 mm and poured into a glass column (diameter 10 mm) with 1 ml of glass beads at the bottom. 1 ml of glass beads were also added on top.

3.0 ml of the IgG fraction of a rabbit antiserum was passed through the column. TESA buffer was used to wash the column through until the protein level in the eluate

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was insignificant. 1 M propionic acid, pH 3.0, was then used to wash the column through and 7-8 ml fractions were collected. Those with significant protein levels were pooled, neutralized and dialysed against N-saline at 4^{o} for 24 hours. The pooled eluate was concentrated to 3 ml with a PM30 ultrafiltration membrane (Amicon Corporation) and stored at -20^{o}.

CHAPTER 3 FUNDAMENTAL ASPECTS OF THE NEISSER-WECHSBERG PHENOMENON

CHAPTER 3.1 Preamble

The Neisser-Wechsberg phenomenon refers to a deviant effect observed in complement-mediated bactericidal systems. In particular, the presence of excess antibody inhibits a bactericidal reaction, which occurs readily with lower levels of the same antibody and under otherwise identical conditions. This chapter deals with experiments, designed to establish some fundamental properties of this phenomenon. The system used consisted of *S. typhimurium* bacteria, specific rabbit antiserum and complement from various sources.

From the first description of the NW phenomenon (Neisser and Wechsberg, 1901) onwards, various authors have suggested that a fluid-phase reaction is responsible. The explanation by Neisser and Wechsberg was made without a thorough understanding of the role of complement in the bactericidal reaction. They suggested that complement is deviated by excess antibody in the fluid-phase. The suggestion of complement deviation was taken up by later workers. Coombs (Muschel, 1965) thought that soluble bacterial antigen could be responsible but offered no evidence. Muschel *et al.*, (1969) claimed that fluid-phase anticomplementary factors were partly responsible, as the prozoning activity of antiserum was found to be enhanced by heat-inactivation of its complement (56^{o} for 30 minutes).

These workers and Normann *et al.*, (1972) both showed that target bacterial cells do participate in the NW phenomenon. Bacteria were sensitized with a prozoning

level of antiserum and then washed. These cells were not killed when subsequently incubated with complement. Bacteria that were first sensitized with higher (normally bactericidal) levels of antiserum were killed.

The work, described in this chapter, sought to establish the contributions of fluid-phase and cell surface interactions to the NW phenomenon. The fundamental experiment, in which sensitized bacteria were washed, was repeated in our system to confirm the earlier findings. The role of fluid-phase factors in the phenomenon was examined in several ways: (i) the effect of heat on the prozoning activity of an antiserum was determined, (ii) complement consumption, when the complement source was incubated with a prozoning level of antiserum, was measured, and (iii) the effect of preincubating an antiserum with the complement source, on the prozoning activity of that antiserum, was also determined.

In vivo studies of the NW phenomenon have not been reported. The possible effects on the immune response of a hyperimmune animal, when challenged with the bacterium, are thus unknown. It is important, however, to gauge whether the NW phenomenon does exist *in vivo*. The phenomenon assumes greater immunological importance if it does. Joos and Hall (1968) showed that fresh whole blood from hyperimmunized rabbits does not kill the target cells in a bactericidal assay. The present study used fresh hyperimmune antiserum as the diluent, the antibody source and the complement source in a similar experiment. For the NW phenomenon to occur *in vivo*, it must also occur under these conditions.

CHAPTER 3.2 RESULTS

A. Target cell involvement in the Neisser-Wechsberg phenomenon

3.2.1 The incorporation of bacteria, sensitized with prozoning levels of antiserum and then washed, in the bactericidal assay

A standard bactericidal assay was set up with *S. typhimurium* M206 cells (5×10^7) and various dilutions of a hyperimmune rabbit antiserum. The same number of cells were also presensitized with the same antiserum dilutions, washed once and then tested in a parallel bactericidal assay with HuC.

The results of one such experiment are shown in Table 3.1. There were prozoning and killing phases demonstrated with both pretreated and untreated M206 cells. 1:50 to 1:200 dilutions of antiserum displayed strong prozoning activity. The significantly higher % survival values with the untreated bacteria were probably due to additional nutrients, provided by the antiserum dilutions and present in the final reaction mixtures. The control tube, lacking antiserum and therefore these additional nutrients, showed a similar survival value as the pretreated bacteria in the prozoning situation. This tube also indicated that the complement source did not contain a bactericidal level of specific antibody.

The 1:1000 dilution of antiserum displayed significant prozoning activity with the pretreated cells but none with the untreated cells. The preincubation of M206 cells and antiserum, and the washing, seemed to enhance the prozoning

TABLE 3.1

The effect of using sensitized and then washed *S. typhimurium* M206 target cells on the prozone phenomenon

Final antiserum dilution	% survival of bacteria[a]	
	untreated bacteria[b]	pretreated bacteria[c]
1:50	324	219
1:100	446	160
1:200	197	183
1:1000	13	61
1:5000	11	13
Nil	226	NT[d]

(a) The mean value of bacteria added per tube = 3.2×10^7

(d) Not tested

A standard bactericidal assay[b], with various dilutions of heat-treated A-7 antiserum, 1.2 CH50 units of HuC-15 and 5×10^7 washed M206 cells, was begun from 4° instead of 37°. A parallel assay[c] was performed with M206 cells that were first incubated with the same antiserum dilutions (4° for 15 minutes) and then washed once in Mg^{2+} saline. These sensitized bacteria were resuspended, complement was added and the assay was begun.

activity of that antiserum rather than decrease it.

There was considerable but not efficient killing of the pretreated and untreated M206 cells at the 1:5000 antiserum dilution. The incomplete killing may have been the result of washing the cells in the cold and beginning the bactericidal assays in the cold. Bacteria are known to be most sensitive to the bactericidal action of complement when they are in the log-phase of growth (Davis and Wedgwood, 1965) but the majority of target cells must have been in a stationary-phase. The killing of the pretreated cells indicated that specific antibody had bound to the cells during the preincubation.

3.2.2 The effect of preincubating target bacteria and antiserum dilutions on the prozoning activity

The previous experiment indicated that preincubation of M206 cells and specific antiserum heightened the prozoning activity of that antiserum. However, the washing step after the preincubation removed serum factors, other than bound antibody. The following experiment retained these other serum factors by omitting the washing step. In this way, a more definitive statement on the effect, that such a preincubation had on the prozoning activity, could be made.

Log-phase M206 cells were preincubated with various dilutions of hyperimmune antiserum at 37^{o} for various times. HuC was then added and standard bactericidal assays were performed.

Table 3.2 clearly shows that the prozoning titre rose from 1:200 to 1:400, after a preincubation period of just

TABLE 3.2

The effect of preincubating target bacteria and antiserum dilutions on the prozoning activity

Final antiserum dilution	% survival of bacteria[a] preincubated for: 0 min	15 min	30 min	45 min
1:200	77	254	257	195
1:400	2	52	95	87
1:800	0	0	1	2
1:1600	0	0	0	0
1:3200	0	0	0	0
Nil[b]	100	90	73	63

(a) The mean value of bacteria added per tube = 6.2×10^3

(b) Control tube, lacking antiserum but containing HuC

5×10^3 log-phase M206 cells were added to four sets of C-8 antiserum dilutions in Mg^{2+} saline. The mixtures were incubated at 37^{o} for 0, 15, 30 and 45 minutes before 1.2 CH50 units of HuC-15 were added. Samples were immediately taken for viable counts, and standard bactericidal assays were continued over 60 minutes.

15 minutes. This type of experiment has been repeated several times showing a similar rise. The % survival value for the 1:400 dilution of antiserum also increased significantly between the 15 and 30 minutes preincubation periods. There was no significant change in prozoning activity between the 30 and 45 minutes preincubation periods.

Conditions, favouring the union of target bacteria and specific antibody in the absence of complement, heightened prozoning activity. This effect was apparent for up to 30 minutes.

The control tubes, lacking antiserum, showed decreased % survival values with increased preincubation periods. The target cells became more susceptible to the bactericidal action of the complement source, by preincubating them in Mg^{2+} saline. Colony counts at 0 minutes revealed that the target cells in these control tubes had not been killed during the preincubations; therefore non-lethal structural changes had occurred.

B. Fluid-phase involvement in the Neisser-Wechsberg phenomenon

3.2.3 The effect of heat treatment of antiserum on its prozoning activity

The role of fluid-phase interactions in the NW phenomenon was first investigated by determining the effect of heat treatment on the prozoning activity of an anti-M206 antiserum. It is common for antiserum to be heated at 56^{o} for 30 minutes to destroy its complement activity. It was

possible that this heat treatment could have generated fluid-phase factors that were at least partly responsible for the prozoning activity of the antiserum.

A suitable dilution of anti-M206 antiserum was heated at 56° for various times and then double-diluted. All dilutions were then tested in standard bactericidal assays with log-phase M206 cells and HuC.

The results (see Table 3.3) clearly showed that there was no significant effect on the prozoning activity, even after the initial antiserum dilution had been heated at 56° for 90 minutes. Prozoning titres were 1:800, 1:1600 and 1:800 for heat treatments lasting 0, 30 and 90 minutes, respectively. However, the % survival figures for the 1:1600 dilution all indicated some prozoning activity and did not differ markedly. Further dilutions all showed efficient killing.

There appeared to be no increase in the prozoning activity of an antiserum, after the heat-inactivation of complement components at 56° for up to 90 minutes.

3.2.4 Haemolytic complement consumption on incubation of prozoning antiserum with the complement source, used in bactericidal assays

This experiment investigated whether fluid-phase factors, originating from the hyperimmune antiserum, could deplete the available haemolytic complement in a prozoning reaction mixture. Various dilutions of an anti-M206 antiserum were incubated with absorbed and unabsorbed HuC at 37° for 60 minutes. The haemolytic complement activity, remaining in the mixtures, was titrated so that the % loss

TABLE 3.3

The effect of heating at 56° on the prozoning activity of antiserum specific for *S. typhimurium* M206

Final antiserum dilution	% survival of bacteria[(a)] with antiserum: unheated	heated for 30 min	heated for 90 min
1:800	179	132	181
1:1600	29	57	36
1:3200	2	2	2
1:6400	0	1	0
Nil	163	NT	NT

[(a)] The mean value of bacteria added per tube = 2.9×10^3

A 1:80 dilution of P-5 antiserum in Mg^{2+} saline was heated at 56°C for 0, 30 and 90 minutes. Doubling dilutions were then made up and tested in standard bactericidal assays with 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-14.

could then be calculated. A standard bactericidal assay, with similar dilutions of the antiserum and absorbed HuC, was performed in parallel.

Results of the bactericidal assay (see Table 3.4) indicated that the prozoning titre of the antiserum was 1:1600. This particular dilution showed a % survival value of over 50% while the 1:3200 dilution showed complete killing. The control tube, lacking antiserum, also showed efficient killing and this indicated a presence of bactericidal antibody in the dilution of HuC used.

The 1:200 and 1:800 dilutions of antiserum, included in the complement consumption assay, were thus prozoning dilutions. When unabsorbed HuC was used as the complement source, there was a small but similar loss of haemolytic activity with both prozoning and bactericidal dilutions of antiserum. A similar loss of activity occurred in the control tube, lacking antiserum, due only to experimental conditions. Therefore, there was no loss of haemolytic complement that could be attributed to the anti-M206 antiserum, when prozoning or bactericidal dilutions were incubated with the complement source.

Results were slightly different when the absorbed HuC was used. There were higher losses of haemolytic activity, ranging from 10% to 17%, that could not be attributed to just experimental conditions. The loss of haemolytic activity increased as the antiserum dilution was lowered. The nature of the complement source obviously influenced these results. Some bacterial components eg., LPS, probably remained in the HuC after the absorption with live M206 cells so that, on incubation with antiserum,

TABLE 3.4

Haemolytic complement consumption on incubation of various dilutions of prozoning antiserum with complement

Final antiserum dilution	% loss of haemolytic complement[(a)]		% survival of bacteria[(b)]
	unabsorbed HuC	absorbed HuC	
1:200	18	35	NT
1:400	NT	NT	247
1:800	18	33	266
1:1600	NT	NT	53
1:3200	18	28	0
Nil	15	18	5

(a) 4.0 CH50 units of HuC-12, both absorbed (live M206 cells; see 2.4.1) and unabsorbed, and various dilutions of heat-treated P-5 antiserum were incubated at 37^{o} for 60 minutes. The remaining haemolytic activity, in each 2.0 ml of reaction mixture, was measured by a haemolytic titration (see 2.4.7). The complement consumption was expressed as % loss of haemolytic activity.

(b) A standard bactericidal assay was conducted with 5×10^{3} log-phase M206 cells, various dilutions of heat-treated P-5 antiserum and 2.0 CH50 units of HuC-12 (absorbed) in 1.0 ml reaction volumes. The mean value of bacteria added per tube = 5.6×10^{3}.

the antigen-antibody complexes formed also activated a small fraction of the available complement. Note that even with the lowest dilution of antiserum tested (1:200), 1.3 CH50 units of HuC remained per ml of reaction mixture. A minimum of 0.6 CH50 units has repeatedly been shown to be required for the efficient killing of 5×10^3 M206 cells, under standard bactericidal assay conditions. Depletion of haemolytic complement in the fluid-phase could therefore not explain the prozoning activity of this dilution of antiserum, as a bactericidal level remained.

Retention of bacterial antigens in absorbed complement sources is an important consideration in studies such as this. Table 2.2 showed that there was a considerable loss of haemolytic activity when a complement source, absorbed with acetone-dried M206 cells, was incubated with dilutions of anti-M206 antiserum. This was probably due to a high retention of bacterial antigens, especially O-somatic antigens, in the complement source. The results in Table 3.4 constituted evidence to support this.

3.2.5 Bactericidal complement consumption on incubation of prozoning antiserum with the complement source, used in bactericidal assays

The previous section of work established that haemolytic complement was not lost, when a complement source and prozoning levels of antiserum were incubated under bactericidal assay conditions. The following experiments investigated whether bactericidal complement was lost under these conditions.

S. minnesota R5, a rough Rc strain derived from

S. minnesota 218S (Luderitz *et al.*, 1966) was used as the target bacterium. The parent strain (0:21,22) is serologically unrelated to *S. typhimurium* M206 (0:1,4,5,12). The minimum level of complement, required to efficiently kill 5 x 10^3 log-phase R5 cells, was first determined in a standard bactericidal assay. 0.125 CH50 units of the complement source (HuC-12) was found to be required to kill >90% of the target R5 cells, whether specific antiserum (anti-218S antiserum) was present or not. This meant that the complement source contained a bactericidal level of antibody, specific for the R5 cells. Anti-218S antiserum was therefore not required to provide this antibody and was omitted from the main experiment.

Levels of complement, close to the minimum required for efficient killing, were incubated with various dilutions of a hyperimmune rabbit antiserum, specific for *S. typhimurium* M206. A standard bactericidal assay was then performed, on the addition of log-phase R5 cells.

Table 3.5 shows that 0.125 CH50 units of complement did not kill the target cells, whether prozoning antiserum was included in the preincubation or not (the control tube). However, 0.25 CH50 units uniformly killed the R5 bacteria.

Some complement was lost during the preincubation due to the physical conditions to which it was exposed. Thus, when the minimum concentration required for killing (0.125 CH50 units) was added to the assay, the target cells survived. When 0.25 CH50 units were added, at least half of the complement activity remained after the pre-incubation and this was sufficient to kill the target cells.

1:200 and 1:800 dilutions of the anti-M206 antiserum,

TABLE 3.5

Bactericidal complement consumption on incubation of various dilutions of prozoning antiserum with complement

antiserum dilution	% survival of bacteria[a]	
	CH50 units of HuC added	
	0.25	0.125
1:200	6	114
1:800	2	105
1:3200	3	141
Nil	8	112

[a] The mean value of bacteria added per tube = 5.2×10^3

0.25 and 0.125 CH50 units of HuC-12 and various dilutions of heat-treated P-5 antiserum (reaction volume of 0.9 ml) were incubated at 37° for 60 minutes. A standard bactericidal assay was then performed on the addition of 5×10^3 log-phase *S. minnesota* R5 cells (0.1 ml).

used in the experiment, were known to have strong prozoning activity in the presence of 2 CH50 units of complement (see Table 3.4). From the first description of this prozone phenomenon (Neisser and Wechsberg, 1901), it has been known that prozoning activity is heightened by limiting complement concentrations. The two stated dilutions of anti-M206 antiserum could therefore be regarded as prozoning dilutions in this experiment, as 0.25 CH50 units were added. These prozoning dilutions did not remove even 0.125 CH50 units during the preincubation. This was an insignificant figure when one considered that 1.2 or more CH50 units were commonly used in bactericidal assays with *S. typhimurium* M206 cells.

3.2.6 The effect of preincubating the complement source and antiserum dilutions on the prozoning activity

The effect of preincubating M206 bacteria and antiserum dilutions on the prozoning titre of that antiserum was investigated in 3.2.2. An increase in prozoning activity was noted. The following experiment investigated the effect of preincubating the complement source and antiserum dilutions in a bactericidal assay.

In parallel experiments, two series of the same dilutions of anti-M206 antiserum were tested in standard bactericidal assays, with *S. typhimurium* M206 cells. However, one series of dilutions was preincubated with the complement source at 37^{o}, immediately before the target cells were added.

The results (see Table 3.6) showed that the prozoning titre of the antiserum was 1:800, whether it was

TABLE 3.6

The effect of preincubating the complement source and antiserum dilutions on the prozoning activity

Final antiserum dilution	% survival of bacteria[(a)]	
	preincubation omitted	preincubation included
1:400	206	211
1:800	77	111
1:1600	4	6
1:3200	1	1
Nil	not tested	4

(a) The mean value of bacteria added per tube = 7.2×10^3

2.0 CH50 units of HuC-12 and various dilutions of heat-treated P-5 antiserum (reaction volume of 0.9 ml) were incubated at 37^{o} for 30 minutes. 5×10^3 log-phase M206 cells (in 0.1 ml) were added to these mixtures and to an identical series, in which the complement source had just been added. Standard bactericidal assays then followed.

incubated with the complement source or not. The % survival value obtained with the 1:800 dilution was slightly higher, when the preincubation was included. A small fraction of the complement was possibly lost by the physical conditions of the preincubation. This would explain the slightly increased prezoning activity in this series.

To summarise, there was no significant effect on the prozoning activity of the antiserum, by preincubating it at various dilutions with the complement source. The preincubation afforded conditions that favoured fluid-phase interaction between the antiserum and the complement source. Therefore this type of interaction did not seem to be significant in the prozone phenomenon.

C. The *in vivo* occurrence of the Neisser-Wechsberg phenomenon

3.2.7 The prozoning activity of fresh hyperimmune anti-M206 antiserum

Most bactericidal assays performed in this study involved testing dilutions of heat-treated rabbit antisera and an independent complement source, ie., human or guinea pig serum. The following experiment investigated the prozoning activity of a hyperimmune rabbit antiserum, itself being the source of antibody and complement. It was an attempt to gauge the *in vivo* response of a hyperimmunized animal to challenge from the bacterium used in the vaccination.

The freshly-collected anti-M206 antiserum and also GpC were titrated for haemolytic complement activity

(see 2.4.7). The antiserum, with and without added GpC, was then tested in a standard bactericidal assay with M206 cells. The specific antibody in the antiserum was also titrated in a HA assay with C5 LPS-sensitized SRBCs (see 2.7.).

Table 3.7 shows that final antiserum dilutions of 1:1.1 to 1:50, with complement levels of 13.5 to 0.3 CH50 units, were unable to kill the M206 cells in one bactericidal assay. The HA titre of this antiserum (1:2560) proved that there was a high level of specific antibody present in the undiluted antiserum. The minimum level of HuC to kill 5×10^3 M206 cells in a standard bactericidal assay had been shown to be 0.6 CH50 units. Thus, the undiluted antiserum probably also contained a bactericidal level of complement.

The addition of GpC (18 CH50 units) in the second bactericidal assay confirmed that the "no-kill" situation, just described, constituted a prozoning situation, especially with the lower antiserum dilutions. Even with the added GpC, the antiserum had to be diluted out to 1:2000 before killing was evident. Complement was shown not to be the factor limiting the bactericidal reaction, because high levels were present in mixtures with low antiserum dilutions (eg., 30 CH50 units with 1:1.25) but they did not kill the target cells.

CHAPTER 3.3 DISCUSSION

The major question, that needed to be settled at the commencement of this study, was the relative importance of fluid-phase and cellular-phase interactions in the Neisser-

TABLE 3.7

The prozoning activity of fresh rabbit hyperimmune antiserum specific for *S. typhimirium* M206

Antiserum[a]		% survival of bacteria[b]	
Final dilution	CH50 units provided	No additional C	Additional GpC[c]
1:1.1	13.5	154	NT
1:1.25	12	NT	156
1:2	7.5	291	255
1:10	1.5	194	334
1:20	0.8	215	326
1:50	0.3	357	277
1:1000	0.1	NT	161
1:2000	0.1	NT	0

(a) HA titre 1:2560; haemolytic activity 15 CH50 units per ml

(b) The mean value of bacteria added per tube = 7.7×10^3

(c) 18 CH50 units

A sample of serum was collected from rabbit G;within a few hours, the complement level in this antiserum and absorbed GpC was determined by the haemolytic complement titration (see 2.4.7). The antiserum was tested for anti-M206 antibody in the HA assay (see 2.7.1).

The neat antiserum and various dilutions in Mg^{2+} saline were tested in a standard bactericidal assay, with 5×10^3 log-phase M206 cells and in 1.0 ml reaction volumes. In a parallel assay, another series of the same antiserum dilutions was tested with a final 1:20 dilution of GpC.

Wechsberg phenomenon. A detailed investigation of the factors operating in the phenomenon could then proceed.

The results, presented in this chapter, firmly supported the central role of a cell surface interaction. The union of specific antibody to target bacterial cells was shown to be fundamental to the NW phenomenon. *S. typhimurium* M206 cells, incubated with dilutions of hyperimmune rabbit antiserum and then washed, still displayed the prozone phenomenon in the presence of a bactericidal level of complement (see Table 3.1). The eventual killing phase showed that specific antibody had bound to the target cells, during the preincubation period. These results are in agreement with those of other workers (Daguillard *et al.*, 1968; Muschel *et al.*, 1969; Normann *et al.*, 1972). The sensitization and washing procedure slightly increased the prozoning activity rather than reduced it. This indicated that the specific antibody involved in the NW phenomenon possessed high avidity. The work of Normann *et al.*, (1972) supports this observation, but Muschel *et al.*, (1969) reported a reduced prozoning activity after the washing procedure. The differing conditions of the sensitization and washing procedures or differing avidity of specific antibody may explain the conflicting results.

The results of another experiment indicated that experimental conditions, favouring the efficient binding of specific antibody to the target bacteria, enhanced the prozone phenomenon. Hyperimmune antiserum displayed a higher prozoning activity when preincubated with M206 cells, prior to the addition of complement in a bactericidal

assay (see Table 3.2). On the other hand, preincubation of the antiserum with the complement source had no effect on the prozoning activity (see Table 3.6). This indicated that interactions between the hyperimmune antiserum and the complement source ie., fluid-phase interactions, were relatively unimportant in the NW phenomenon.

Muschel *et al.*, (1969) postulated that fluid-phase interactions played an important role in the NW phenomenon. They claimed that hyperimmune antiserum, especially when heated to inactivate complement, contains anticomplementary factors that deplete bactericidal complement in the prozoning situation; they observed an increase in the prozoning activity of hyperimmune antiserum when heat-treated and a consumption of haemolytic complement in the prozoning situation. Sera with elevated γ-globulin levels, eg., hyperimmune antisera, have been known to be anticomplementary for some time (Davis *et al.*, 1944). Nørgaard (1955) associated this property with the γ-globulin component. Henny and Stanworth (1965) demonstrated the aggregation of isolated IgG, when this was heated at 56^{o}. Soltis *et al.*, (1979) directly attributed an anti-complementary factor, that formed on heating sera (particularly those with elevated IgG levels) at 56^{o}, to aggregated IgG.

Results, presented in this chapter, indicated that such serum anticomplementary factors played no significant role in the NW phenomenon. There was negligible consumption of bactericidal (see Table 3.5) or haemolytic (see Table 3.4) complement activity when the complement source was incubated with prozoning dilutions of heat-treated

antiserum. Anticomplementary factors in the antiserum therefore did not significantly inactivate the complement source. The heating of antiserum at 56° for up to 90 minutes did not affect the prozoning activity of that antiserum. It seems possible that the reported increase, by Muschel *et al.*, (1969) was artifactual. They showed efficient killing (93%) and no killing of *S. typhi* cells with an initial 1:5 dilution of unheated and heat-treated (56° for 30 minutes) rabbit antiserum, respectively. The next dilution of antiserum tested (1:25) showed similar incomplete killing values, while subsequent dilutions (1:125 and 1:625) showed efficient killing. It seems that the additional complement, supplied by the initial dilution of rabbit antiserum, and not anticomplementary factors in the heated antiserum, may have been responsible for the difference in killing with the initial dilution of antiserum. The addition of complement has been known to abrogate the NW phenomenon since the discovery of the latter (Neisser and Wechsberg, 1901). This explanation was supported by the partial killing seen with the next dilution of unheated antiserum (1:25), as less rabbit complement was provided and therefore seemed to be unable to abrogate the partial prozoning state.

Coombs (Muschel, 1965) suggested that soluble bacterial antigen plays an important role in the NW phenomenon. He did not provide experimental evidence but suggested that the antigen fixes specific antibody and complement in a prozoning mixture. Complement is thus deviated from the bacterial cells, in this scheme. The soluble antigen may originate from the bacterial culture in the assay or from

the complement source, which is commonly absorbed with acetone-dried or live bacteria. The bactericidal assays reported, in the present study, were performed with 1:10,000 dilutions of two hour broth cultures of Salmonellae or with washed suspensions of such cultures. It therefore seems unlikely that sufficient soluble bacterial antigen was present in assay mixtures to have deviated complement in this way. Further, we showed that the complement source did not contain significant levels of bacterial antigen, provided it was absorbed with washed, live bacteria (see Table 2.2).

Table 3.7 showed that a freshly-collected hyperimmune rabbit antiserum was unable to kill target M206 cells in a standard bactericidal assay, in which the antiserum acted both as a complement and antibody source. A dilution of guinea pig serum, with a similar haemolytic complement level, was able to kill the target cells only with high dilutions of the antiserum. The inability of the whole antiserum to kill the target cells therefore seemed to be due to the NW phenomenon. It also gave a strong indication that the phenomenon does exist *in vivo*, in undiluted plasma. Muschel *et al.*, (1969) doubted whether the phenomenon could exist *in vivo* because of the high level of complement in undiluted plasma. Joos and Hall (1968), however, showed that fresh whole blood from hyperimmunized rabbits was unable to kill *Br. abortus* cells in an *in vitro* assay. This correlated with the existence of prozoning activity in the serum, demonstrated in a normal bactericidal assay system with an extrinsic complement source. Thus, experimental evidence suggests the NW phenomenon to be an *in vivo* phenomenon also.

The work, so far reported, establishes the importance of bacteria-antibody interactions ie., cell surface interactions, in the NW phenomenon. The next chapter presents results of the investigations into the types of antibody involved and their immunological properties.

CHAPTER 3.4 SUMMARY

3.4.1 The interactions between bacterial cells and specific antibody are an important component of the Neisser-Wechsberg phenomenon. Bacterial cells, when sensitized and then washed, displayed this phenomenon when subsequently incubated with complement. The higher prozoning activity, when antiserum was preincubated with the target cells, was further supportive evidence.

3.4.2 Fluid-phase interactions ie., interactions between antiserum and complement leading to loss of the latter, do not play a significant role in the NW phenomenon. Heat treatment of antiserum or preincubation of antiserum with the complement source did not affect the prozoning activity. There was also no significant loss of bactericidal or haemolytic complement activity when prozoning dilutions of antiserum were incubated with complement, under otherwise normal bactericidal assay conditions.

3.4.3 The *in vivo* occurrence of the NW phenomenon was supported by *in vitro* studies. Whole fresh hyperimmune antiserum, acting both as a complement and antibody source, displayed the phenomenon in a standard bactericidal assay.

CHAPTER 4 CHARACTERIZATION OF THE IMMUNOGLOBULIN INVOLVED IN THE NEISSER-WECHSBERG PHENOMENON

CHAPTER 4.1 Preamble

The central role that specific antibody plays in the Neisser-Wechsberg phenomenon was established in Chapter 3. The phenomenon occurs because of the interaction of bacterial cells and specific antibody, derived from a hyperimmune antiserum. The results to follow in this Chapter concern the nature of this antibody, termed prozoning antibody.

Neisser and Wechsberg (1901) recognized the need for a prolonged vaccination programme: prozoning activity was more evident in hyperimmune antisera than in early antisera. This has been accepted or shown by other workers, although an extensive periodic assessment of the humoral immune response during a vaccination programme has not been undertaken. The results of such a study, on two rabbits, will follow in this Chapter.

The appearance of the NW phenomenon in hyperimmune antiserum correlates with its appearance in the sera of human patients, during the convalescent phase of Gram-negative bacterial infections (Thomas *et al.*, 1943; Hall, 1950; Waisbren and Brown, 1966; Gower *et al.*, 1972; Griffiss, 1975). This indicates that prozoning antibodies may be of the IgG or IgA class, or both. Specific IgM antibodies generally peak early in response to vaccination or infection, whilst specific IgG and serum IgA antibodies peak later.

Two groups of workers (Daguillard and Edsall, 1968; Muschel *et al.*, 1969) have directly associated prozoning activity in the bactericidal reaction with both IgM and IgG antibodies. Others have associated it with only IgG antibody (Normann, 1972; Taylor, 1972) or only IgA antibody (Hall *et al.*, 1971; Griffiss, 1975; Griffiss and Bertram, 1977). IgA and IgG antibodies may participate in the NW phenomenon by binding to the target bacterial cells and inhibiting complement fixation. There is considerable evidence to suggest that IgA antibody cannot fix complement in cytolytic reactions (Ishizaka *et al.*, 1965; Eddie *et al.*, 1971; Knop *et al.*, 1971; Colten and Bienenstock, 1974; Heddle *et al.*, 1975). Russell-Jones *et al.*, (1980) also showed that specific IgA antibody can inhibit the complement-mediated lysis of sheep red cells (SRBCs) sensitized with IgG antibody. Some IgG subclasses of various mammalian species are also known not to fix complement eg., human IgG_4 (Ishizaka *et al.*, 1970; Spiegelberg, 1974) and mouse IgG_1 (Klaus *et al.*, 1979; Ey *et al.*, 1979; Ey *et al.*, 1980).

The second part of this chapter deals with the results of serum fractionation protocols, applied to antisera with high prozoning activity. This study sought to establish whether prozoning activity was restricted to a particular class or subclass of immunoglobulin, and whether this class or subclass could interfere with the bactericidal action of other classes, eg., IgM.

CHAPTER 4.2 Results

A. The immune response during an immunization schedule

4.2.1 Bactericidal and haemagglutinating antibody profiles of rabbits vaccinated with *S. typhimurium* M206 cells

Two rabbits (C and A) were vaccinated over 7 weeks with heat-killed *S. typhimurium* M206 cells (see 2.3.2(b)). Sera, collected at 7 day intervals, were tested for specific antibody levels by the standard complement-mediated bactericidal assay and the HA assay with C5 LPS-sensitized SRBCs. Further details and the results are listed in Tables 4.1 and 4.2.

There were differences in the immune responses of the two rabbits, but there were also important similarities. Bactericidal titres quickly rose from 1:100 on day 0 to 1:10,000 on day 7, for both rabbits. The bactericidal activity then remained relativley static for both rabbits, even 85 days after the last vaccination for one rabbit (A). The other rabbit (C) died during this period and so a parallel observation could not be made.

The patterns of the prozoning activity did not follow those of the bactericidal activity. Prozoning activity was significantly higher for one rabbit (A). The activity had not significantly dropped on day 56 - 14 days after the last vaccination - but the titre then dropped from 1:1600 to 1:200 on day 127, for that rabbit (A). Prozoning activity took longer to appear and then peak than bactericidal activity but the decline, on cessation of the vaccinations, was more abrupt. Repeated vaccinations seemed to be essential for the development of high prozoning activity.

TABLE 4.1

The immune response of rabbit A to vaccination with heat-killed *S. typhimurium* M206 cells

Serum No	Day	Prozoning titre	Bactericidal titre	Haemagglutinating titre without 2ME	Haemagglutinating titre with 2ME
1	0	<1:10	1:100	<1:10	<1:10
2	7	<1:10	1:10,000	1:20	<1:10
3	14	1:10	1:100,000	1:80	<1:10
4	21	1:800	1:100,000	1:640	1:80
5	28	1:3200	1:10,000	1:1280	1:320
6	35	1:3200	1:10,000	1:1280	1:640
7	42(a)	1:1600	1:10,000	1:1280	1:640
8	56	1:1600	1:10,000	1:320	1:80
9	127	1:200	1:10,000	1:80	1:20

(a) last vaccination administered

Two-fold and ten-fold dilutions of heat-treated samples of rabbit sera, collected during a vaccination schedule with 10^8 heat-killed M206 cells, were tested in standard bactericidal assays. 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-13 were included. These assays established prozoning and bactericidal titres for the sera.

Heat-treated samples of the sera were tested in the HA assay ci C5 LPS-sensitized SRBCs, in the presence and absence of 2ME (see 2.7.1). Absorption of the sera with unsensitized SRBCs was included.

TABLE 4.2

The immune response of rabbit C to vaccination with heat-killed *S. typhimurium* M206 cells

Serum No	Serum Day	Prozoning titre	Bactericidal titre	Haemagglutinating titre without 2ME	Haemagglutinating titre with 2ME
1	0	<1:10	1:100	<1:10	<1:10
2	7	1:10	1:10,000	1:160	<1:10
3	14	1:50	1:100,000	1:320	1:20
4	21	1:200	1:100,000	1:1280	1:40
5	28	1:400	1:100,000	1:640	1:80
6	35	1:400	1:100,000	1:640	1:80
7	42[(a)]	1:400	1:100,000	1:640	1:80
8	56	1:200	1:100,000	1:320	1:80

(a) last vaccination administered

Two-fold and ten-fold dilutions of heat-treated samples of rabbit sera, collected during a vaccination schedule with 10^8 heat-killed M206 cells, were tested in standard bactericidal assays. 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-16 were included. These assays established prozoning and bactericidal titres for the sera.

Heat-treated samples of the sera were tested in the HA assay with C5 LPS-sensitized SRBCs, in the presence and absence of 2ME (see 2.7.1). Absorption of the sera with unsensitized SRBCs was included.

The patterns of the prozoning activity related better to the patterns of the HA activity. The latter rose gradually for one rabbit (A), but more rapidly for the other (C); there was a similar trend with the prozoning activity. HA activity peaked on day 28 (rabbit A) and day 21 (rabbit C). Titres remained steady until vaccinations were halted. They then dropped between days 42 and 56, for both rabbits, and dropped further between days 56 and 127, for the surviving rabbit (A). Although peak HA titres were similar for both rabbits, the prozoning activity was significantly higher for the surviving rabbit during this period.

The patterns of prozoning activity correlated best with 2ME-resistant HA activity. Both activities were first detected at the same time, for both rabbits. Both activities peaked at the same time; peak activities were significantly higher for one rabbit (A). There was a significant drop in both activities after the last vaccination, especially by day 127.

IgM immunoglobulin is denatured by 0.1 M 2ME. IgG and IgA immunoglobulins are not denatured and so constitute 2ME-resistant antibody. Specific IgG and IgA antibodies generally appear and peak later than IgM antibodies in the immune response. This was also the case with the prozoning activity. For these reasons, prozoning activity seemed to be associated with specific IgA or IgG antibody, or both. Serum fractionation studies were required to further explore these possibilities.

B. Hyperimmune antiserum fractionation studies

4.2.2 Prozoning activity of the IgG fraction

(a) Fractionation details

A hyperimmune rabbit antiserum, specific for *S. typhimurium* M206, was chromatographed on a Sepharose column (see 2.9.1(b)). Further details of the method and the elution profile are shown in Fig. 4.1. Both the resulting pH 3 and pH 8 eluants contained high concentrations of protein, according to the OD_{280nm} values. Before the pH 3 eluant could be said to represent the IgG fraction of this antiserum, it needed to be tested for IgG concentration and purity. The pH 8 eluant and the whole antiserum also needed to be tested for IgG concentration and the presence of other immunoglobulins.

(b) The immunochemical testing of fractions

The antiserum and its fractions were first tested for immunoglobulin class content by the comparative DID method (see 2.9.3(a)). Precipitin lines in Fig. 4.2A and 4.2B indicated that the antiserum and its pH 8 eluant contained IgG and IgM immunoglobulins plus at least one other protein. The concentration of IgG in the pH 8 eluant seemed to be low because the precipitin line, formed with the anti-rabbit γ-chain antiserum, was thin and faint. The pH 3 eluant seemed to contain a high level of IgG but no IgM as a thick precipitin band was formed with only that antiserum (see Fig. 4.2C). Note that IgA was not detected on any of the three slides in Fig. 4.2. This was due to the low potency of the anti-rabbit γ-chain antiserum used. Another batch was subsequently purchased.

FIGURE 4.1

Elution profile of a hyperimmune rabbit antiserum on a protein A-Sepharose column

The IgG fraction was purified from S-6 antiserum, by adsorption of the serum to a protein A-Sepharose column at pH 8.0 and elution at pH 3.0. 2.0 ml of antiserum was diluted in 2.0 ml phosphate buffer, pH 8.0, and applied to the column. This was washed first with phosphate buffer and then with citrate buffer, pH 3.0. 2 ml fractions were collected every 5 minutes; those eluted at pH 3.0 were collected in tubes containing 2.0 ml of Tris-HCl buffer, pH 8.0. The optical density at 280 nm (OD280) was measured, after which fractions were pooled as indicated (pH 8, pH 3 eluants) and dialysed. An aliquot of each was then concentrated.

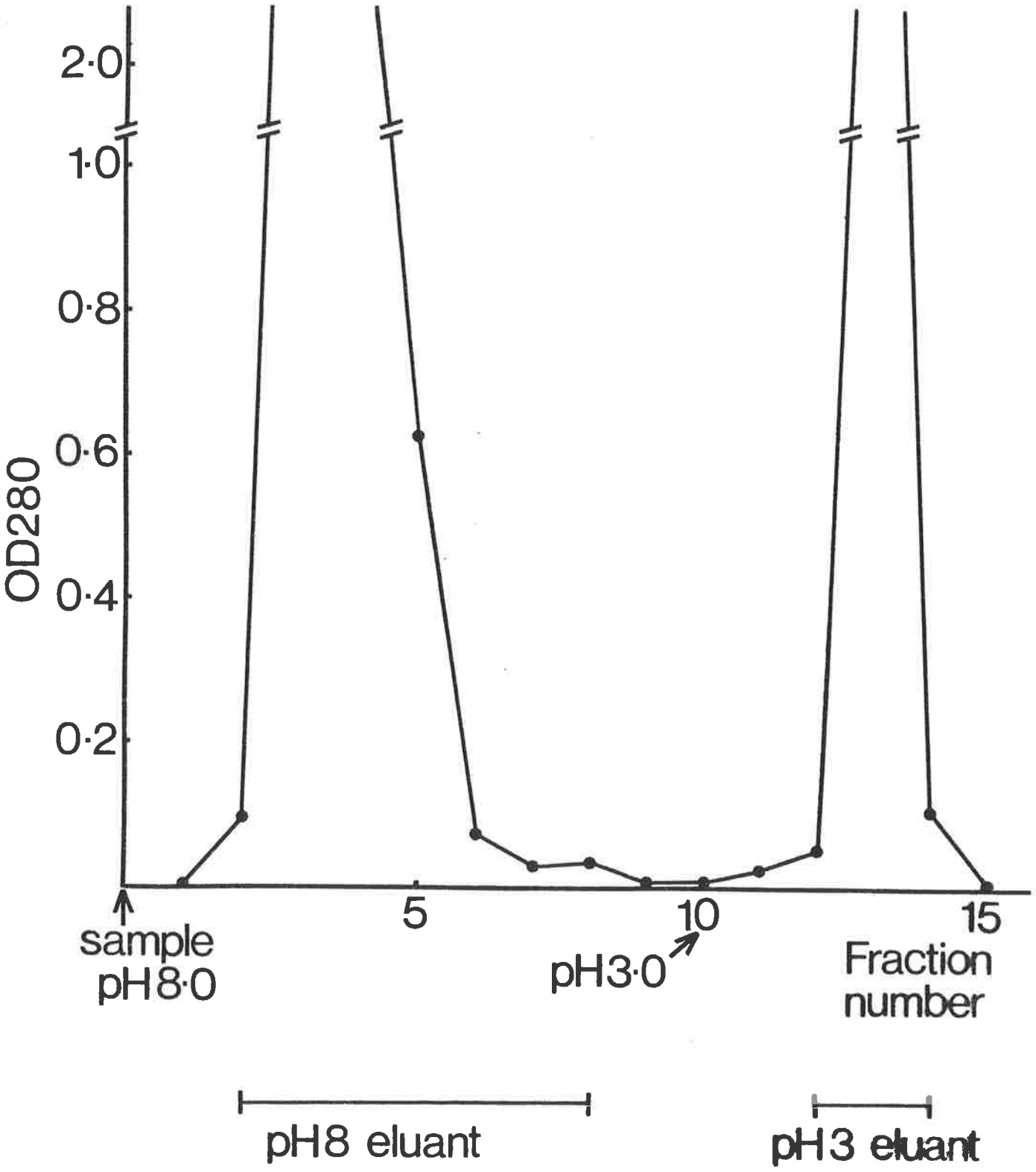
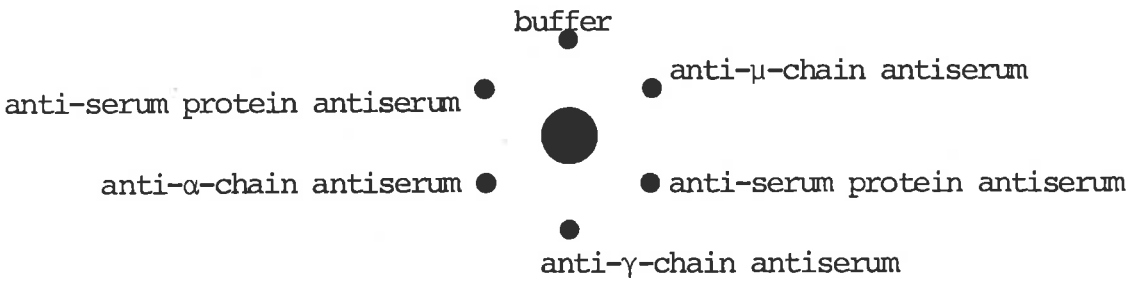
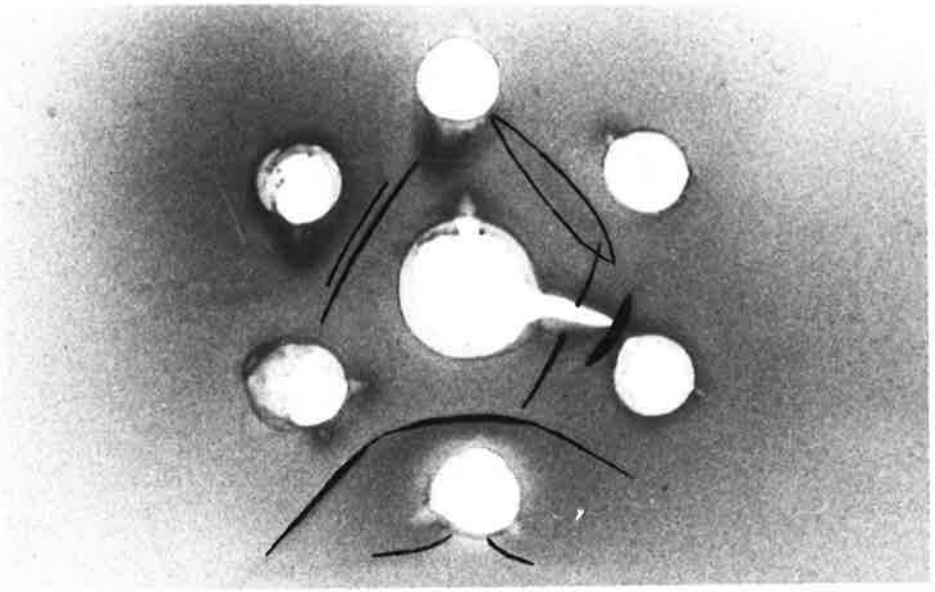

2·0
1·0
0·8
0·6
0·4
0·2
OD280
5
10
15
sample
pH8·0
pH3·0
Fraction
number
pH8 eluant
pH3 eluant

FIGURE 4.2

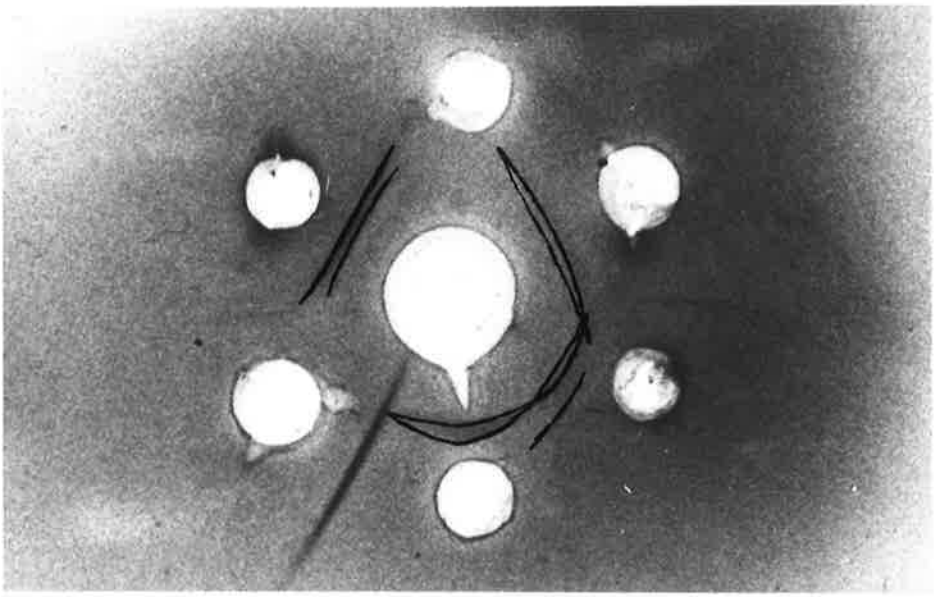
Comparative double immunodiffusion patterns of a hyperimmune rabbit antiserum and the eluants resulting from protein A-Sepharose chromatography



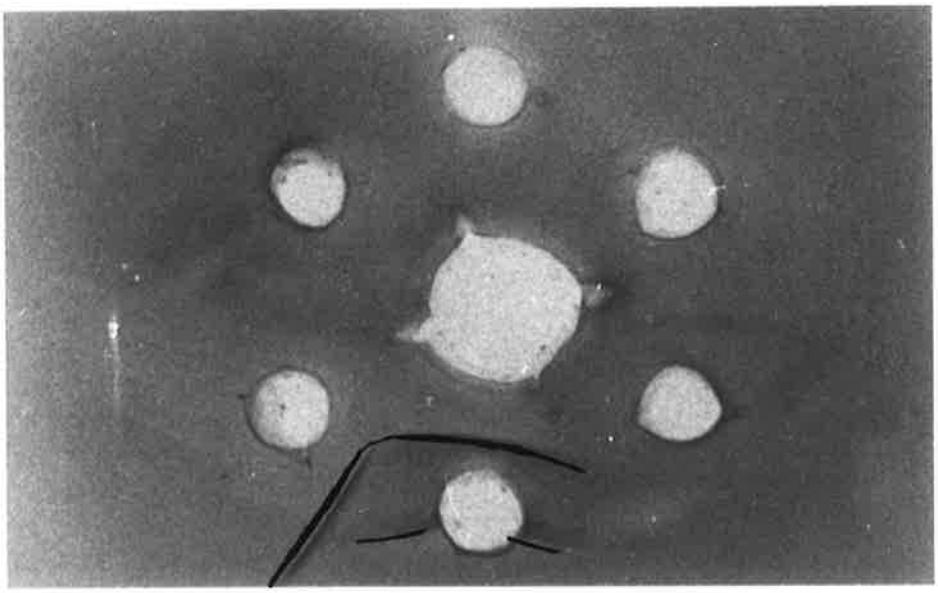
Samples of the whole S-6 antiserum (10 μl) and the two eluants (15 μl) were tested for immunoglobulin content by comparative DID. The central well (4 mm diameter) contained these samples (antiserum-A, pH 8 eluant-B, pH 3 eluant-C) whilst the peripheral wells (2 mm diameter) contained 5 μl of the commercial antisera or buffer.



A



B



C

It was tested with the hyperimmune rabbit antiserum and its two eluants. Fig. 4.3 shows that the rabbit antiserum and the concentrated (2/5 the original antiserum volume) pH 8 eluant contained measurable IgA. This figure also shows that the pH 3 eluant, even when concentrated to ¼ the original antiserum volume, contained no measurable IgA.

Comparative DID tests therefore showed that the pH 3 eluant of the hyperimmune rabbit antiserum, chromatographed on a protein A-Sepharose column, contained most of the IgG immunoglobulin. It contained no other measurable serum protein. The pH 8 eluant contained all measurable IgM and IgA, plus a small fraction of the serum IgG.

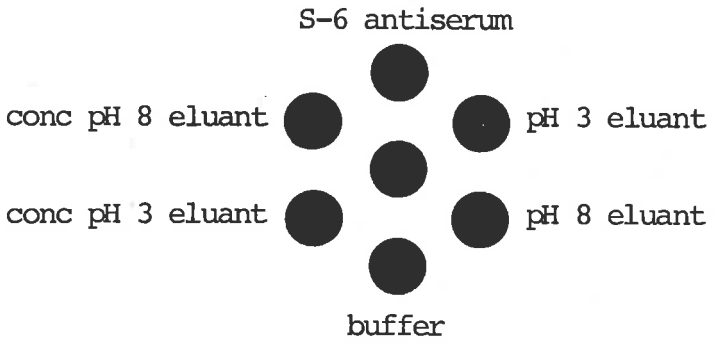
The protein content of both eluants was also analysed by IEP (see 2.9.3(b)). Fig. 4.4A shows that the pH 3 eluant formed only one thick arc of precipitation and that was with the anti-rabbit γ-chain antiserum. This result again indicated that this eluant contained a high level of IgG, but no other serum protein. The pH 3 eluant showed no arc with the anti-serum protein antiserum; the latter probably contained antibody specific for rabbit IgG in too low a concentration.

The pH 8 eluant formed a number of arcs of precipitation with the anti-rabbit serum protein antiserum and also a faint arc with the anti-rabbit γ-chain antiserum (see Fig. 4.4A). These results indicated that the pH 8 eluant contained the bulk of the serum proteins of the chromatographed antiserum, but only a small fraction of the IgG.

The IgG concentration of the antiserum and its two

FIGURE 4.3

Comparative double immunodiffusion patterns of the hyperimmune antiserum and its fractions with antiserum specific for rabbit IgA



Samples of the whole S-6 antiserum (10 µl), the two eluants (15 µl) and the concentrated eluants (15 µl) were tested for IgA content by comparative DID. The central well (4 mm diameter) contained 10 µl of anti-rabbit α-chain antiserum and the peripheral wells (4 mm diameter) contained S-6 antiserum and its eluants.

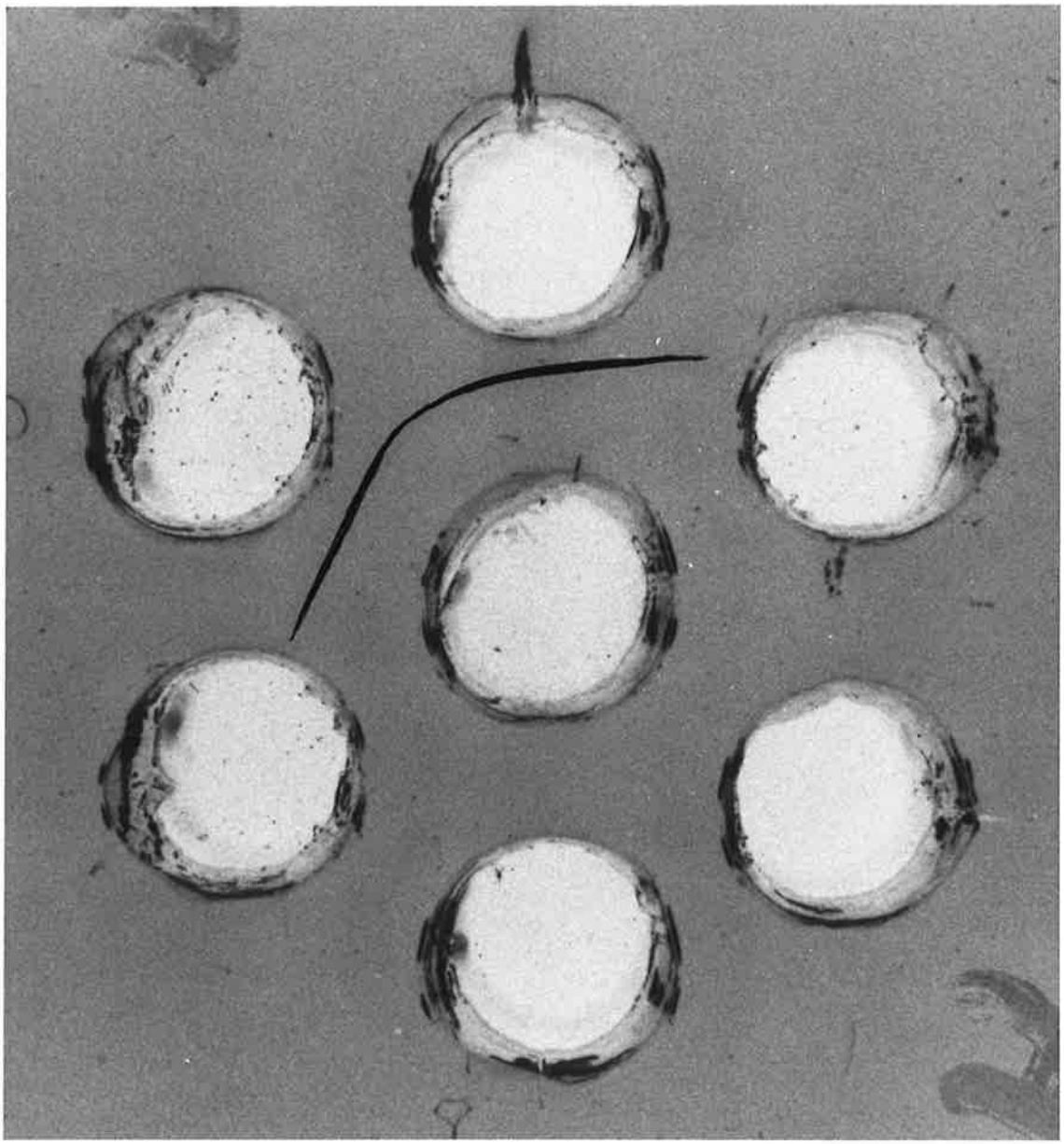
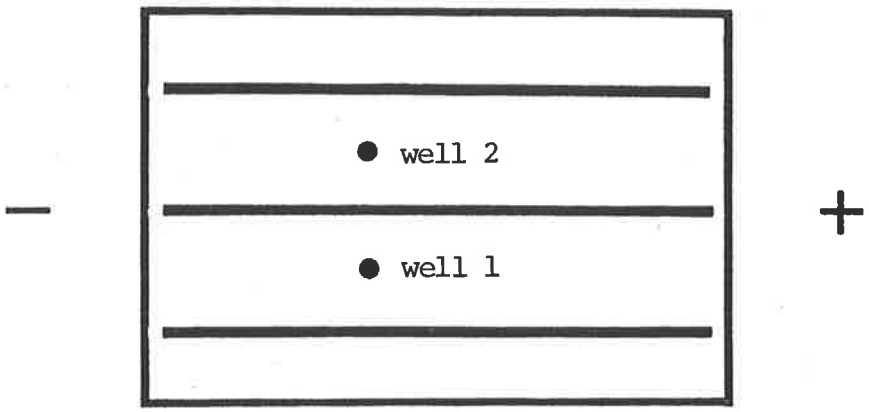
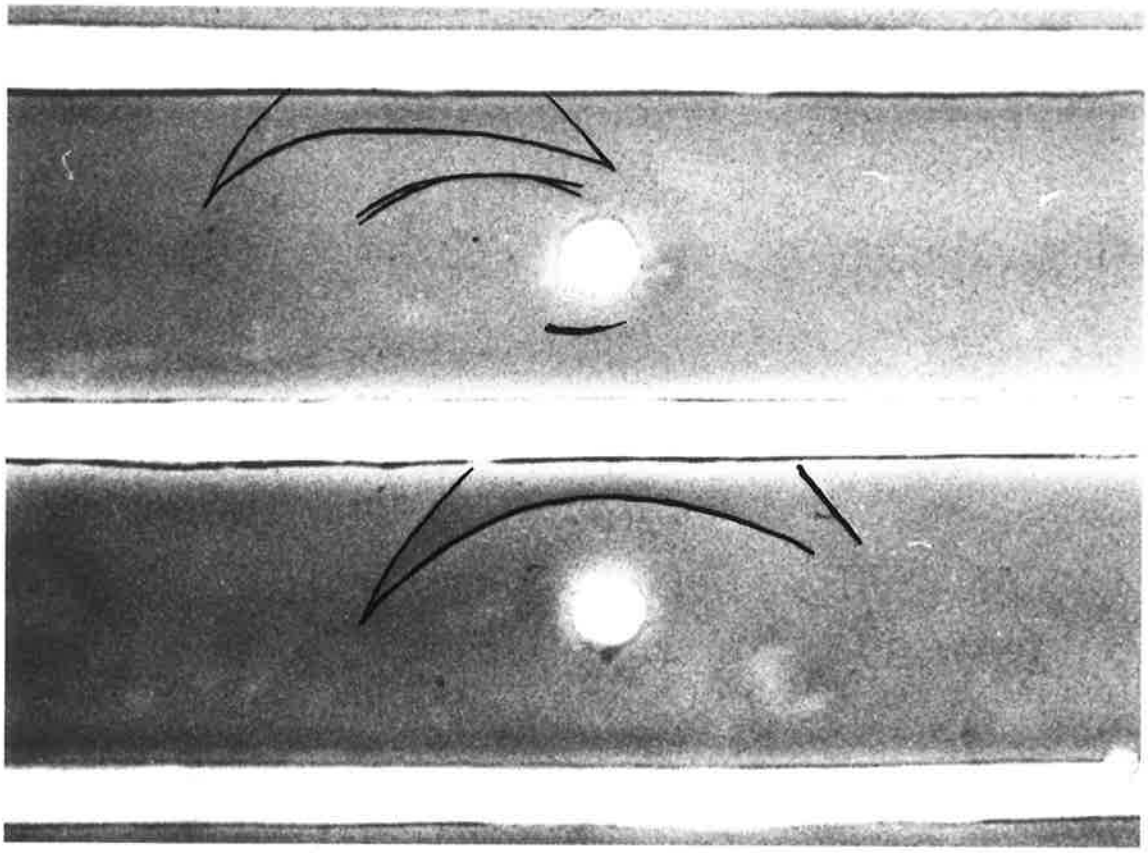


FIGURE 4.4

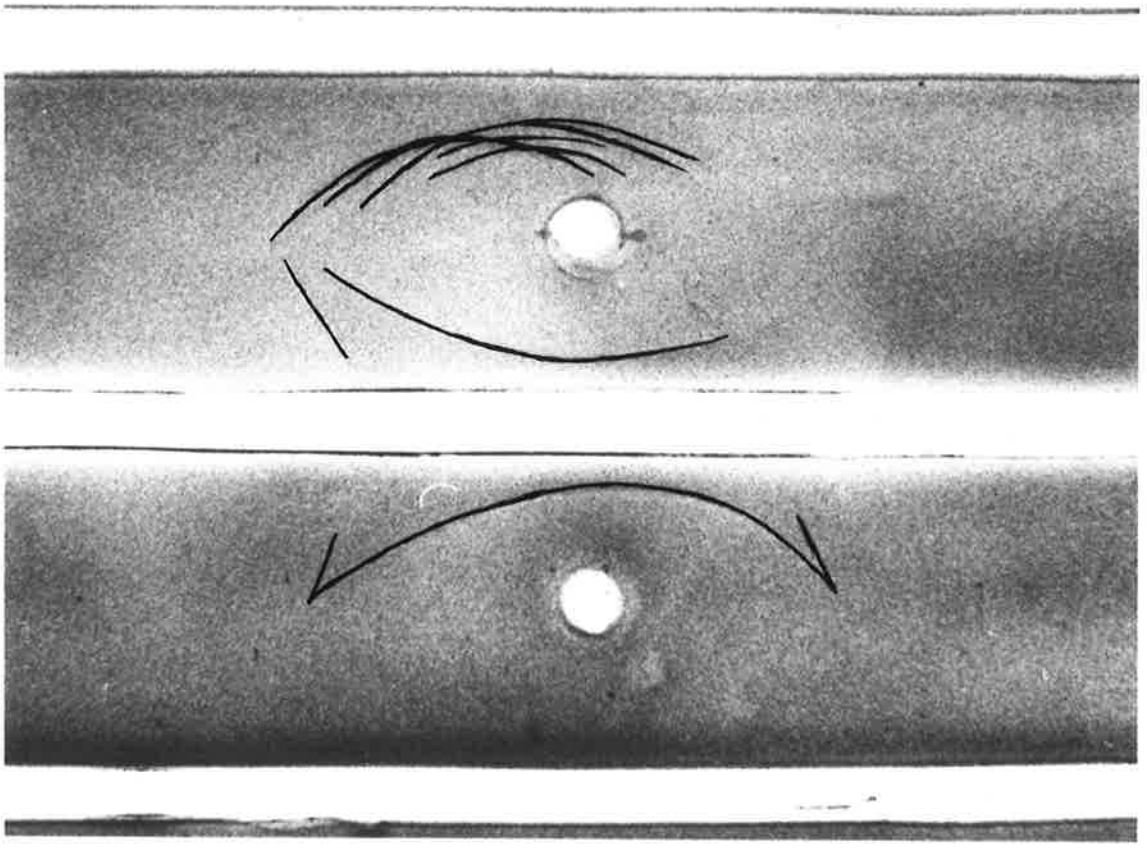
Immunoelectrophoretic patterns of eluants, derived from the hyperimmune rabbit antiserum, and of the standard IgG preparation



The protein content of the eluants (A), resulting from the protein A-Sepharose chromatography of S-6 antiserum, and of the standard IgG preparation (B) was determined by IEP. Samples of the pH 3 eluant and the standard preparation were loaded into well 1, whilst samples of the pH 8 eluant and the whole serum, from which the standard preparation was derived, were loaded into well 2. After electrophoresis, anti-rabbit serum protein antiserum was placed in the outer troughs while anti-rabbit γ-chain antiserum (1:16) was placed in the middle trough.



A



B

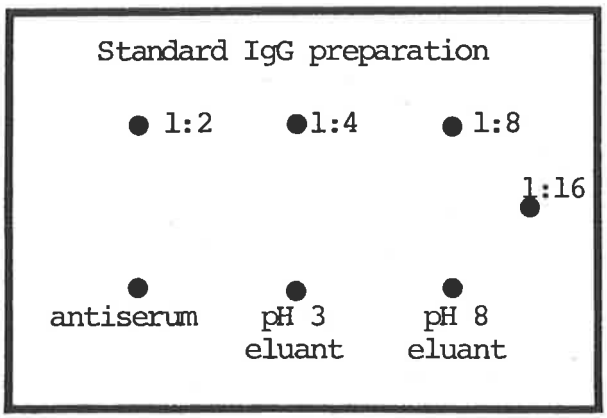
fractions was determined by SRID (see 2.9.3(c)). A standard IgG preparation, with a known IgG concentration, was utilized. This preparation consisted of the pH 3 eluant of a normal rabbit serum, chromatographed on the protein A-Sepharose column (see 2.9.1(b)). IEP showed that it contained IgG as the only serum protein (see Fig. 4.4B). The concentration of IgG was calculated to be 2.6mg per ml by the extinction coefficient method and 3mg per ml by the sulphosalicylic acid method (see 2.9.1(d)). The first figure was chosen to represent the IgG concentration, as the extinction coefficient method is the more accurate one.

Dilutions of the standard IgG preparation were tested with various dilutions of the hyperimmune antiserum and the two eluants in the SRID test. The resulting haloes of precipitation are shown in Fig. 4.5. A linear plot of IgG concentration versus $(\text{diameter})^2$ for the standard preparation then allowed the IgG concentrations of the three unknowns to be calculated.

The average values of IgG concentration were thus 1.8mg per ml for the pH 3 eluant and 12.0 mg per ml for the whole antiserum. The 12 ml of the pooled pH 3 eluant therefore contained 21.6mg of IgG. The 2.0 ml of antiserum, chromatographed on the protein A-Sepharose column, contained 24.0mg and so about 90% of IgG was retrieved in the pH 3 eluant. If 10% of IgG remained in the pH 8 eluant, the concentration there would have been only 0.15mg per ml. This probably explains why no halo of precipitation was observed around the well, containing this eluant, in Fig. 4.5. Precipitin lines, indicating

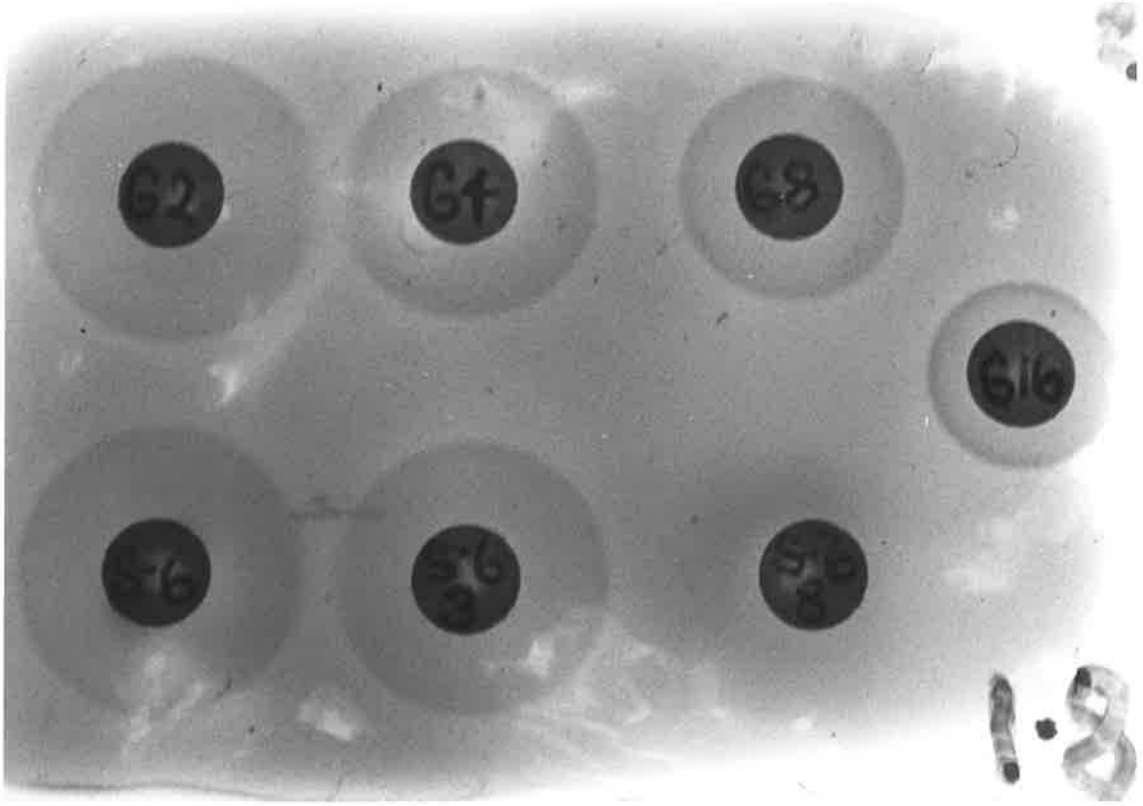
FIGURE 4.5

Calculation of the IgG concentration of the hyperimmune antiserum and its fractions by single radial immunodiffusion



A SRID test was performed to determine the IgG concentration of S-6 antiserum and its two eluants. 15 µl of anti-rabbit γ-chain antiserum was incorporated into the agarose. 5 µl samples of 1:2 to 1:16 dilutions of the standard IgG preparation, S-6 antiserum (1:10), the pH 3 eluant (1:2) and the undiluted pH 8 eluant were loaded into the wells shown above.

Diameters of the resulting haloes of precipitation were measured. The IgG concentration of the standard preparation was known to be 2.6 mg per ml. A linear plot of IgG concentration vs $(\text{diameter})^2$ for the standard preparation then allowed the concentrations of the three unknowns to be calculated ie., 1.2 mg per ml, 0.9 mg per ml, and an undetectable level for the 1:10 S-6 antiserum, 1:2 pH 3 eluant and undiluted pH 8 eluant, respectively.


G2
G4
G8
G16
1:8

the presence of IgG, were demonstrated in the two qualitative immunochemical tests only because they were more sensitive.

(c) Haemagglutinating antibody activity

The hyperimmune antiserum and the two eluants, obtained by protein A-Sepharose chromatography, were tested for haemagglutinating antibody specific for *S. typhimurium* M206. Details of the assay used are given in 2.7.1; the reducing agent 2ME and the absorption step with SRBCs were incorporated.

Titres, in the absence of 2ME, were 1:640, 1:40 and 1:80 for the antiserum, the pH 3 eluant and the pH 8 eluant, respectively. 2ME affected only the antiserum and the pH 8 eluant titres: they dropped to 1:160 and 1:10, respectively, in its presence.

The HA titre of the pH 3 eluant was high and resistant to 2ME. The latter finding indicated that there was no measurable specific antibody of the IgM class in this eluant, as only IgM immunoglobulin is denatured by the concentration of 2ME used. Because the total volume of the pH 3 eluant was six times that of the chromatographed antiserum, it also contained most of the 2ME-resistant HA activity. These results were consistent with the pH 3 eluant containing only IgG and, in particular, specific antibody of this class.

The HA titre of the pH 8 eluant was also high but sensitive to 2ME. All measurable 2ME-sensitive activity therefore appeared in this eluant. This was consistent with the pH 8 eluant containing all the specific antibody

of the IgM class. The low level of 2ME-resistant activity in this eluant was probably due to the combined activity of residual specific IgG and of specific IgA antibody.

(d) Bactericidal antibody activity

Various dilutions of the hyperimmune antiserum and the two eluants were tested in normal bactericidal assays, with 5×10^3 *S. typhimurium* M206 cells. The prozoning and bactericidal titres of each were the immediate aims of these assays.

The results, as shown in Table 4.3, were important. The prozoning titre of the antiserum was 1:200, whilst that of the pH 3 eluant was 1:20. There was some prozoning activity even with the 1:40 dilution of this eluant. Because its total volume was six times that of the chromatographed antiserum, the pH 3 eluant seemed to account for the majority of the prozoning activity of the antiserum.

The prozoning titre of the $pH_{\wedge}^{8}$ eluant was <1:5, although some activity was evident at this lowest dilution tested. The total volume of the pH 8 eluant was eight times that of the chromatographed antiserum, and so its contribution to the prozoning activity was seen to be small.

The results, so far, indicated that the pH 3 eluant contained the bulk of the IgG immunoglobulin as the only serum protein and possessed most of the prozoning activity of the chromatographed antiserum. Specific antibody of the IgG class was therefore thought to be the antibody responsible for the NW phenomenon, in the system studied. The low level of prozoning activity seen in the pH 8 eluant may have been due to specific antibody of the IgA or IgM

TABLE 4.3

The prozoning and bactericidal activities of the hyperimmune antiserum and the eluants, resulting from one step protein A-Sepharose chromatography

Dilution of antibody source	% survival of bacteria[a]		
	antiserum	pH 3 eluant	pH 8 eluant
1:5		307	20
1:10		235	1
1:20		216	0
1:40		16	0
1:80		0	0
1:160		0	0
1:320		40	
1:50	233		
1:100	260		
1:200	222		
1:500	2	42	
1:1000	0	235	0
1:10,000	6		170
1:100,000	28		189
1:1,000,000	290		

(a) The mean value of bacteria added per tube = 4.0×10^3 (antiserum), 4.9×10^3 (pH 3 eluant) and 3.8×10^3 (pH 8 eluant).

Various dilutions of S-6 antiserum and the two eluants, resulting from protein A-Sepharose chromatography, were tested in three standard bactericidal assays. 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-13 were added to the reaction mixtures.

classes, but it may also have been due to the low level of specific IgG antibody present.

The bactericidal titres reflected the immunoglobulin compositions of the two eluants. The pH 3 eluant, containing only the bulk of the IgG of the chromatographed antiserum, showed a significantly lower titre (1:160) than the pH 8 eluant (1:1000). The latter contained all the measurable serum IgM. Specific antibody of this class, being more efficient in the complement-mediated bactericidal reaction than that of the IgG class (Robbins *et al.*, 1965; Daguillard and Edsall, 1968; Eddie *et al.*, 1971; Schulkind *et al.*, 1972), often accounts for the majority of the bactericidal antibody activity of an antiserum. This appeared to be the case with the chromatographed antiserum.

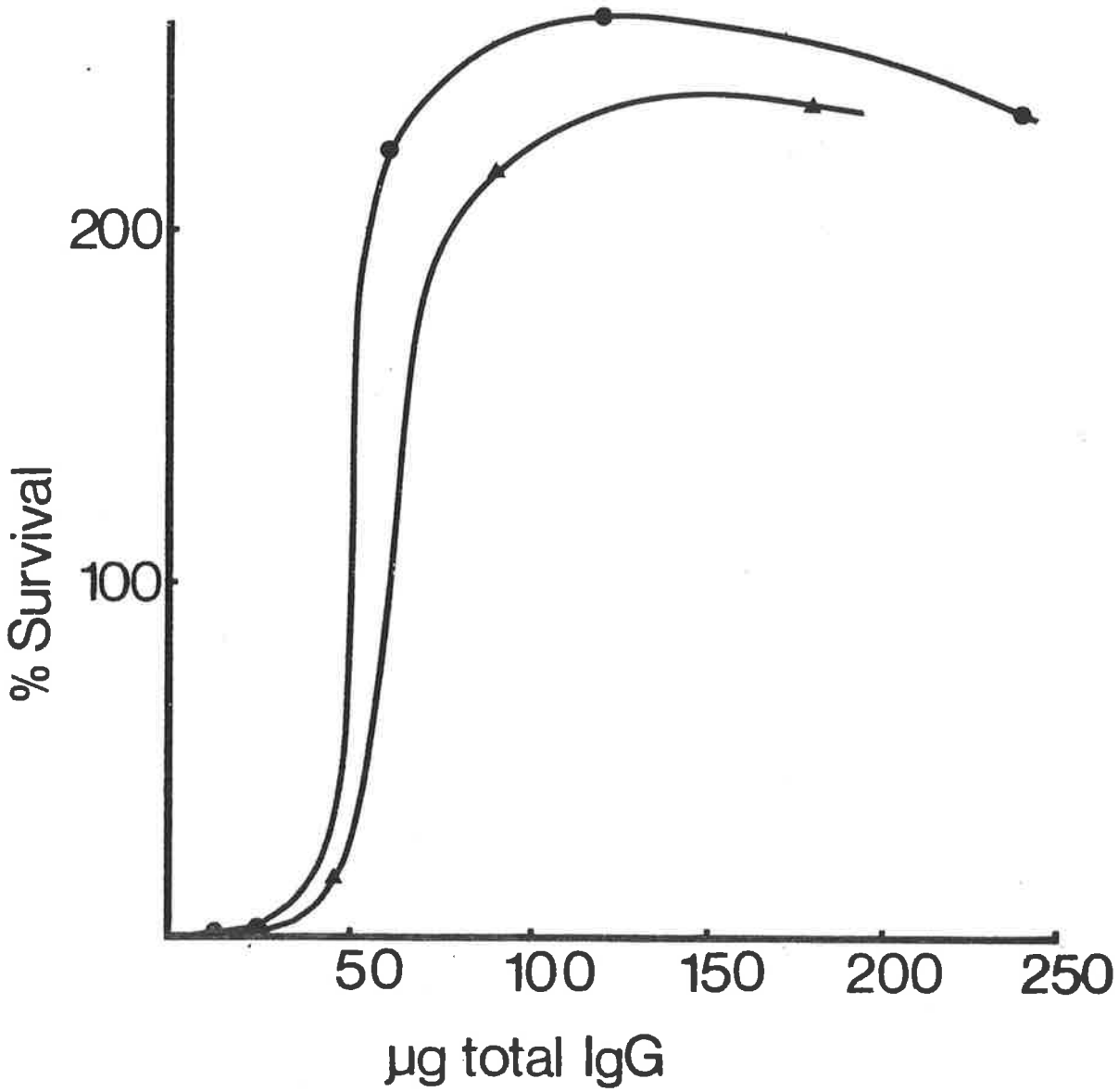
Stronger evidence, that specific IgG antibody was responsible for the prozoning activity of the antiserum, followed. The lower dilutions of the chromatographed antiserum and its pH 3 eluant, already tested in bactericidal assays, were expressed as µg of total IgG. The concentrations of the undiluted antiserum and pH 3 eluant were already calculated (see 4.2.2(b)). Fig. 4.6 shows the plot of % survival values vs the µg of total IgG added, in these low dilutions. For both the antiserum and its pH 3 eluant, good prozoning activity was evident when the total IgG in the reaction mixture was above 50 µg. It was reasonable to assume that the specific antibody fraction of the total IgG was similar for the antiserum and its pH 3 eluant, as protein A binds to the Fc and not the Fab fragments of rabbit IgG molecules (Grov, 1973; Endresen,

FIGURE 4.6

The prozoning activities of the hyperimmune antiserum and its pH 3 eluant in relation to the total IgG content

The dilutions of S-6 antiserum and its pH 3 eluant, appearing in Table 4.3 as prozoning dilutions, were expressed as total IgG values and were plotted vs the corresponding % survival values. The undiluted antiserum and its pH 3 eluant were taken to contain 12.0 and 1.8 mg per ml of total IgG, respectively (see 4.2.2(b)).

S-6 antiserum ● , pH 3 eluant ▲ .


200
100
% Survival
50
100
150
200
250
µg total IgG

1978). This indicated that prozoning activity was evident, with both the antiserum and the pH 3 eluant, when the level of specific IgG antibody in the reaction mixture was above the same value. This correlation strongly indicated that specific antibody of the IgG class was solely responsible for the prozoning activity of the chromatographed antiserum.

4.2.3 Prozoning activity of subpopulations of the IgG fraction

(a) Fractionation details

A hyperimmune rabbit antiserum, specific for *S. typhimurium* M206, was chromatographed on a protein A-Sepharose column, with washes incorporating buffers of increasing acidity (see 2.9.1(c)). Details of the elution profile are shown in Fig. 4.7.

The resulting pH 8, pH 6, pH 5 and pH 4 eluants contained reasonable concentrations of protein, according to the optical density (OD_{280}) values. The pH 7 eluant contained little protein while no protein was eluted at pH 3.0. The pH 7, pH 6, pH 5 and pH 4 eluants were believed to contain only IgG, and the pH 8 eluant was believed to contain all other serum proteins.

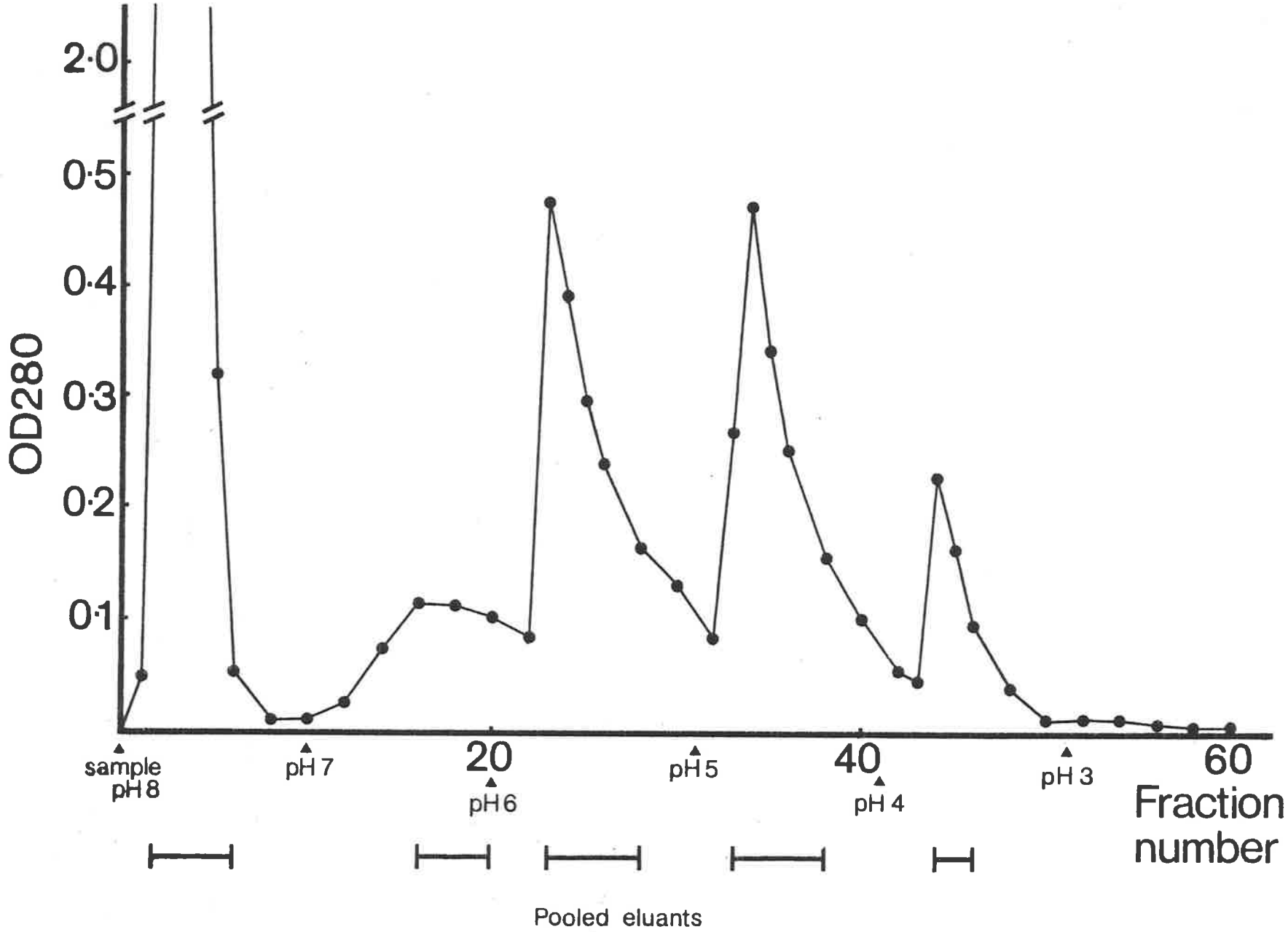
(b) The immunochemical testing of fractions

The antiserum and its five eluants were first tested for immunoglobulin class content by the comparative DID method (see 2.9.3(a)). All six showed a precipitin line with anti-rabbit γ-chain antiserum, each line joined that formed with anti-rabbit serum protein antiserum. Only the

FIGURE 4.7

Stepwise elution profile of hyperimmune rabbit antiserum on a protein A-Sepharose column

A number of IgG fractions were purified from C-8 antiserum, by adsorption to a protein A-Sepharose column at pH 8.0 and by stepwise elution at decreasing pH values. 2.0 ml of the antiserum was diluted in 2.0 ml of phosphate buffer, pH 8.0, and applied to the column. This was washed first with phosphate buffer and then with citrate buffers of pH 7.0, 6.0, 5.0, 4.0 and 3.0. 2.5 ml fractions were collected every 6 minutes until the OD_{280nm} (OD280) value was insignificant. The column was then washed with the buffer of the next acidic pH. Fractions were pooled as indicated (pH 8, pH 7, pH 6, pH 5 and pH 4 eluants). The resulting eluants were quickly neutralized and dialysed.


2·0
0·5
0·4
0·3
0·2
0·1
OD280
sample
pH 8
pH 7
20
pH 6
pH 5
40
pH 4
pH 3
60
Fraction
number
Pooled eluants

antiserum and its pH 8 eluant showed multiple lines with the anti-serum protein antiserum. These results indicated that each of the five eluants contained a measurable fraction of the serum IgG. The pH 6 and pH 5 eluants, in particular, showed thick and long precipitin lines with the anti-γ-chain antiserum; this indicated high IgG levels. The corresponding line was faint and short for the pH 8 eluant; this indicated a low IgG level.

Fig. 4.8 shows that only the pH 8 eluant contained detectable levels of the serum IgA and IgM immunoglobulins, because only the concentrated pH 8 eluant showed precipitin lines with the anti-α- chain and the anti-μ-chain antiserum, respectively. This constituted evidence to suggest that the pH 7, pH 6, pH 5 and pH 4 eluants contained IgG, with differing reactivities for protein A, but no IgA or IgM.

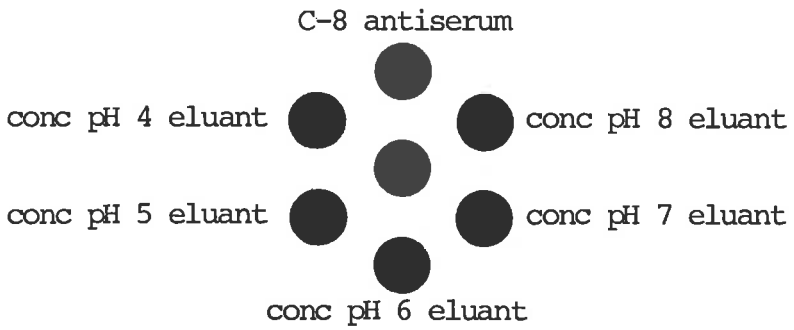
Because of the dilution factors inherent in this fractionation procedure, the IgG concentration in the eluants was believed to be too low for the accurate determination by single radial immunodiffusion. The extinction coefficient method (see 2.9.1(d)) was preferred; IgG concentrations of 0.15, 0.34, 0.33 and 0.22 mg per ml were calculated for pH 7, pH 6, pH 5 and pH 4 eluants, respectively. These values were in agreement with the predicted relative levels, from the comparative DID tests.

(c) Bactericidal and other antibody activities

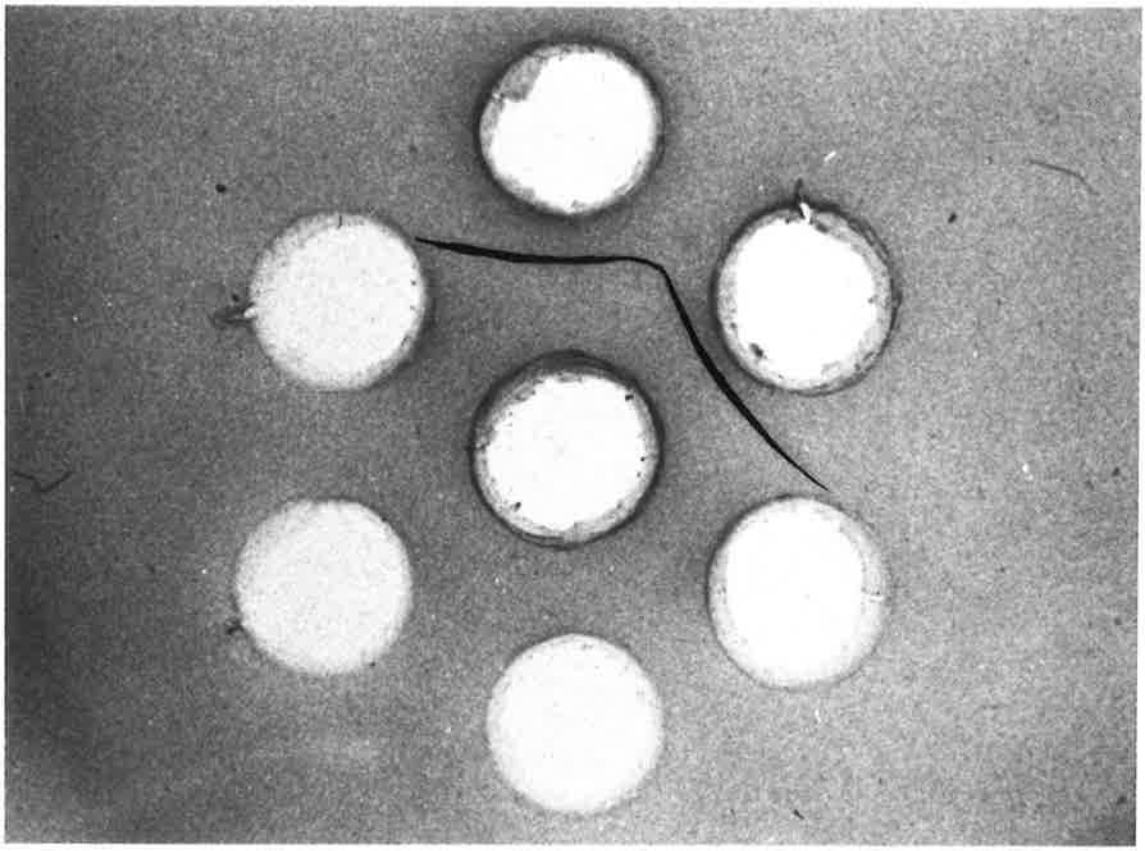
Standard bactericidal assays, with 5×10^3 *S. typhimurium* M206 cells, revealed that the prozoning activity of the whole antiserum was distributed amongst the pH 7, pH 6, pH 5 and pH 4 eluants (see Table 4.4). The

FIGURE 4.8

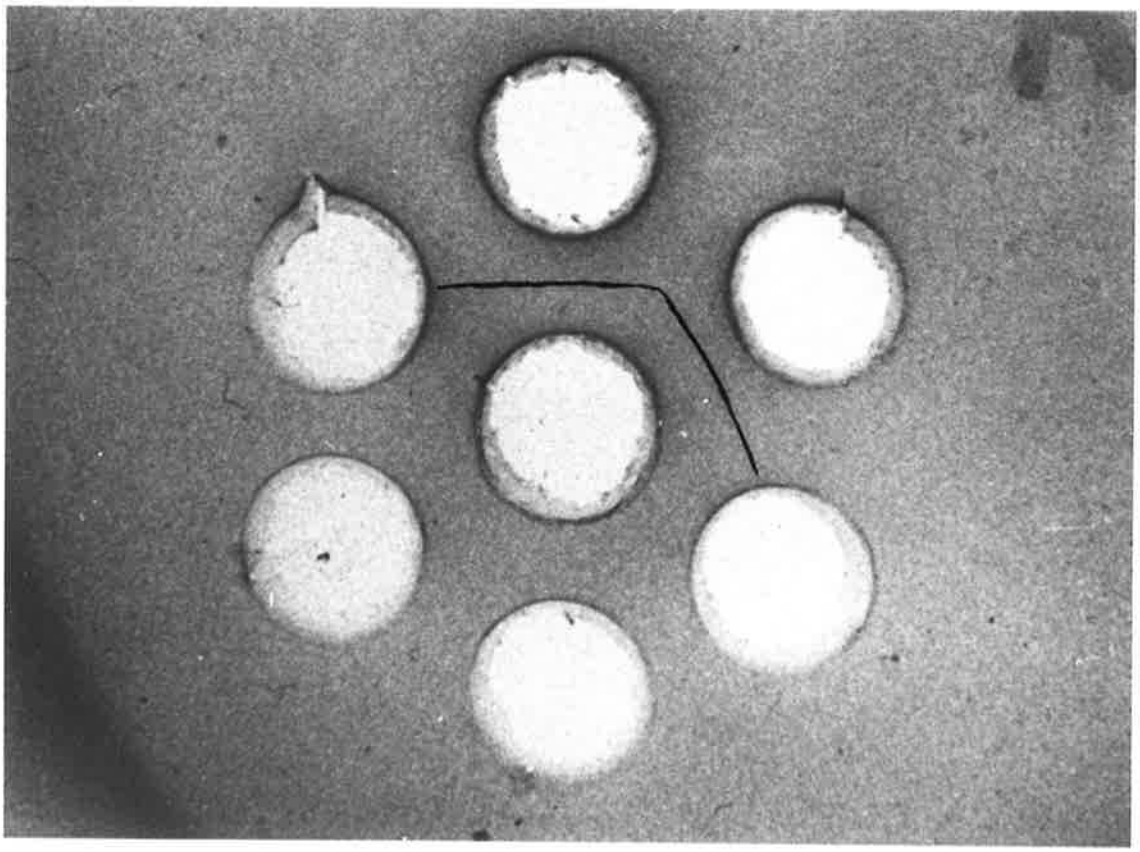
Comparative double immunodiffusion patterns of a hyperimmune antiserum and the eluants resulting from stepwise protein A-Sepharose chromatography



Samples of the whole C-8 antiserum and its eluants were tested for IgA and IgM content by comparative DID. The central well (4 mm diameter) contained 15 µl of anti-rabbit µ- chain antiserum (A) or anti-rabbit α-chain antiserum (B). The peripheral wells (4 mm diameter) contained 15 µl of antiserum and its eluants. The latter were concentrated by the use of a B15 Minicon unit (Amicon Corporation, Mass., USA) (X25 for pH 4 eluant; X5 for all other eluants).



A



B

TABLE 4.4

Specific antibody activities of a hyperimmune antiserum and the five fractions obtained from stepwise protein A-Sepharose chromatography

Antibody source	IgG concn. (mg per ml)	Titre of antibody activity: prozoning[(a)]	bactericidal[(a)]	haemagglutination[(b)]	immune haemolytic[(c)]
Antiserum	NT	1:400	1:100,000	1:320	1:1280
Eluants: pH 8	NT	<1:3	1:2560	1:32	1:160
pH 7	0.15	1:3	1:40	1:1	<1:5
pH 6	0.33	1:10	1:640	1:2	1:10
pH 5	0.32	1:5	1:640	1:2	1:5
pH 4	0.24	1:3	1:160	1:2	<1:5

(a) Prozoning and bactericidal titres were determined by testing doubling dilutions of C-8 antiserum and its eluants in standard bactericidal assays, with 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-14.

(b) Doubling dilutions of the antiserum and its eluants (25 µl) were incubated with VBS (50 µl) and C5 LPS-sensitized SRBCs (25 µl) at room temperature (see 2.7.1).

(c) The semi-quantitative assay (see 2.6.1(a)). Doubling dilutions of the antiserum and its eluants (25 µl), VBS (25 µl), a 1:10 dilution of GpC (25µl) and C5 LPS-sensitized SRBCs (25 µl) were incubated at 37°.

pH 6 and pH 5 eluants contained the highest prozoning activity. Although the stated titre of the pH 5 eluant was 1:5, there was still inefficient killing with the 1:10 dilution and so the difference between these two eluants was not as great as shown in the table. The pH 7 and pH 4 eluants contained lower prozoning activities. Again, although the stated titre of the pH 7 eluant was 1:3, there was inefficient killing at 1:5. Of these four eluants, the pH 6 and pH 5 eluants also showed the highest bactericidal titres. The titre of the pH 4 eluant was significantly higher than that of the pH 7 eluant; therefore the bactericidal activity seemed to more accurately reflect the total IgG concentration in each eluant than the prozoning activity.

The pH 8 eluant, which contained very little IgG but all other measurable serum proteins, efficiently killed target M206 cells even at a final 1:3 dilution and so did not show any prozoning activity. However, the bactericidal activity was very high and was consistent with the presence of high levels of specific IgM antibody in this eluant.

These results again indicated that specific antibody of the IgG class was responsible for the prozone phenomenon. Furthermore, the stepwise elution of IgG from protein A-Sepharose could not link this specific antibody to any fraction, with a narrow range of reactivity for protein A.

Table 4.4 reveals that the pH 8 eluant contained the bulk of the specific HA activity of the antiserum. This was consistent with the observed high levels of IgM and IgA immunoglobulins (see Fig. 4.8) in this eluant. The

pH 6, pH 5 and pH 4 eluants showed lower HA activity whilst the pH 7 eluant showed activity only when undiluted. The titres of these four eluants reflected their total IgG content.

The pH 8 eluant also contained the bulk of the specific immune haemolytic activity of the antiserum. This was presumably due to the high IgM content in this eluant. The pH 6 and pH 5 eluants showed lower haemolytic activity, whilst little haemolysis was evident even with the initial 1:5 dilutions of the pH 7 and pH 4 eluants. The titres of these four eluants closely paralleled their prozoning titres.

To summarise, protein A-Sepharose chromatography did allow IgG fractions of differing protein A reactivity to be isolated from a hyperimmune rabbit antiserum with prozoning activity. All of these fractions contained prozoning activity and all contained specific IgG antibody capable of fixing complement. Although the pH 7 and pH 4 eluants did not show immune haemolytic activity, all four eluants showed significant bactericidal activity. No evidence of non-complement-fixing IgG antibody was seen.

4.2.4 The effect of a prozoning IgG fraction on the bactericidal reaction mediated by specific IgM antibody

(a) IgM fractionation details

The method, whereby the IgM fraction of a rabbit antiserum was isolated from other serum proteins, is given in 2.9.2. Briefly, the antiserum, collected early in a vaccination schedule with *S. typhimurium* M206 cells, was

first chromatographed on a Sephadex G-200 column. The elution profile is shown in Fig. 4.9. Fraction 17 (pool I) was thought to contain mostly lipoprotein, as an aliquot precipitated with a heparin/manganous chloride solution. Fractions 19 to 21 (pool II) and fractions 22 to 27 (pool III) were thought to contain the bulk of the serum IgM and IgG, respectively. Fractions 28 to 32 (pool IV), constituting the major protein peak, were thought to contain the serum albumins.

Pool II was then chromatographed on a protein A-Sepharose column, (as described in 2.9.1(b)) to remove contaminating serum IgG. Most of the protein in the pool was eluted at pH 8.0, according to the OD_{280} values in Fig. 4.9. The resulting pH 8 eluant was believed to contain the serum IgM and no other serum proteins, especially not IgG or IgA. Some protein was eluted from the column at pH 3.0. Thus, IgG was present in pool II, but presumably only in a low concentration.

The pH 3 and pH 8 eluants were concentrated to half and the same volume of the original serum, respectively.

(b) The immunochemical testing of fractions

The antiserum, pools I, III and IV (from G-200 chromatography), and the concentrated pH 3 and pH 8 eluants (from protein A-Sepharose chromatography) were first tested for immunoglobulin class content by the DID method (see 2.9.3(a)). Fig. 4.10A shows that IgM was detected only in the concentrated pH 8 eluant and the whole antiserum. All the serum IgM appeared to be present in this eluant.

FIGURE 4.9

Sephadex G-200 and protein A-Sepharose chromatography to isolate the IgM fraction of an antiserum, collected early in a vaccination schedule

2.0 ml of A-3 antiserum was passed through a glass column (40cm x 2.5 cm) packed with Sephadex G-200. Phosphate buffer, pH 8.0, was used to wash the column through and 5 ml fractions were collected every 10 minutes. A total of 40 fractions were collected and those, constituting protein peaks, were mixed as pools I, II, III and IV.

Pool II was then passed through the protein A-Sepharose column. It was washed through with phosphate buffer and 11 x 2 ml fractions were collected. Fractions with elevated protein levels were pooled as the pH 8 eluant. This 18 ml was concentrated to 2 ml using a Minicon B15 concentrator. Citrate buffer, pH 3.0, was then used to wash the column through and 7 x 2 ml fractions were collected. Fractions with elevated protein levels were pooled as the pH 3 eluant. This was then concentrated to 1 ml.

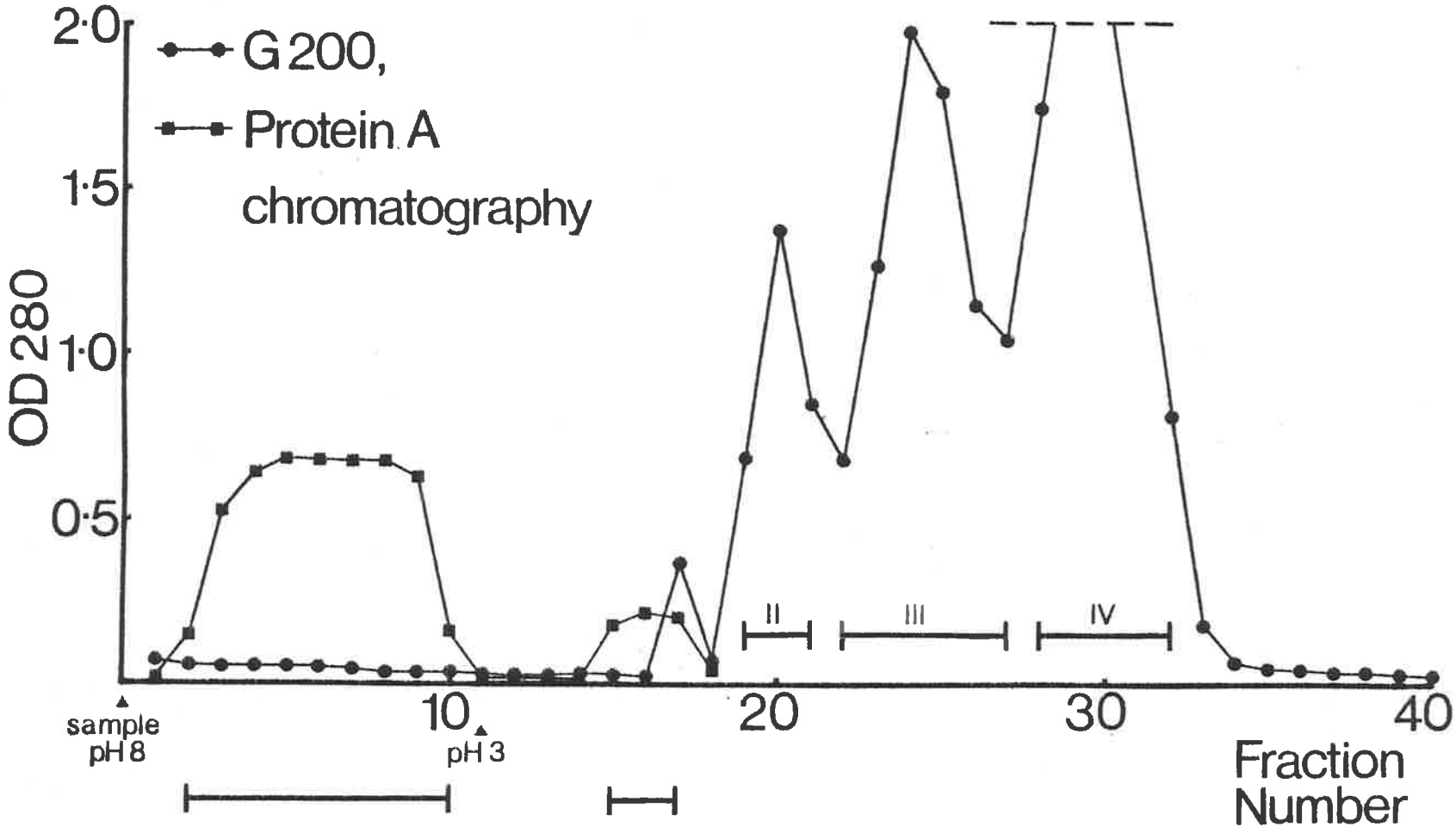
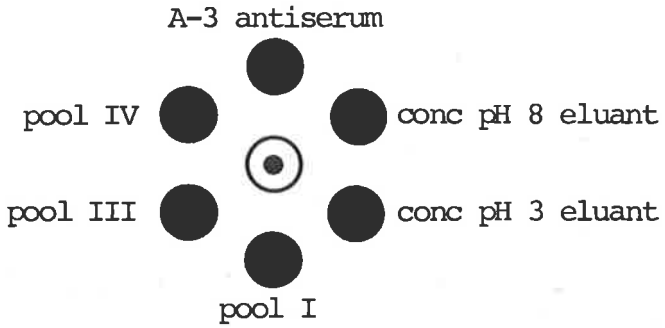
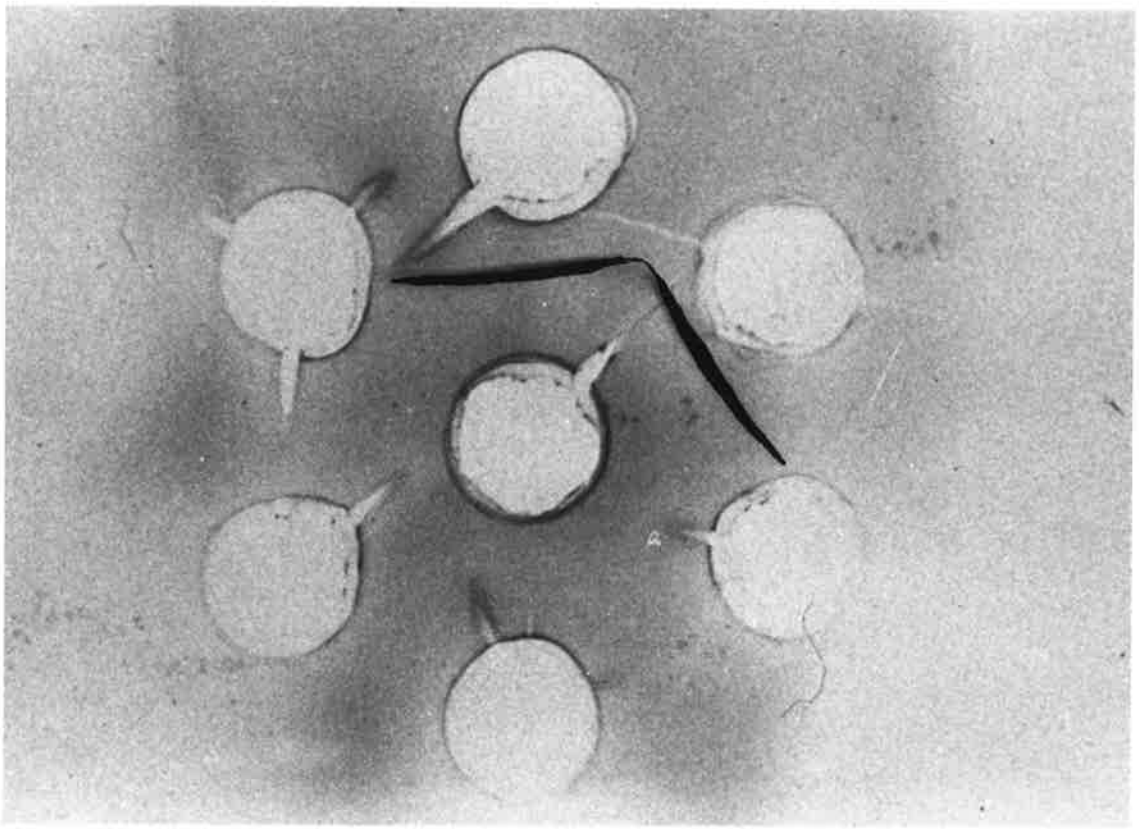

G 200,
Protein A
chromatography
OD 280
2·0
1·5
1·0
0·5
II
III
IV
sample
pH 8
10
pH 3
20
30
40
Fraction
Number

FIGURE 4.10

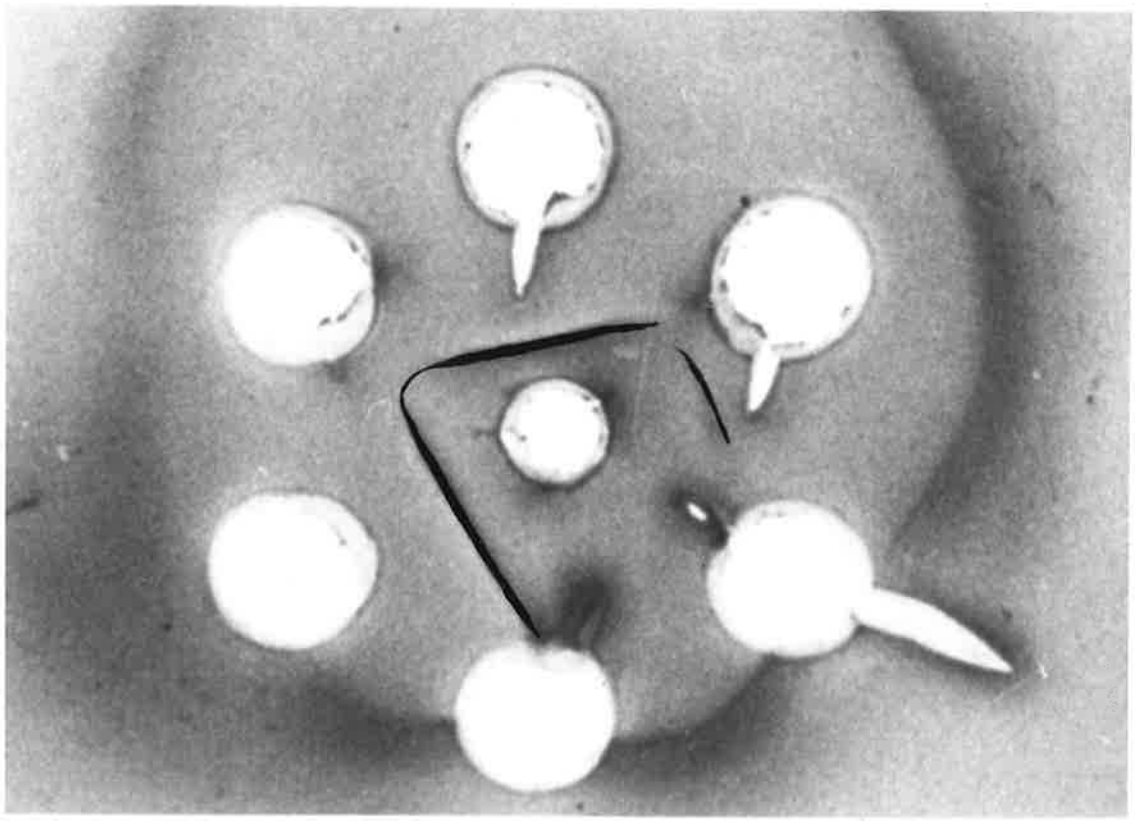
Comparative double immunodiffusion patterns of an antiserum and the fractions resulting from Sephadex G-200 and protein A-Sepharose chromatography



Samples of the whole A-3 antiserum and its fractions were tested for IgM and IgG content by comparative DID. The central well (4 mm or 2 mm diameter) contained 10 µl of anti-rabbit µ-chain antiserum (A) or 5 µl of anti-rabbit γ-chain antiserum (B). The peripheral wells (4 mm diameter) contained 10 µl of whole antiserum and the concentrated eluants; and 15 µl of pools I, III and IV.



A



B

The serum IgG was mainly detected in pool III, as shown in Fig. 4.10B. Some also seemed to be present in the concentrated pH 8 eluant! Although the concentrated pH 3 eluant did not show a precipitin line with the anti-γ-chain antiserum on this occasion, this line was seen in other testings. Thus, IgG seemed to be present in the concentrated pH 3 eluant, but only at a low level.

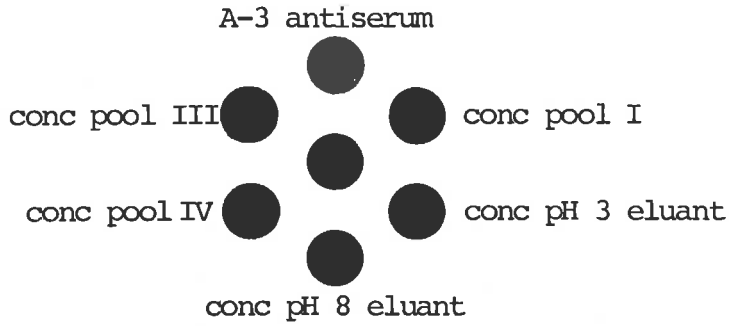
Aliquots of pools I, III and IV were concentrated 5X, 10X and 10X, respectively, in a Minicon B15 unit. These concentrated pools, the concentrated pH 3 and pH 8 eluants, and the whole antiserum were tested for IgA by the DID test. Fig. 4.11 shows that only the concentrated pool III and the antiserum showed an identical precipitin line with the anti-γ-chain antiserum. All the serum IgA appeared to be present in pool III.

The DID test with anti-γ-chain antiserum seemed to indicate that the concentrated pH 8 eluant contained some IgG. The SRID technique (see 2.9.3(c)) was used to quantitate the IgG level in the concentrated pH 8 eluant, as well as in other serum fractions.

Fig. 4.12 shows the results when just the concentrated pH 3 and pH 8 eluants were tested. A small halo of precipitation around the well containing the concentrated pH 3 eluant indicated that this eluant contained some IgG but at a concentration lower than 100 μg per ml. The large but very faint halo around the well containing the concentrated pH 8 eluant was an important finding. This eluant formed a faint precipitin line with the same anti-γ-chain antiserum in the DID test (see Fig. 4.10B). This antiserum appeared to contain a low level of antibody

FIGURE 4.11

Comparative double immunodiffusion patterns of the antiserum and its fractions with antiserum specific for rabbit IgA



Samples of the whole A-3 antiserum (10 µl) and its concentrated fractions (15 µl) were tested for IgA content by DID. The central well (4 mm diameter) contained 10 µl of anti-rabbit α- chain antiserum whilst the peripheral wells (4 mm diameter) contained the antiserum and its fractions. The slides were photographed when unstained.

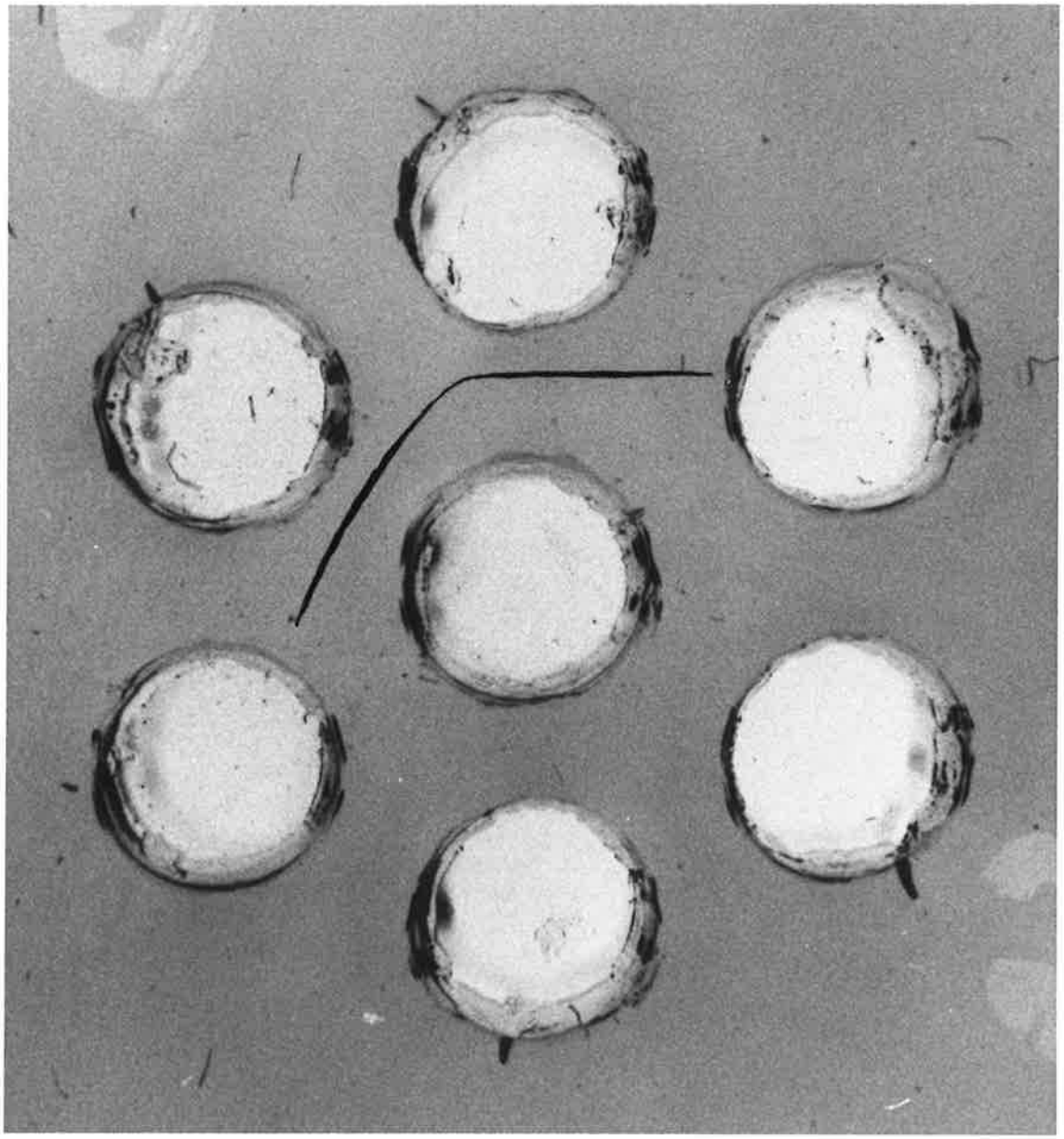
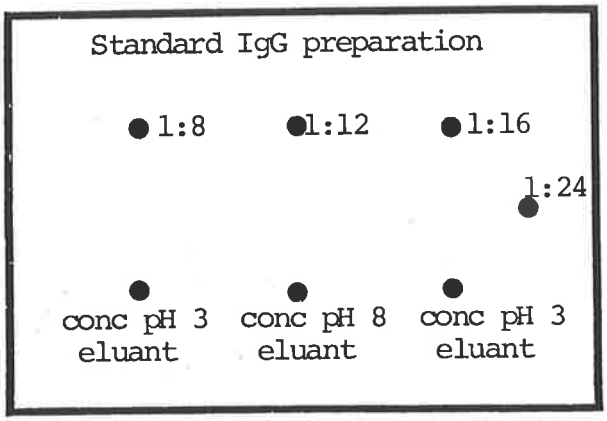


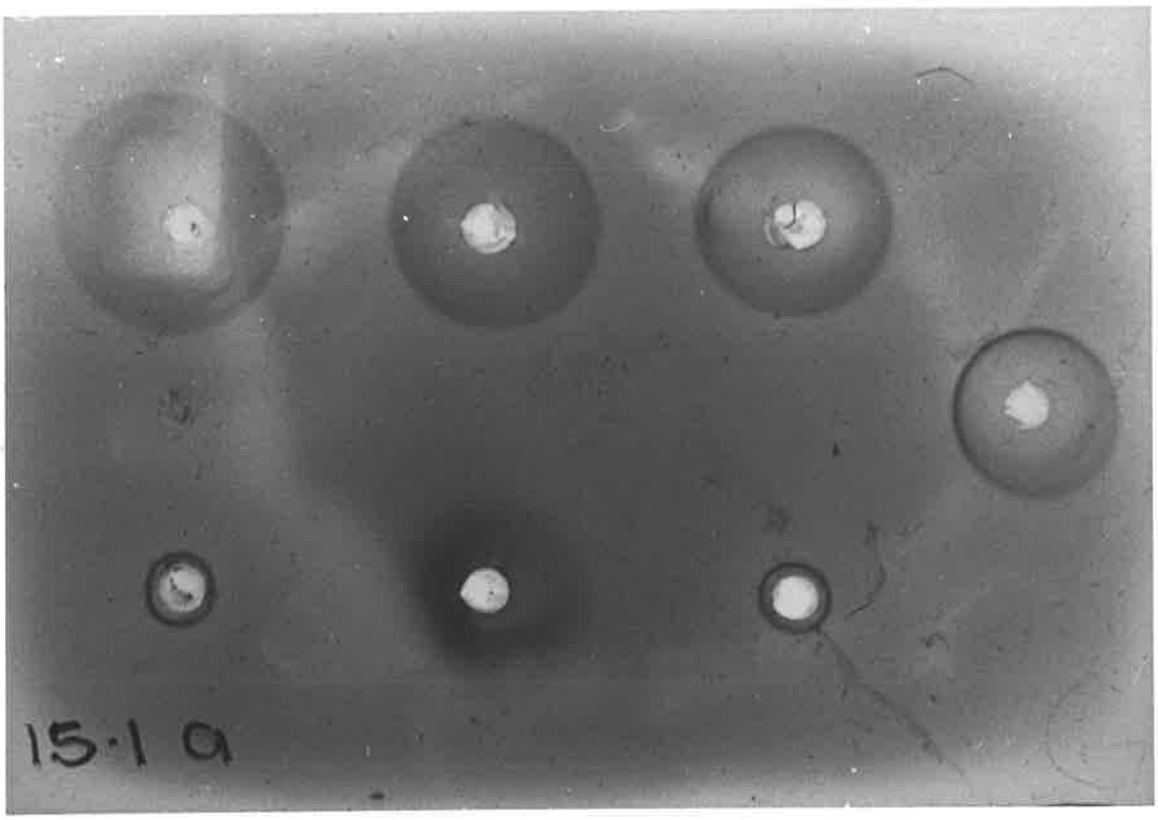
FIGURE 4.12

Calculation of the IgG concentration of the concentrated pH 3 and pH 8 eluants by single radial immunodiffusion



A SRID test was performed to determine the IgG concentration of the concentrated pH 3 and pH 8 eluants of A-3 antiserum. 15 µl of anti-rabbit γ-chain antiserum was incorporated into the agarose. 5 µl samples of 1:8 to 1:24 dilutions of the standard IgG preparation and the undiluted eluants were loaded into the wells shown above.

Diameters of the resulting haloes of precipitation were measured. The IgG concentration of the standard preparation was known to be 2.6 mg per ml. A linear plot of IgG concentration vs $(\text{diameter})^2$ for the standard preparation then allowed the concentrations of the conc pH 3 and pH 8 eluants (<100 µg per ml) to be calculated.


15·1 a

specific for rabbit IgM. The precipitation observed in both tests was presumably due to the interaction of this contaminating antibody with the high level of IgM in the pH 8 eluant. It was concluded that the pH 8 eluant contained no detectable serum IgG.

The IgG concentration of the whole antiserum and pool III was calculated to be 10.7 and 0.8 mg per ml, respectively, from other radial immunodiffusion tests. The total volume of pool III was approximately 26 ml; this meant that between 95% and 100% of the serum IgG was collected in this pool.

The two immunochemical tests established that the pH 8 eluant contained all of the detectable serum IgM, but no IgG or IgA. For the remainder of this section, the concentrated eluant will be referred to as the IgM fraction.

(c) Bactericidal activity of the IgM fraction

Various dilutions of the IgM fraction were tested in a standard bactericidal assay with 5×10^3 *S. typhimurium* M206 cells. Table 4.5 shows that, although the three lowest dilutions tested did not completely kill the target cells, there was no clear prozoning activity in the IgM fraction. The prozoning titre was thus <1:5 compared with the very high bactericidal titre of 1:100,000.

Table 4.1 shows that the prozoning and bactericidal titres of the whole antiserum were calculated to be 1:10 and 1:100,000, respectively. The IgM fraction consisted of the pH 8 eluant, concentrated to the original volume of chromatographed antiserum. The IgM fraction lacked the clear prozoning activity of the whole antiserum but

TABLE 4.5

The bactericidal activity of the IgM fraction resulting from Sephadex G200 and protein A-Sepharose chromatography

Dilution of IgM fraction	% survival of bacteria[(a)]
1:5	17
1:10	11
1:20	11
1:40	0
1:100	0
1:1000	0
1:10,000	0
1:100,000	4
1:1,000,000	32
Nil	124

(a) the mean value of bacteria added per tube = 6.6×10^3

Various dilutions of the IgM fraction, derived from A-3 antiserum, were tested in a standard bactericidal assay, with 5×10^3 log-phase M206 cells and 1.2 CH50 units of HuC-14.

contained most of its bactericidal activity. Again, specific IgM antibody, present in high concentration, was shown not to mediate the prozone phenomenon.

(d) The interaction of a prozoning IgG fraction and the bactericidal IgM fraction in the bactericidal reaction with *S. typhimurium* M206

This important study established whether prozoning dilutions of the IgG fraction of a hyperimmune antiserum (see 4.2.2) could interfere with the bactericidal action of the IgM fraction. Log-phase M206 cells were first sensitized with several dilutions of this IgM fraction. The bacteria were then exposed to various dilutions of the IgG fraction, before the addition of complement and the performance of a standard bactericidal assay.

The important finding was that there was no significant difference in the prozoning activity of the IgG fraction, whether the target bacteria had been presensitized with low or high bactericidal levels of the IgM fraction (see Table 4.6). Prozoning titres were 1:71, 1:53, 1:71 and 1:53 when the target cells were presensitized with no, 1:100, 1:1000 and 1:10,000 dilutions of the IgM fraction. Even these slight differences were misleading because significant numbers of target cells survived at the 1:71 dilution, whatever dilution of the IgM fraction was added.

Table 4.6 also shows that the three dilutions of the IgM fraction were bactericidal in the absence of the IgG fraction. These results agreed with previous findings (see Table 4.5). The IgG fraction of the hyperimmune antiserum showed strong prozoning activity at 1:30, 1:40, 1:53

TABLE 4.6

The effect of prozoning IgG antibody on the bactericidal action of the IgM fraction on *S. typhimurium* M206 cells

IgG fraction[b] dilution	% survival of bacteria[a]			
	IgM fraction dilution			
	1:100	1:1000	1:10,000	Nil
1:30	220	285	222	231
1:40	202	185	242	197
1:53	107	110	118	166
1:71	48	92	41	75
1:80	1	0	1	1
Nil	0	0	4	170

(a) The mean value of bacteria added per tube = 3.8×10^3

(b) The pH 3 eluant, derived by the protein A-Sepharose chromatography of S-6 antiserum (see 4.2.2)

3×10^4 log-phase M206 cells were sensitized (4°, 15 minutes) with IgM fraction dilutions of 1:100, 1:10,000 and 1:100,000 in a total reaction volume of 6.0 ml. The cells were then centrifuged down at 4° and resuspended as 5×10^4 per ml. Aliquots (0.1 ml) were added to tubes containing Mg^{2+} saline (0.7 ml) and various dilutions of the IgG fraction from a hyperimmune antiserum (0.1 ml). The mixtures were incubated at 4° for 20 minutes, before the addition of 1.2 CH50 units of HuC-14 (0.1 ml) and the commencement of a standard bactericidal assay.

and 1:71 dilutions, in the absence of the IgM fraction. The 1:80 dilution was the only bactericidal one. These results were also consistent with previous findings (see Table 4.3). The control tube, lacking both antibody sources, showed no killing and so indicated that the complement source was free of bactericidal antibody.

The study showed that specific IgG antibody, with prozoning activity, could block the bactericidal action of specific IgM antibody, even after the latter had been incubated with, and had bound to, target M206 cells. If one specific IgM molecule was assumed to sensitize each M206 cell at the 1:100,000 dilution ie., the highest bactericidal dilution (see Table 4.5), then 1000 molecules possibly sensitized each cell at the 1:100 dilution. Nevertheless, killing did not occur.

CHAPTER 4.3 Discussion

The work, described in Chapter 3, established that the Neisser-Wechsberg phenomenon is mediated by the interaction of target bacterial cells and specific antibody. One immediate question to be resolved was the nature of this antibody. In particular, it was important to establish whether prozoning activity was restricted to a certain class or subclass of immunoglobulin, and whether a particular property of such a class or subclass was responsible. Accordingly, the appearance of prozoning activity, in relation to other specific antibody activities, was closely followed during a vaccination schedule, involving rabbits and heat-killed *S. typhimurium* M206 cells. Antisera, collected early and late in this vaccination schedule,

were also fractionated into various immunoglobulin classes. Specific antibody activities, including prozoning activities of these were assayed. All evidence suggested that specific IgG antibody, appearing late in the vaccination schedule, is that responsible for the Neisser-Wechsberg phenomenon, in this system.

Since the NW phenomenon was discovered, a long vaccination schedule has been recognized to be a requirement for high prozoning activity in any antiserum collected. Normann (1972) provided some supportive evidence by comparing the bactericidal activities of two rabbit antisera, that were collected at different times, during a vaccination schedule with *S. typhimurium* 395 MRO. The data, described in 4.2.1, represented a more detailed study. Two rabbits were bled once a week, prior to the intravenous injection of 10^8 heat-killed M206 bacteria, for at least 8 weeks.

Prozoning activity first appeared after a delay of two weeks and then continued to rise steadily to a maximum level after four weeks (see Tables 4.1 and 4.2). It remained at this level until the vaccinations ceased; it then declined rapidly. Specific IgM antibody generally peaks earlier than IgG or IgA antibody in immune responses. These observations therefore indicated that prozoning activity was restricted to either IgG or IgA antibody, or both, but not to IgM antibody. They also indicated that a continuous antigenic stimulus was required for the maintenance of prozoning activity.

The bactericidal activity peaked after two weeks and did not decline, even 80 days after the last vaccination. Specific HA activity also appeared early but it rose slowly,

and then declined quickly after the last vaccination. HA activity, resistant to 2ME, appeared one to two weeks later than the general activity. This represented the combined IgG and IgA antibody specific for the M206 strain, as IgM immunoglobulin is susceptible to reduction by 0.1 M 2ME (Deutsch and Morton, 1957) but IgG in whole serum is not susceptible (Capel *et al.*, 1980). The prozoning activities of both series of sera correlated very well with their 2ME-resistant HA activities. Both activities peaked significantly higher for one rabbit. Specific IgG or IgA antibody, or both, were again implicated as being responsible for the prozoning activity of the antisera.

Several points need to be remembered when assessing the data from the vaccination schedules. Bactericidal and haemagglutination reactions are secondary antibody interactions. Specific antibody of the IgM class is significantly more efficient than that of the IgG class in the bactericidal reaction with Salmonella bacteria as target cells (Robbins *et al.*, 1965; Daguillard and Edsall, 1968; Eddie *et al.*, 1971; Schulkind *et al.*, 1972). IgA antibody is generally believed to be unable to mediate this reaction (Adinolfi *et al.*, 1966; Eddie *et al.*, 1971; Knop *et al.*, 1971; Heddle *et al.*, 1975). Specific IgM antibody is also more efficient than specific IgG (Benedict, 1965) and specific IgA (Ishizaka *et al.*, 1965) antibody in HA reactions. The contribution of specific IgG and IgA antibody to the total population of anti-M206 antibody in the antisera was higher than that manifested in the bactericidal and HA titres.

The dominant immunogens in vaccines comprising Gram-

negative bacteria are the O-somatic antigens ie., the polysaccharide side-chains of the outer membrane LPS. They are regarded as leading to antibody responses that are, at least initially, of the IgM class only. The switch in the production of IgM antibody to IgG antibody is much less pronounced and the secondary response does not result in the marked rise in antibody level, noted with protein antigens (Britton and Moller, 1968; Holmgren, 1970; Holmgren *et al.*, 1971). Specific IgG antibody appears later in the primary response and is heightened by repeated vaccinations (Holmgren, 1970; Gupta and Reed, 1971; Schulkind *et al.*, 1972). The results obtained from the sera, collected throughout our vaccination schedules, were consistent with these previous findings. Specific IgM antibody was present throughout, but the IgG antibody (ie., 2ME-resistant antibody) seemed to appear later.

The avidity of specific IgM and IgG antibodies, together with their secondary activities, increase during the immune response to such vaccines (Schulkind *et al.*, 1972; Holmgren and Svennerholm, 1972; Ahlstedt *et al.*, 1973). Specific antibody of the IgG class also has higher avidity than that of the IgM class, in any one antiserum (Robbins *et al.*, 1965; Holmgren and Svennerholm, 1972). These findings indicated that prozoning antibody, suspected to be of the IgG class, was very avid as it appeared relatively late in the vaccination schedules.

The fractionation of antiserum with high prozoning activity was a crucial step in assigning the activity to specific antibody of the IgG class. The protein A-Sepharose chromatography method of Goding (1976) presented

itself as a simple yet effective way of separating IgG from all the other serum proteins. The predicted yield of IgG was doubtful, due to the reported low reactivity of rabbit IgG with Staphylococcal protein A (Sjöquist *et al.*, 1967; Forsgren and Sjöquist, 1967; Grov, 1973; Endresen, 1978). Several prozoning antisera were fractionated on the protein A-Sepharose column, in the present study. These fractionations all resulted in the majority (about 90%) of the serum IgG being collected in the pH 3 eluant and all other serum proteins being collected in the pH 8 eluant. The yield of IgG from the one step fractionation (10.8 mg per ml of serum) was a little higher than that quoted by Goding (1976) and significantly higher than those quoted by Grov (1973) and Endresen (1978). These workers fractionated normal rabbit serum and the technique of the latter two workers was significantly different. They first precipitated the globulins from serum with ammonium sulphate and fractionated these on a DEAE-cellulose column, before loading the partially purified IgG fraction onto a protein A-Sepharose column. Some serum IgG must have been lost in the preliminary fractionations.

The pH 3 eluant, from the one step fractionation, contained IgG as the only serum immunoglobulin and protein (see 4.2.2(b)). Its specific HA activity was resistant to 2ME (see 4.2.2(c)) and this was consistent with specific activity of the IgG class. In contrast, the HA activity of the pH 8 eluant was reduced eight-fold by 2ME; this was consistent with specific activity of the IgM class. The residual low activity was due to the combined specific IgG and IgA antibody. Very little IgG remained in the pH 8

eluant therefore this 2ME-resistant activity was probably due to the specific IgA antibody. The contribution of specific IgA antibody to the HA activity of the whole serum therefore seemed to be small.

The most important finding in the fractionation study was that the pH 3 eluant contained practically all of the prozoning activity of the antiserum (see Table 4.3). The level of total IgG, that was required to cause a prozone phenomenon, was similar, for both the whole antiserum and it's pH 3 eluant (50 µg). In contrast, the pH 8 eluant contained most of the bactericidal activity of the antiserum. This was consistent with the demonstrated presence of the serum IgM. The slight prozoning activity, shown by a very low dilution of the pH 8 eluant (see Table 4.3), was probably due to the low level of specific IgG antibody, as a little IgG was demonstrated in this eluant by immuno-electrophoresis (see Fig. 4.4A).

The results unequivocally showed that specific antibody of the IgG class, and not that of the IgM or IgA classes, is responsible for the Neisser-Wechsberg phenomenon. The results also agreed with those obtained by Normann (1972) and Normann *et al.*, (1973), involving the fractionation of rabbit antisera specific for *S. typhimurium* MRO 395 and *S. enteriditis*, respectively. They also agreed with the results obtained by Taylor (1972), who fractionated the prozoning sera of two human patients, convalescing from *Proteus mirabilis* infections. Two groups of workers (Daguillard and Edsall, 1968; Muschel *et al.*, 1969) reported prozoning activity in the IgM as well as IgG fractions of rabbit antisera specific for *S. typhi*. The

fractionation techniques had serious deficiencies, as described in 1.2.1, that may explain why prozoning activity was detected in the IgM fractions.

Although the results of this fractionation study clearly indicated that specific antibody of the IgA class did not possess prozoning activity, this antibody has been implicated in other studies. Thus, colostral IgA from rabbits vaccinated with *S. typhimurium* cells can inhibit the bactericidal activity of the serum IgG or IgM antibody (Eddie *et al.*, 1971). Hall (1950) reported that sera from humans chronically infected with, or convalescing from an acute infection with, *Brucella abortus*, are unable to kill this bacterium. Rabbits, hyperimmunized with this bacterium, display prozoning activity in their blood (Joos and Hall, 1968). Serum fractionation studies indicated that the IgA fraction is responsible (Hall *et al.*, 1971), although the contamination of this fraction by IgG and other serum proteins weakened the argument. Sera from patients convalescing from *Neisseria meningitidis* infection or from rabbits hyperimmunized with this bacterium, are also known to have prozoning activity (Thomas *et al.*, 1943; Griffiss, 1975). Griffiss reported that the IgA fraction from convalescing patients is not bactericidal and that it specifically inhibits the bactericidal activity of both the IgM and IgG fractions. He stated, however, that significant inhibition was observed only when an IgA level, far in excess of that in serum, was added to the bactericidal fractions. Recently, Russell-Jones and coworkers (1980) reported that monomeric (serum) and polymeric (secretory) IgA can inhibit the complement-mediated lysis of

red blood cells sensitized with IgG antibody. The polymeric form appeared to be 100 times more efficient as an inhibitor, on a molar basis, and this gave credence to the major finding of Eddie *et al.*, (1971).

These sources of evidence suggest that specific IgA antibody does inhibit the bactericidal action of IgG and IgM antibody, and complement-mediated cytolytic reactions, in general. This activity is presumably due to the failure to fix complement (Ishizaka *et al.*, 1965; Eddie *et al.*, 1971; Knop *et al.*, 1971; Colten and Bienenstock, 1974; Heddle *et al.*, 1975). However, it seems doubtful that specific IgA antibody is responsible for the prozoning activity of hyperimmune antisera; specific IgG antibody seems to be responsible, instead. The fractionation procedures in these previous studies were conducive to the formation of anti-complementary products or the concentration of IgA required to inhibit bactericidal IgG or IgM antibody was far in excess of what occurs in the whole serum (Griffiss, 1975).

Mouse IgG subclasses (IgG_1, IgG_{2a} and IgG_{2b}) can be simply isolated from whole serum by sequential acid elution from a protein A-Sepharose column (Ey *et al.*, 1978). The IgG_1 subclass has been shown to consist of two isotypes, one able and the other unable to fix complement upon association with antigen (Ey *et al.*, 1979; Ey *et al.*, 1980). The second isotype can interfere with the sensitization of target cells by the complement-fixing IgG_2 antibodies.

It seemed possible that the rabbit IgG fraction, found to be responsible for the NW phenomenon in the present study, could contain a subpopulation or even a subclass of IgG that

is unable to fix complement. Subclasses have not yet been defined for rabbit IgG, although distinct fractions have been prepared and characterized (Schlamowitz *et al.*, 1975). Specific antibody of this particular subpopulation or subclass may then compete with bactericidal IgM and IgG antibody to effect the prozone phenomenon. The sequential acid elution technique of Ey *et al.*, was used, in the present study, to isolate three (pH 6, pH 5 and pH 4 eluants) and possibly four (pH 7 eluant) IgG fractions from a prozoning antiserum (see Fig. 4.7). All showed prozoning, bactericidal, specific haemagglutinating and specific haemolytic activities (see Table 4.4). The fraction, containing all other serum proteins including IgM (pH 8 eluant), showed no prozoning activity but every other activity. Thus, although IgG subpopulations with different protein A binding characteristics could be isolated, they were all able to fix complement. The NW phenomenon cannot be caused by the competition between non-complement-fixing IgG antibody and bactericidal antibody for bacterial antigens. A form of hindrance eg., electrostatic or steric, of complement components by cell-bound IgG antibody, seems a likely explanation.

The effect of prozoning IgG antibody on the bactericidal activity of IgM antibody was also determined (see Table 4.6). M206 cells were first sensitized with the IgM fraction of an early antiserum. The IgM fraction possessed bactericidal activity but no prozoning activity. The same dilution of the prozoning IgG fraction was then able to inhibit the complement-mediated killing of these sensitized cells as was required to show prozoning with unsensitized cells.

The prozoning activity of the IgG fraction was therefore unaffected by the presence of bactericidal IgM antibody on the target cells. It emphasised the efficiency with which prozoning IgG antibody can convert a potentially lethal situation to a prozoning situation. This antibody behaved as a very avid antibody. It may have saturated antigenic sites about the already bound IgM antibody. However, the prozoning IgG antibody was more likely to displace the bound IgM antibody, due to its greater avidity and the equilibrium state between antibody and bacterial surface antigens.

Essentially, this chapter established that complement-fixing antibody of the IgG class, and of high avidity, is responsible for the Neisser-Wechsberg phenomenon. It is proposed that the phenomenon in systems, other than the one investigated in this study, is also due to this class of antibody. The next chapter discusses the various investigations into the nature and involvement of bacterial antigens in the phenomenon.

CHAPTER 4.4 SUMMARY

4.4.1 Specific IgG antibody, peaking in rabbit serum late (28 days) in the vaccination schedule with heat-killed *S. typhimurium* M206 bacteria, is responsible for the Neisser-Wechsberg phenomenon. Continuous vaccination is required for the maintenance of high prozoning activity and the antibody seems to be very avid.

4.4.2 Subpopulations of rabbit IgG exist with differing affinities for protein A. All, isolated from prozoning antiserum, fix complement and also exhibit prozoning and bactericidal activities. Hindrance of complement components by cell-bound IgG antibody seemed to be a likely mechanism of the NW phenomenon.

4.4.3 Specific antibody of the IgA or IgM class is not responsible for the NW phenomenon, although the inhibitory action of IgA antibody on the complement-mediated bactericidal reaction is acknowledged.

4.4.4 Prozoning IgG antibody efficiently inhibits the complement-mediated killing of target M206 cells, sensitized with bactericidal IgM antibody. It is suggested that the more avid IgG antibody displaces the less avid IgM antibody from the bacterial surface.

CHAPTER 5 INVOLVEMENT OF BACTERIA AND THEIR ANTIGENS IN THE NEISSER-WECHSBERG PHENOMENON

CHAPTER 5.1 Preamble

The two previous Chapters established that the Neisser-Wechsberg phenomenon was associated with target bacterial cells, rather than the fluid-phase, and that it was mediated by specific antibody solely of the IgG class. The investigations, described in the present Chapter, concern the bacterial antigens that interact with this antibody to cause the phenomenon.

Normal serum participates in the complement-mediated bactericidal reaction via the classical pathway and specific antibody, but another pathway also seems to be involved (Wardlaw and Pillemer, 1956; Götze and Müller-Eberhard, 1971; Root *et al.*, 1972; Reed and Allbright, 1974). Immune antiserum also participates in the reaction principally via the classical pathway. Bacterial LPS and, in particular, the O-somatic antigens of the polysaccharide component are recognized as the initial combining sites for specific antibody (Cundiff and Morgan, 1941; Adler, 1953; Muschel, 1960). This Chapter describes several experiments that were designed to show whether these bacterial antigens have a similar role in the NW phenomenon.

Mutant strains of *S. typhimurium* (0:1,4,5,12) and *S. minnesota* (0:21,26), possessing incomplete outer membrane LPS, were employed. Hyperimmune antiserum, specific for *S. typhimurium*, was adsorbed with whole cells or a cell wall component. The effect of such adsorptions, on the prozoning and bactericidal activities of the antiserum,

was then measured. The effect on the prozoning activity of a hyperimmune antiserum was also determined, after exposing target bacterial cells to EDTA, a chelating agent that modifies outer membrane LPS structure.

These investigations showed that the O-somatic antigens were the bacterial antigens participating in the NW phenomenon. This knowledge was then utilized to isolate specific antibody from an antiserum with good prozoning activity. The whole antiserum, or its IgG fraction, was adsorbed to and eluted from fixed bacterial cells or their LPS. Normann (1972) indicated problems associated with this procedure, although Daguillard and Edsall (1968) used it with apparent success. It was expected that the specific IgG antibody preparation would possess good prozoning activity and would give quantitative data on the prozone phenomenon. In particular, whether a finite number of specific IgG molecules were required to bind to target bacterial cells to cause this phenomenon; the number could then be calculated.

Normann *et al.*, (1972) reported that the prozoning titre of an antiserum is insensitive to bacterial numbers. This important finding has basic implications for the mechanism of the NW phenomenon. It seems to refute steric hindrance as the underlying mechanism. We have repeated and extended the work of Normann *et al.*, to include the prozoning IgG fraction as well as the whole antiserum, and the results are presented in this Chapter.

CHAPTER 5.2 RESULTS

A. Involvement of bacterial lipopolysaccharide in the Neisser-Wechsberg phenomenon

5.2.1 The effect of adding lipopolysaccharide to a prozoning mixture

In this experiment, various levels of LPS from *S. typhimurium* M206 were included with log-phase M206 cells in a bactericidal assay. Three dilutions of hyperimmune rabbit antiserum, covering prozoning and bactericidal situations in the absence of LPS, were tested.

Normally, 1:50 and 1:250 antiserum dilutions displayed prozoning whilst the 1:5000 dilution displayed killing (see Table 5.1). However, the addition of 25 µg resulted in only the 1:50 antiserum dilution showing significant prozoning activity. The 1:250 dilution showed complete killing. The situation changed as more LPS was added: 100 µg resulted in both the 1:50 and 1:250 antiserum dilutions showing complete survival of target cells.

The results again indicated that bacterial LPS has an important role in the NW phenomenon, as well as the bactericidal reaction. 25 µg of purified LPS was required to substantially reduce the prozoning activity of the antiserum with 5×10^3 target cells. A competition for the available antibody, between the added LPS and the target cells, was the likely explanation for the diminution in prozoning activity. This may have resulted in: (i) a level of specific antibody, lower than that required to cause prozoning, binding to the target cells, or (ii) that population of specific antibody with prozoning activity preferentially

TABLE 5.1

The effect of adding bacterial LPS to normally prozoning mixtures

M206 LPS added (μg)	% survival of bacteria[a]		
	Final antiserum dilution		
	1:50	1:250	1:5000
Nil	450	493	0
5	350	462	NT[b]
25	300	0	NT
50	200	8	NT
100	400	269	NT

[a] The mean value of bacteria added per tube = 5.2×10^3

[b] not tested

Three dilutions of G antiserum, covering prozoning and killing situations, were tested in a bactericidal assay with undiluted guinea pig complement. 5×10^3 log-phase M206 cells and various levels of M206 LPS were added to the tubes and the assay was begun.

binding to the free LPS. The first explanation implies steric hindrance whilst the second implies a more active role for antibody in the prozone phenomenon.

The complete survival of target bacteria, on addition of 100 µg of LPS, was probably due to all the available bactericidal antibody binding to the free LPS. There was insufficient antibody left to trigger the classical complement pathway on the target cells.

5.2.2 The effect of the chelating agent EDTA on the Neisser-Wechsberg phenomenon

(a) The minimum concentration of EDTA required to strip a significant level of LPS from the cell wall of *S. typhimurium* M206.

Aliquots of a suspension of log-phase M206 cells were incubated with various concentrations of EDTA. Viable counts were done at 0 and 15 minutes. The mixtures were then centrifuged and the level of released LPS (µg per ml), present in the supernatants, was determined by the haem-agglutination inhibition test (see 2.7.2).

The results (see Table 5.2) showed that M206 cells could be incubated in 0.0005 M EDTA/Tris buffer (at room temperature, for 15 minutes) with the removal of about half the LPS from the outer cell membrane, but without significant effect on cell viability. This 50% removal of LPS was the maximum figure obtained.

(b) The prozoning activity with M206 cells, pretreated with EDTA and subsequently incubated in nutrient broth

This study sought to establish whether the removal of

TABLE 5.2

The effect of various concentrations of EDTA on the LPS of the outer membrane of *S. typhimurium* M206

Final molarity of EDTA	% survival of bacteria[(a)]	LPS present in supernatant (µg/ml)
Nil	111	3.1
0.00025	104	6.3
0.0005	97	12.5
0.001	72	12.5
0.002	29	12.5
0.004	33	12.5

[(a)] The mean value of bacteria in each tube = 1.3×10^{10}

0.5 ml of log-phase M206 cells (1×10^{10}), suspended in Tris buffer + 1% BSA (pH 7.2), was added to 0.5 ml of 0.0005 M to 0.008 M solutions of EDTA in the same buffer. The mixtures were incubated at room temperature for 15 minutes, with viable counts being performed on dilutions in N-saline, at 0 and 15 minutes. The mixtures were then centrifuged at 10,000 g for 10 minutes at 20^{o}. The LPS level (µg/ml) in the supernatants was measured by the haemagglutination inhibition test, as described in 2.7.2. A 1:60 dilution of C-7 antiserum represented 2 haem-agglutination units.

part of the bacterial outer membrane LPS by EDTA would have a significant effect on the prozoning activity of antiserum, tested with the treated bacteria.

Log-phase M206 cells were preincubated with 0.0005 M EDTA/Tris buffer and Tris buffer alone. Viable counts were determined at 0 and 15 minutes. On dilution of the bacteria in nutrient broth (maintained at 37°), bactericidal assays were performed with a range of dilutions of hyperimmune antiserum. The broth containing EDTA-treated M206 cells was maintained at 37° for 90 minutes and then the bactericidal assay was repeated.

The concentration of EDTA used was sufficient to strip LPS from the bacteria, without reducing their viability (see Table 5.2). The dilution of these cells into broth ensured that the stripping of LPS was halted and that complement activity in the bactericidal assays was not impaired by EDTA.

Viable counts, before and after the preincubations in EDTA/Tris buffer and Tris buffer alone revealed over 100% survival of M206 cells (103% and 136%, respectively). Only the 1:800 antiserum dilution showed any prozoning activity in the three subsequent bactericidal assays (see Table 5.3). However, the survival of bacteria that had simply been diluted in nutrient broth was minimal (12% and 6%). The survival of bacteria, diluted and then incubated for 90 minutes, was complete (210%).

This difference was presumably due to the initial loss of outer membrane LPS during the preincubations with EDTA and the subsequent restoration of normal LPS structure during the incubation in nutrient broth. Thus, M206 cells

TABLE 5.3

Prozoning activity of an antiserum with *S. typhimurium* M206 cells, pretreated with EDTA and then incubated in nutrient broth

Final antiserum dilution	% survival of bacteria		
	Preincubation in Tris/dilution in nutrient broth[(a)]	Preincubation in EDTA/dilution in nutrient broth[(b)]	Preincubation in EDTA/incubation in nutrient broth[(c)]
1:800	6	12	210
1:1200	0	0	0
1:1600	0	0	1
1:2400	0	0	1
Nil	274	160	344

The mean value of bacteria added per tube = (a) 2.1×10^3
(b) 2.1×10^3
(c) 2.2×10^3

10^8 log-phase M206 cells were suspended in 10.0 ml of 1% BSA/Tris buffer, pH 7.2, and 0.0005 M EDTA/BSA/Tris buffer. Both suspensions were incubated at room temperature, with viable counts taken at 0 and 15 minutes. 1.0 ml of both was then diluted (1:200) in nutrient broth, warmed to 37°. Standard bactericidal assays were immediately performed with bacteria from both broths. Various dilutions of P-5 antiserum were tested with 1.2 CH50 units of HuC-14 in these assays.

The EDTA-treated cells in broth were incubated at 37° for 90 minutes. A standard bactericidal assay was then performed with the bacteria (diluted 1:4 in Mg^{2+} saline).

with a normal LPS showed higher prozoning activity than those with an incomplete LPS.

The difference in prozoning activity appeared to be two-fold. Although not large, it clearly indicated that the removal of LPS significantly reduced the prozoning activity of the antiserum, ie., a higher level of specific antibody was required to effect the prozone phenomenon.

5.2.3 The effect on the prozoning activity of anti-M206 antiserum of adsorbing with acetone-dried cells and cell wall protein of several Salmonella strains

The abrogation of the prozone phenomenon, by the addition of bacterial LPS to a bactericidal assay mixture (see 5.2.1), indicated that outer membrane LPS is involved in the phenomenon and that prozoning antibody is specific for the LPS. The following experiments sought to pinpoint the antigens within the LPS and to establish the involvement of other cell wall structures.

(a) Adsorption with acetone-dried cells

An aliquot of prozoning anti-M206 antiserum was first incubated with acetone-dried cells of *S. typhimurium* M206. After intensive centrifugation, the supernatant was diluted and tested, together with the unadsorbed antiserum, in a standard bactericidal assay with M206 cells.

Table 5.4 shows that the adsorbed antiserum had a prozoning titre of <1:200, whereas the unadsorbed antiserum had a titre of 1:600. The absorption step significantly reduced the prozoning activity.

The adsorption procedure with prozoning anti-M206 antiserum was repeated, using acetone-dried cells of

TABLE 5.4

Prozoning activity of an anti-M206 antiserum adsorbed with acetone-dried cells of *S. typhimurium* M206

Final antiserum dilution	% survival of M206 bacteria[a]	
	unadsorbed antiserum	adsorbed antiserum
1:200	150	6
1:400	100	2
1:600	73	3
1:800	9	1
1:1000	0	1
1:10000	0	0

[a] The mean value of bacteria added per tube = 6.7×10^3

An aliquot of G-antiserum was incubated with acetone-dried M206 cells (10 mg per ml) at 4^{o} for 60 minutes. The mixture was then centrifuged in the cold at 10,000 g for 10 minutes. The supernatant was diluted and tested together with the unadsorbed antiserum in a standard bactericidal assay. A 1:2.6 dilution of guinea pig serum was the complement source; 5×10^3 log-phase *S. typhimurium* M206 were added to each tube.

S. minnesota R595. Table 5.5 shows that, in this case, the adsorbed antiserum had a prozoning titre of 1:2000 whereas the unadsorbed antiserum had a titre of 1:1000. The adsorption step did not reduce the prozoning activity; if anything, it appeared to increase the activity.

The adsorption step with R595 cells presumably removed antibody specific for the common structures (cytoplasmic membrane, cell wall glycopeptide, and the lipid and KDO of the outer membrane LPS). Because there was no reduction in prozoning activity, the antibody responsible was not specific for these common structures. The rise in activity was an unexpected result. It presumably related to the removal of bactericidal antibody specific for the common structures or the consumption of complement by bacterial antigen-antibody complexes, remaining after the adsorption step.

The bactericidal activity of the adsorbed antiserum was also determined in a bactericidal assay with R595 cells. Final dilutions of 1:10 and 1:100 were unable to kill the target cells and so the absorption step removed all bactericidal antibody specific for the R595 strain. However, a final 1:10,000 dilution of anti-R595 antiserum efficiently killed the R595 cells (0% survival) in this assay, so proving that sufficient complement was present to kill the target cells.

The adsorption procedure with anti-M206 antiserum was again repeated, using acetone-dried cells of *S. typhimurium* TV119. Table 5.6 also shows that the prozoning titre of the antiserum (1:250) was unchanged by this procedure. Both adsorbed and unadsorbed antisera were also tested in

TABLE 5.5

Prozoning activity of an anti-M206 antiserum adsorbed with acetone-dried cells of *S. minnesota* R595

Final antiserum dilution	% survival of M206 bacteria[a]	
	unadsorbed antiserum	adsorbed antiserum[b]
1:500	150	150
1:1000	209	100
1:2000	20	216
1:4000	0	15
1:8000	0	0

[a] The mean value of bacteria added per tube = 6.1×10^3

[b] Final 1:10 and 1:100 dilutions of adsorbed antiserum showed 120% and 98% survival in a bactericidal assay with 7.1×10^3 *S. minnesota* R595 bacteria.

An aliquot of G-antiserum was incubated with acetone-dried R595 cells (10 mg per ml) at 4^{o} for 60 minutes. The mixture was then centrifuged in the cold at 10,000 g for 10 minutes. The supernatant was diluted and tested together with the unadsorbed antiserum in a standard bactericidal assay. A 1:2.6 dilution of guinea pig serum was used as the complement source; 5×10^3 log-phase *S. typhimurium* M206 cells were added to each tube.

TABLE 5.6

Prozoning activity of an anti-M206 antiserum adsorbed with acetone-dried cells of *S. typhimurium* TV119

Final antiserum dilution	% survival of M206 bacteria[a]	
	unadsorbed antiserum	adsorbed antiserum
1:50	414	411
1:100	601	430
1:250	506	641
1:500	3	3
1:1000	0	0
1:2000	0	0
1:4000	0	0
Nil	0	NT[b]

[a] The mean value of bacteria added per tube = 4.6×10^3

[b] not tested

An aliquot of G-antiserum was incubated with acetone-dried TV119 cells (15 mg per ml) at 4^{o} for 60 minutes. The mixture was then centrifuged in the cold at 10,000 g for 10 minutes. The supernatant was diluted and tested together with the unadsorbed antiserum in a standard bactericidal assay. Undiluted guinea pig serum was used as the complement source; 5×10^3 log-phase M206 cells were added to each tube.

a HA assay (see 2.7.1) with SRBCs, sensitized with C5 LPS and R595 cell wall protein (CWP). The unadsorbed antiserum showed titres of 1:2048 and 1:4096 with C5 LPS-SRBCs and R595 CWP-SRBCs, respectively. The corresponding titres for the adsorbed antiserum were 1:4096 and 1:2.

The HA assay indicated that, adsorption of the antiserum with this strain, removed antibody specific for outer membrane CWP. Antibody, specific for other cell wall antigens and common to both M206 and TV119 strains, was presumably removed. Antibody, specific for the O-somatic polysaccharide of the M206 strain, was presumably not removed. The similar haemagglutination titres with C5 LPS-SRBCs (before and after adsorption) was proof of this, as O-somatic polysaccharide antigens are the major antigens of the LPS.

This particular experiment provided evidence that the O-somatic antigens of the M206 strain were those interacting with the anti-M206 antiserum in the prozone phenomenon. Prozoning antibody appeared to be specific for these antigens. Adsorption of an antiserum with M206 cells had previously reduced its prozoning activity; adsorption with TV119 cells had no such effect. The TV119 strain lacks only the O-somatic antigens of the M206 strain, and so adsorption with TV119 cells did not remove antibody specific for these.

(b) Adsorption with cell wall protein

An aliquot of prozoning anti-M206 antiserum was incubated with *S. minnesota* R595 CWP. After centrifugation, the resulting supernatant and the original antiserum were tested in HA and passive haemolysis tests (see 2.7.1 and

2.6.1), with SRBCs that were sensitized with C5 LPS or R595 CWP.

Table 5.7 shows that both HA and haemolytic antibody, specific for C5 LPS, were unaffected by the adsorption step. This was expected because the adsorbing R595 CWP preparation was supposedly free of LPS, especially that of *S. typhimurium*. The results indicated that haemagglutinating antibody, specific for R595 CWP, was completely removed by the adsorption step. Haemolytic antibody, already present at a low level prior to adsorption, was significantly removed.

These preliminary tests showed that the adsorption step was successful in removing antibody, specific for the outer cell wall protein common to all Salmonellae, from the anti-M206 antiserum. The adsorption step did not remove antibody specific for the LPS of the M206 strain.

A standard bactericidal assay was performed with M206 cells to assess the prozoning activity of both the adsorbed and unadsorbed antiserum. Table 5.8 shows that the titres were identical (1:250). This meant that the removal of antibody, specific for CWP, did not affect the prozoning activity. Thus, the CWP was shown to be unimportant as an antigen in the prozone phenomenon.

5.2.4 A study of the Neisser-Wechsberg phenomenon with smooth and rough variants of a mutant *Salmonella typhimurium* strain

S. typhimurium G30 cannot synthesise galactose from glucose, nor can it metabolize galactose. Thus, when grown in the absence of this sugar, the outer membrane LPS lacks

TABLE 5.7

Specific antibody activity of anti-M206 antiserum adsorbed with cell wall protein of *S. minnesota* R595

Anti-M206 antiserum	Haemagglutination titre		Passive haemolytic titre	
	C5 LPS-SRBC	R595 CWP-SRBC	C5 LPS-SRBC	R595 CWP-SRBC
unadsorbed	1:2048	1:8192	1:1024	1:64
adsorbed	1:2048	<1:2	1:2048	1:16

2.0 ml of G-antiserum was incubated with the outer CWP of the R595 strain (20 mg) at 4^{o} for 60 minutes. The mixture was then centrifuged in the cold at 20,000 g for 30 minutes. The supernatant was diluted and tested together with the unadsorbed antiserum in haemagglutination (see 2.7.1) and passive haemolysis tests (see 2.6.1). SRBCs, sensitized with both C5 LPS and R595 CWP, were used in the tests.

TABLE 5.8

Prozoning activity of an anti-M206 antiserum adsorbed with cell wall protein of *S. minnesota* R595

Final antiserum dilution	% survival of M206 bacteria[(a)]	
	unadsorbed antiserum	adsorbed antiserum
1:50	200	167
1:100	200	200
1:250	168	161
1:500	20	20
1:1000	0	0
1:2000	0	0
Nil	0	4

(a) The mean value of bacteria added per tube = 7.2×10^3

A standard bactericidal assay was performed on various dilutions of G-antiserum, both unadsorbed and adsorbed with CWP of the R595 strain (see table 5.7). Undiluted guinea pig serum was used as the complement source; 5×10^3 log-phase *S. typhimurium* M206 cells were added to each tube.

the O-somatic polysaccharides and the terminal sugars (N-acetyl glucosamine, glucose and galactose) of the core polysaccharide. When grown in the presence of galactose, this strain contains all these sugars in its LPS and is therefore smooth.

(a) Prozoning and bactericidal activities of an anti-M206 antiserum

A prozoning anti-M206 antiserum was tested for prozoning and bactericidal activities, in bactericidal assays with both variants of the G30 strain as the target cells. The smooth variant was repeatedly shown to be resistant to the bactericidal action of complement, when Mg^{2+} saline was used as the diluent. However, this variant was shown to be susceptible in Tris buffer as diluent, in agreement with Reynolds and Rowley (1969). Tris buffer was therefore used as diluent in the assays.

Results obtained with the two variants were vastly different (see Table 5.9). With the rough variant, the antiserum showed no prozoning activity (titre <1:50) but a high bactericidal titre ($1:10^6$). With the smooth variant, the antiserum showed a high prozoning titre (1:1000) and a low bactericidal titre ($1:10^4$). The control tube, lacking antiserum, showed greater killing with the rough variant. This was in agreement with the general finding that rough strains of Gram-negative bacteria are more susceptible to the bactericidal action of complement.

The undetectable prozoning activity with the rough variant indicated that the prozoning antibody in the anti-M206 antiserum was specific for the polysaccharides,

TABLE 5.9

Prozoning and bactericidal activities of an anti-M206 antiserum with two variants of *S. typhimurium* G30

Final antiserum dilution	% survival of G30 bacteria grown in:	
	the absence of galactose[(a)]	the presence of galactose[(b)]
1:50	1	237
1:1000	7	111
1:10,000	3	0
1:100,000	2	64
1:1,000,000	7	107
1:10,000,000	48	99
Nil	38	91

The mean value of bacteria added per tube was (a) 1.5×10^4 and (b) 1.6×10^4

G30 cells were grown in brain heart infusion broth with and without 0.5% galactose. The prozoning and bactericidal activities of A-7 antiserum were determined by testing tenfold dilutions in bactericidal assays, with 10^4 log-phase cells of both types. 2.0 CH50 units of HuC-17 were added and the diluent was 0.1 M Tris buffer, pH 7.2.

missing from this variant but present in the smooth variant. These were the O-somatic polysaccharides. The higher bactericidal titre, shown with the rough variant, was consistent with the greater susceptibility of the variant, viz., a lower concentration of bactericidal antibody was required for killing.

(b) Exposure of the rough variant of *S. typhimurium* G30 to galactose and the effect on the prozoning activity of an anti-M206 antiserum

G30 cells were first grown up in the absence of galactose. A galactose fermentation test (see 2.1.4), performed on this initial culture, was negative. Log-phase cells were centrifuged down and suspended (5×10^8 per ml) in broth containing 0.5% galactose and broth lacking galactose. Both were then incubated at 37^{o}. Aliquots, taken at 0, 15 and 60 minutes, were diluted to provide the target cells in normal bactericidal assays with various dilutions of prozoning anti-M206 antiserum.

Galactose fermentation, acriflavine agglutination and 4-0 agglutination tests (see 2.1.4) were performed on both broth cultures after the 60 minute incubation. The fermentation tests were again negative, confirming the inability of this strain to metabolize galactose. The cells from the broth culture lacking galactose showed a positive acriflavine test and a negative 4-0 agglutination test. This was consistent with a rough strain of *S. typhimurium*, lacking at least the 4-0 (somatic) antigen. It presumably lacked the remaining ones also (1, 5 and 12). The cells from the broth culture containing galactose showed the opposite results. This was consistent with a smooth strain,

containing the 4-0 antigen, as well as the other somatic antigens.

These agglutination tests showed that, during the 60 minute incubation of the broth culture containing galactose, the G30 cells assembled the missing O-somatic antigens on the outer membrane LPS. They changed from the rough to the smooth variant.

Results of the bactericidal assays (see Table 5.10) were very clear and significant. There was no prozoning activity evident, when G30 cells were immediately suspended (0 minutes) in warm broth with or without added galactose. The titre was <1:50. However, after the cells had been incubated for only 15 minutes in the presence of galactose, significant prozoning activity (titre 1:500) was demonstrated. This activity did not appear to change, when the cells were incubated for a further 45 minutes in the presence of galactose. By this time, the O-somatic antigens were completely assembled on the outer membrane of the target cells, according to the results of the agglutination tests. These antigens were probably assembled after only 15 minutes of incubation, as the prozoning activity did not significantly change after this time.

Note that target cells, incubated in broth containing galactose for 15 and 60 minutes, survived in the control tube lacking antiserum. This was consistent with the transformation to the smooth variant, as smooth strains are more resistant to the bactericidal action of complement than rough strains (Dlabac, 1968; Muschel, 1965; Rowley, 1968). The G30 cells, that were incubated in the absence of galactose for 60 minutes, did not show any prozoning

TABLE 5.10

The effect on the prozoning activity of anti-M206 antiserum when log-phase *S. typhimurium* G30 cells are exposed to galactose

Final antiserum dilution	% survival of G30 bacteria incubated: in absence of galactose for:		in presence of galactose for:		
	0 min	60 min	0 min	15 min	60 min
1:50	1	1	0	318	237
1:500	1	2	0	316	427
1:5,000	0	0	0	1	0
1:50,000	0	0	0	19	4
Nil	0	1	2	261	227

Mean value of bacteria added per tube ($X10^{-3}$)				
7.0	5.3	5.6	5.7	4.6

2×10^9 log-phase G30 cells, grown in BH1B, were suspended in 2.0 ml of BH1B and BH1B + 0.5% galactose. The suspensions were then incubated at 37°. They provided the target cells in standard bactericidal assays: at 0 minutes both were diluted 1:10,000 in N-saline; at 15 minutes only the suspension in BH1B + galactose was so diluted; at 60 minutes both were diluted 1:40,000. Tenfold dilutions of A-7 antiserum were tested in the bactericidal assays with 2.0 CH50 units of HuC-17. Galactose fermentation tests were performed on the original and two subsequent cultures at 60 minutes. Acriflavine and 4-0 antiserum agglutination tests were also performed on the two subsequent cultures.

activity with the anti-M206 antiserum.

The overall results clearly indicated that the antibody, responsible for the prozone phenomenon in the antiserum, was specific for the O-somatic antigens (1, 4, 5 and 12). This prozoning antibody was also required to bind to the cell-bound antigens to cause the prozone phenomenon.

B. The attempted isolation of specific antibody with prozoning activity

5.2.5 Elution from whole bacterial cells and insolubilized outer membrane LPS

With the knowledge that prozoning activity was of the IgG class and was specific for the outer membrane LPS (the O-somatic antigens, in particular), the isolation of specific antibody with prozoning activity was attempted. Three isolation methods (see 2.10) were tried on either anti-M206 antiserum, with good prozoning activity, or the IgG fraction, from protein A-Sepharose chromatography (see 2.9.1(b)).

Specific antibody was efficiently adsorbed to the three types of particulate antigen source because on centrifugation, supernatants displayed markedly decreased titres in various assays. However, the bound antibody was eluted from the adsorbants only with great difficulty. Buffer of pH 3.0 was able to retrieve only a small fraction of the prozoning and bactericidal activities of the starting material (serum or its IgG fraction). These results implied that the antigen-antibody bond was very firm and therefore that the specific antibody with prozoning activity was very avid.

C. Influence of bacterial numbers on the prozone phenomenon

5.2.6(a) Whole antiserum

The effect of varying bacterial numbers on the prozoning activity of an antiserum was first determined. Doubling dilutions of an antiserum were tested with 5×10^3 and 5×10^5 cells of *S. typhimurium* M206, in separate bactericidal assays.

Table 5.11 shows that the prozoning titre of the antiserum was 1:800 with both numbers of target cells. % survival figures were very similar throughout the dilution series. The difference in cell numbers was actually 70-fold and yet the prozoning activity was unaffected.

Control tubes, lacking antiserum, showed incomplete killing with both numbers of target cells. This indicated that the complement source had retained some bactericidal antibody after absorption with live M206 cells (see 2.4.1).

(b) The IgG fraction of antiserum

A similar experiment to that in (a) was performed with doubling dilutions of the IgG fraction of a prozoning antiserum. The fractionation procedure and subsequent antibody tests is thoroughly described in 4.2.2. The prozoning titre of the IgG fraction was previously calculated to be 1:20.

Table 5.12 shows that the prozoning titre of the IgG fraction was 1:40, whether 5×10^3 or 2×10^5 M206 cells were incorporated into the bactericidal assay. The difference in cell numbers was therefore 35-fold and yet the % survival values were very similar throughout the dilution

TABLE 5.11

The effect of varying bacterial numbers on the prozoning activity of whole antiserum

Final antiserum dilution	% survival of bacteria added: 5.4×10^3	3.7×10^5
1:400	244	268
1:800	85	114
1:1600	1	2
1:3200	0	0
Nil	13	26

Doubling dilutions of the heat-treated P-5 antiserum were tested in two bactericidal assays. 2.0 CH50 units of HuC-11, and either 5×10^3 or 5×10^5 log-phase M206 cells were added to tubes.

TABLE 5.12

The effect of varying bacterial numbers on the prozoning activity of the IgG fraction of anti-M206 antiserum

Final dilution of the IgG fraction	% survival of bacteria added: 4.9×10^3	1.7×10^5
1:10	217	259
1:20	280	282
1:40	58	67
1:80	1	0
Nil	274	225

Doubling dilutions of the IgG fraction of S-6 antiserum were tested in two bactericidal assays. (The IgG fraction resulted from chromatography of the antiserum on a protein A-Sepharose column). 1.2 CH50 units of HuC-13, and either 5×10^3 or 2.5×10^5 log-phase M206 cells were added to tubes.

series. On first testing (see 4.2.2), this IgG fraction showed partial survival (16%) of target cells at 1:40. Correlation of the prozoning activity on the two occasions was therefore even closer than the titres suggested.

The prozoning activity of the IgG fraction was unaffected by increasing bacterial numbers. The results paralleled those obtained with whole antiserum.

CHAPTER 5.3 Discussion

The major antigens, that function in the complement-mediated bactericidal reaction with Gram-negative bacteria, are the O-somatic polysaccharides of the outer membrane LPS (Cundiff and Morgan, 1941; Adler, 1953; Muschel, 1960). They are believed to constitute the initial antibody binding sites where the classical pathway of complement is activated.

The outer membrane LPS has been assigned a similar role in the NW phenomenon. Thus, Norman *et al.*, (1972) incubated an antiserum, specific for *S. typhimurium* 395 MRO, with the purified LPS of this bacterium and found that the prozoning activity was significantly reduced (100-fold) by this adsorption. The 395 MRO strain, used in this study, is an Ra mutant ie., the outer membrane lacks O-somatic polysaccharides. For this reason, it was perhaps an unfortunate choice. Lipid A or core polysaccharide components of the LPS were presumably acting as antigens in both bactericidal and prozoning situations.

The present study concentrated on a smooth but serum-sensitive strain of *S. typhimurium* ie., M206, that contains all the components of outer membrane LPS (Jensen, 1929;

Archer and Rowley, 1969). Experimental evidence again suggested that LPS contained the antigens operating in the prozone phenomenon, and these antigens were identified as the O-somatic polysaccharides by the use of rough mutant strains.

The addition of pure M206 LPS (25 µg per ml) to bactericidal assay mixtures significantly reduced the prozoning activity of a hyperimmune rabbit antiserum (see table 5.1). The addition of more LPS (100 µg per ml) eventually abrogated the bactericidal activity as well. These results implicated the outer membrane LPS in both phenomena. There was competition for the available antibody between the fluid-phase LPS and the cell-bound LPS in the assay mixtures.

Anti-M206 antiserum showed lower prozoning activity with M206 cells when they had been preincubated in 0.0005 M EDTA/Tris buffer (see table 5.3). The chelating agent EDTA solubilizes (Gray and Wilkinson, 1965), and therefore removes a significant amount of outer membrane LPS from Gram-negative bacteria (Lieve *et al.*, 1968). The latter are then more susceptible to the bactericidal action of complement (Muschel and Gustafson, 1968; Reynolds and Pruul, 1971a). LPS was again implicated in the prozone phenomenon: M206 cells, whose LPS was partially removed by EDTA, required larger amounts of specific antibody to display the prozone phenomenon.

The prozoning activity was reduced by the EDTA treatment, but not severely reduced or completely abrogated. The relatively short treatment removed approximately 50% of the outer membrane LPS (Reynolds and Pruul, 1971b).

There was little competition between the released (soluble) LPS and cell-bound LPS for available antibody, as the released LPS was diluted by a factor of 2000 prior to the bactericidal assay. The observed reduction in the prozoning activity must therefore be explained by the disorganization caused to the outer membrane of the target cells.

The involvement of outer membrane protein in the NW phenomenon was proven to be insignificant. Adsorption of an anti-M206 antiserum with this protein, purified from *S. minnesota* R595 (Schnaitman, 1974), had no effect on the prozoning activity of that antiserum (see table 5.8). Haemagglutination assays showed that anti-CWP antibody had been efficiently removed but that antibody, specific for LPS components, had not (see table 5.7).

The involvement of O-somatic antigens in the NW phenomenon, in the system studied, was proven in several ways. The adsorption of anti-M206 antiserum with acetone-dried cells of *S. minnesota* R595 (see table 5.5) or *S. typhimurium* TV119 (see table 5.6) failed to reduce its prozoning activity. R595, an Re mutant strain, contains only lipid A, KDO and ethanolamine in the outer membrane LPS (Luderitz *et al.*, 1966). They were obviously not the antigens, operating in the prozone phenomenon. Neither were the antigens present in the inner cell membrane, glycopeptide layer or the glycolipid component of the outer membrane. TV119, an Ra mutant strain, only lacks the O-somatic polysaccharide, present in smooth strains of *S. typhimurium* (Nikaido *et al.*, 1964). Thus, the antigens operating in the phenomenon, in the system studied, were

not even part of the core polysaccharide of the outer membrane LPS.

Normann *et al.*, (1972) used an Ra strain, instead of a smooth strain of *S. typhimurium*, to study the NW phenomenon. Prozoning antibody was presumably specific for lipid A or the core polysaccharide of the LPS. Together with the data presented in this Chapter, it indicates that antibody, with prozoning activity, is specific for the O-somatic or other antigens of the LPS depending on the nature of the immunogen. If O-somatic antigens are present, the antibody will be specific for these.

S. typhimurium G30 is an Rc mutant strain, lacking UDPG-epimerase (Osborn *et al.*, 1964). It cannot synthesise galactose from glucose, nor can it ferment galactose. When grown in the absence of galactose, the outer membrane LPS lacks both O-somatic polysaccharides and the terminal sugars (N-acetyl glucosamine, glucose and galactose) of the core polysaccharide.

Hyperimmune anti-M206 antiserum showed no prozoning activity but high bactericidal activity with this rough variant (see table 5.9). When cells of the rough variant were exposed to galactose in the culture medium, the antiserum then showed high prozoning activity but a lower bactericidal activity (see table 5.10). This change occurred in a short space of time (15 minutes). The G30 cells underwent a change from the rough to the smooth variant, as acriflavine and 4-0 agglutination tests showed. The missing terminal sugars of the core polysaccharide and sugars of the O-somatic polysaccharides were assembled on the incomplete outer membrane LPS. The assembled sugars -

the O-somatic polysaccharides, in particular - obviously functioned as the antigens in the prozone phenomenon in this system, with the smooth M206 strain as immunogen.

Three methods of isolating specific antibody with prozoning activity, from anti-M206 antiserum or its IgG fraction, were attempted. They all involved adsorption to insoluble matrices, containing outer membrane, at neutral pH and subsequent elution at low pHs. Aldehyde-fixed C5 cells, aldehyde-fixed C5 LPS (Eskenazy *et al.*, 1975) and SRBC stromata, sensitized with C5 LPS, were tried as matrices. Antibody assays revealed that specific antibody bound to each matrix very efficiently but was not eluted very efficiently. A maximum 10% of the original prozoning activity was retrieved by elution at pH 3. It was hoped that a preparation of specific IgG antibody could have afforded quantitative data regarding the binding of IgG molecules to target cells, in the prozone situation.

These findings agreed with the opinion of Normann *et al.*, (1972). They did not publish experimental data but stated that the LPS-prozoning antibody complex is not significantly dissociated at pH 2.5. Daguillard and Edsall (1968) adsorbed specific antibody to acetone-dried Salmonella cells and then eluted it at pH 3.5. They subsequently detected both prozoning and bactericidal activities in IgM and IgG antibody fractions. These results were not considered valid due to flaws in experimental technique eg., the prolonged incubation at pH 3.5 to elute specific antibody probably destroyed complement-fixing activity (Stollar *et al.*, 1976) and removed bacterial components.

The observed stability of the O-somatic antigen-

prozoning antibody bond indicated that this antibody was of high avidity. This is consistent with recognized facts regarding the immune response. The avidity of all classes of immunoglobulin increases with time, after primary and secondary immunizations with Gram-negative bacteria (Gupta and Reed, 1971; Schulkind *et al.*, 1972; Holmgren and Svennerholm, 1972; Ahlstedt *et al.*, 1973). Because of the high affinity of prozoning antibody, the frequency of dissociation from target cells in a bactericidal assay would be low. This is an important aspect to consider, when formulating a theory to explain the NW phenomenon.

Normann *et al.*, (1972) made the surprising observation that the prozone phenomenon was independent of bacterial cell concentration. The present work confirmed this: bacterial concentration was varied 70-fold, with no effect on the prozoning activity of anti-M206 antiserum (see table 5.11). Chapter 4 established that specific antibody of the IgG class constituted the antibody with prozoning activity. In the present Chapter (see Table 5.12), bacterial concentration was varied 35-fold, with no effect on the prozoning activity of such an IgG fraction.

This independence of bacterial cell concentration needs to be seriously considered in the formulation of a theory for the NW phenomenon. It seemed to contradict the hypothesis of cell saturation, and steric hindrance of complement components, by specific antibody. However, approximately 25 µg of pure M206 LPS - the amount present on 10^{10} whole M206 cells - was able to abrogate this prozone phenomenon (see table 5.1). An important factor is that less complement, as well as less antibody, was

available per target cell, as the concentration of these cells was increased. Perhaps, the NW phenomenon is dependent on the number of specific antibody molecules, bound per bacterial cell, (instead of the concentration of these molecules in the assay mixture) as well as the complement available per cell. With a larger number of target cells but the same complement concentration, the probability of all components assembling at any one lethal site was reduced. A particular dilution of antiserum or the IgG fraction, that previously saturated such sites, did not do so with the target number of cells. However, less complement was available per target cell. Normann *et al.*, (1972) preferred to abandon the hypothesis of cell saturation altogether and instead proposed that complement is deviated in the NW phenomenon.

A theory for the mechanism of the NW phenomenon can be proposed only after a comprehensive study of all the major parameters of the serum bactericidal reaction. The work so far has focused on the involvement of bacterial cells and antibody. Next, we concentrate on the involvement of complement components.

CHAPTER 5.4 Summary

5.4.1. The outer membrane LPS of *S. typhimurium* was shown to contain the antigens, operating in the NW phenomenon with hyperimmune anti-M206 antiserum. They were further identified as the O-somatic polysaccharides. The phenomenon also seemed to be potentiated when smooth target cells, possessing a maximum level of such antigens in the LPS, were used.

5.4.2. The isolation of specific antibody with prozoning activity, from anti-M206 antiserum or its IgG fraction, was unsuccessful. Several methods, all adopting adsorption to and subsequent elution from fixed bacterial cells or the LPS, were tried. Specific antibody bound so firmly that very severe physical conditions were required to elute it. Prozoning antibody was therefore considered to be very avid.

5.4.3. The prozoning activity of anti-M206 antiserum, and of the IgG fraction, appears to be independent of the number of target cells in a bactericidal assay. This has important consequences, when formulating a theory to explain the NW phenomenon.

CHAPTER 6 THE ROLE OF COMPLEMENT IN THE NEISSER WECHSBERG PHENOMENON

CHAPTER 6.1 Preamble

Work, previously discussed in Chapter 3, established that there was no appreciable consumption of haemolytic or bactericidal complement in the fluid-phase of a prozoning mixture. Thus, anticomplementary serum factors and soluble bacterial antigens were unimportant in this regard. It was necessary to establish whether any such consumption occurred on the sensitized target cells i.e., in the cellular phase. Muschel *et al.*, (1969) reported considerable haemolytic complement consumption in the prozoning situation, but they attributed the consumption to anti-complementary factors, present in the prozoning antiserum. Thomas and Dingle (1943) reported no haemolytic complement consumption, in another example of the NW phenomenon, while other workers (Hall *et al.*, 1971; Griffiss, 1975) have inferred no complement consumption, by implicating non-complement-fixing IgA antibody in this phenomenon. Specific IgG antibody has already been shown to be responsible for the NW phenomenon in the present study (see 4.2.2) and so the extent of activation and consumption of complement by sensitized target bacteria was the first important issue to be resolved.

From the first description (Neisser and Wechsberg, 1901) onwards (Thomas *et al.*, 1943; Thomas and Dingle, 1943; Joos and Hall, 1968; Muschel *et al.*, 1969), workers have recognized that the NW Phenomenon can be abrogated by increasing the concentration of complement in bactericidal assay mixtures. It was important to confirm this finding in the present study and to extend it to show whether (i) there was a direct inverse relationship between the

prozoning titre of an antiserum and the complement concentration, and (ii) a similar relationship could be demonstrated for the IgG fraction of such an antiserum.

Complement consumption, and therefore activation, was shown to occur on sensitized target cells in the prozoning situation. A modification or a deviation of the cytolytic pathway of complement could therefore be assumed to occur in this situation. It was then important to establish at which step in this pathway, the deviation was occurring. According to Muschel and co-workers (1969), the addition of C-EDTA abrogates the prozone phenomenon in the immune haemolytic system. They suggested that the deviation in the haemolytic system occurs after the activation of C1 and C2 of the classical pathway. They also demonstrated immune adherence with prozoning SRBCs and proposed that C3 is activated on such cells. These workers assumed that these conclusions were also relevant to the prozone phenomenon in the serum bactericidal reaction. The present work makes no such assumption. Biological consequences of complement activation, such as immune adherence (Nelson, 1963) and deviated lysis (Rother *et al.*, 1974), were studied in the bactericidal system to prove the activation of specific complement components in the prozone situation. R reagents, complement sources deficient in specific components, were also used in bactericidal assays to achieve this goal.

CHAPTER 6.2 Results

A. Influence of complement concentration on the prozoning activity

6.2.1(a) Whole antiserum

A hyperimmune (rabbit) anti-M206 antiserum was tested

for prozoning activity in standard bactericidal assays, with doubling concentrations of a (human) complement source.

Table 6.1 shows the results. Prozoning titres with 1.0, 2.0, 4.0 and 8.0 CH_{50} units of complement were 1:400, 1:200, 1:50 and <1:50, respectively. A closer inspection of the % survival values revealed that the 1:100 dilution showed significant prozoning activity with 4.0 CH_{50} units of complement. Therefore, not only did the prozoning activity of the antiserum fall with increasing complement concentration, but it appeared to be inversely proportional to complement concentration.

The control tubes, lacking antiserum, showed complete survival with 1.0 CH_{50} unit ranging through to complete killing with 8.0 CH_{50} units. This pattern reflected the level of bactericidal antibody present in the complement source. There was sufficient antibody, in the complement source providing 4.0 and 8.0 CH_{50} units, to cause efficient killing.

(b) An IgG fraction of antiserum

A similar experiment was then performed on doubling dilutions of an IgG fraction (concentrated pH 6 eluant) of the same antiserum. The fractionation procedure and subsequent antibody tests are thoroughly described in 4.2.3. The pH 6 eluant was shown to contain IgG as the only serum protein and to have a prozoning titre of 1:10 (see table 4.4). It therefore contained a significant fraction of the prozoning activity of the antiserum (approximately 1/5).

The pH 6 eluant was concentrated by a factor of 5 before being used in this experiment. The results are shown

TABLE 6.1

The effect of varying complement concentration on the prozoning activity of anti-M206 antiserum

Final antiserum dilution	% survival of M206 bacteria[(a)] CH50 units of complement added:			
	1.0	2.0	4.0	8.0
1:50	240	237	121	12
1:100	257	239	31	0
1:200	275	119	0	0
1:400	153	1	0	0
1:800	1	0	0	1
Nil	141	68	7	0

(a) The mean value of bacteria added per tube = 5.5×10^3.

Doubling dilutions of C-8 antiserum were tested in four bactericidal assays with 5×10^3 log-phase M206 cells. 1.0, 2.0, 4.0 and 8.0 CH50 units of HuC-15 were added to the mixtures.

in table 6.2. With 1.2 CH_{50} units, the concentrated eluant showed a prozoning titre of 1:20 while, with 4.8 CH_{50} units, it showed a titre of <1:10. The prozoning activity was thus reduced fourfold or more, by increasing the complement concentration fourfold. This was consistent with results obtained with the whole antiserum. The experiment also supported the idea that specific IgG antibody was responsible for the prozone phenomenon.

Part of the prozoning activity of the pH 6 eluant seemed to be lost through the concentration procedure, as the titre was expected to be 1:40 with 1.2 CH_{50} units. The control tubes, lacking antiserum, again showed complete survival with the lower complement concentration and complete killing with the higher one. This reflected the level of bactericidal antibody in the complement source.

B. Haemolytic complement consumption in the prozone phenomenon

6.2.2(a) 2×10^8 target cells

In this vital experiment, doubling dilutions of a prozoning anti-M206 antiserum were first tested in a bactericidal assay with log-phase cells of *S. typhimurium* M206 (2×10^8 in a reaction volume of 3.0 ml). The haemolytic complement, remaining in the mixtures, was then determined by centrifuging off the bacteria and then performing a haemolytic titration (see 2.4.7) on the supernatants and a freshly-diluted sample of the complement source.

Figure 6.1 shows the plot obtained in the titration of the human complement source. It is an example of the type obtained in the titration of new complement sources. In

TABLE 6.2

The effect of varying complement concentration on the prozoning activity of an IgG fraction of anti-M206 antiserum

Final dilution of conc. pH 6 eluant	% survival of M206 bacteria[a]	
	CH50 units of complement added:	
	1.2	4.8
1:10	>150	3
1:20	>150	0
1:40	3	0
1:80	0	0
1:160	0	0
Nil	>100	0

[a] The mean value of bacteria added per tube = 1.5×10^4

Doubling dilutions of an IgG fraction (the concentrated pH 6 eluant, from stepwise chromatography on a protein A-Sepharose column) of C-8 antiserum were tested in two standard bactericidal assays with 5×10^3 log-phase M206 cells. 1.2 and 4.8 CH50 units of HuC-15 were added to the mixtures.

FIGURE 6.1

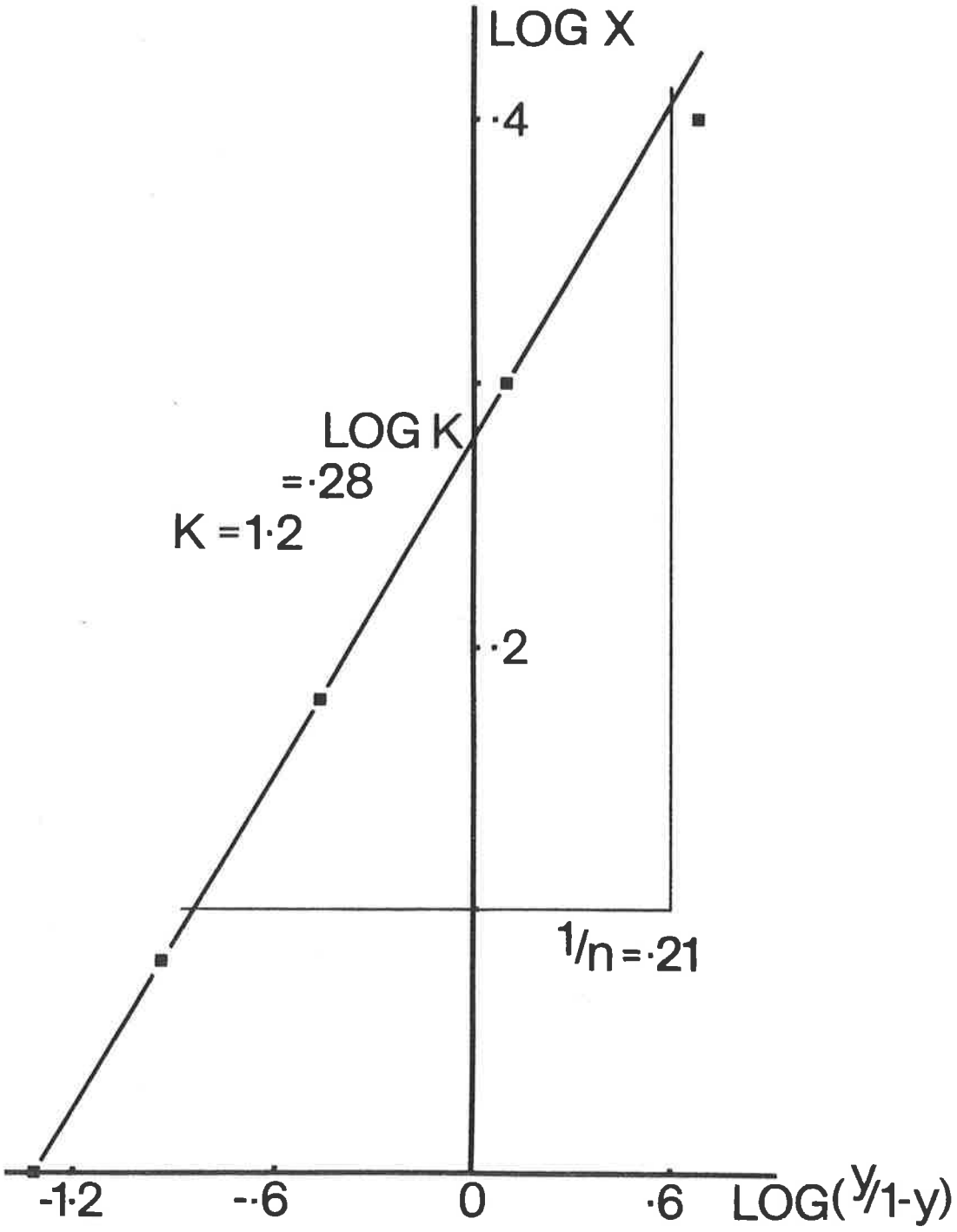
Haemolytic titration of HuC-14

A 1:20 dilution of the HuC-14 strength used in bactericidal assays (a 1:5.2 dilution) was made up in VBS. Five volumes (0.5 ml - 1.25 ml) were tested in a haemolytic complement titration (see 2.4.7). Appropriate control tubes were included and OD_{541nm} values of the supernatants were calculated.

A plot of log X vs log $(^{Y}/1-Y)$ was drawn up, where X = serum dilution and y = the corresponding fraction of SRBCs lysed. The Von Krogh equation,

$$\log X = \log K + {}^{1}/n \log (^{Y}/1-y),$$

was then cited. A value for $^{1}/n$ (the gradient) and log K (the intercept on the X-axis) was then calculated. K represented the volume of the diluted complement to cause 50% haemolysis.


LOG X
·4
LOG K
=·28
K =1·2
·2
1/n =·21
-1·2
-·6
0
·6
LOG(y/1-y)

this instance, the plot yielded an X-intercept (log K) of 0.28 and so the undiluted complement source (HuC-14) contained 54.7 CH_{50} units per ml. This meant that 3.2 CH_{50} units were added to the bactericidal assay tubes. The gradient (1/n) was 0.21 and this was close to the ideal value of 0.20 (Williams and Chase, 1977).

In the bactericidal assay, antiserum dilutions of 1:100 and 1:200 were shown to be strongly prozoning, whilst dilutions of 1:800 and 1:1600 were bactericidal (see Table 6.3). Control tubes, lacking either antiserum or complement, showed complete survival. The lower % survival figure for the tube lacking antiserum was probably due to the presence of a low level of bactericidal antibody in the complement source.

The subsequent haemolytic titrations revealed that there was considerable haemolytic complement consumption in the prozoning and bactericidal situations. Only 0.3 or 0.4 out of 3.2 CH_{50} units remained in the tubes after the bactericidal assay.

Control tubes, lacking either antiserum or bacteria in the bactericidal assay, showed a significantly lower complement consumption or loss. The tube, lacking antiserum, lost 1.4 out of 3.2 CH_{50} units of complement during the bactericidal assay. Exposure of the diluted complement source to the various temperatures in this experiment probably depleted the more temperature-sensitive complement components. There would also have been some consumption of complement by the alternative pathway and by the classical pathway, since the complement source contained some bactericidal antibody.

TABLE 6.3

Haemolytic complement consumption in prozoning and killing situations with 2×10^8 *S. typhimurium* M206 cells

Final antiserum dilution	% survival of M206 bacteria[a]	CH50 units of complement consumed during the bactericidal assay[b]
Nil	145	1.4
1:100[c]	-	1.7
1:100[d]	299	-
1:100	322	2.9
1:200	269	2.9
1:400	14	2.9
1:800	0	2.9
1:1600	1	2.8

(a) The mean value of bacteria added per tube = 1.8×10^8

(b) 3.2 - (CH50 units remaining in the supernatant)

(c) control tube minus M206 cells

(d) control tube minus complement

Doubling dilutions of P-5 antiserum (1:100 to 1:1600) were first tested in a bactericidal assay with 2×10^8 log-phase M206 cells and approximately 3.6 CH50 units of HuC-14, in a total volume of 3.0 ml. At 60 minutes, the tubes were centrifuged at 4° (10,000 g for 20 minutes). Two volumes (1.0 and 1.25 ml) of the supernatants were then tested in a haemolytic complement titration, together with dilutions of HuC-14. Log K values from the Von Krogh equation,

$$\log X = \log K + {}^{1}/_{n} \log ({}^{y}/_{1-y}),$$

were estimated for the supernatants ($^{1}/_{n}$ was calculated to be 0.21). The number of CH50 units of complement remaining in each supernatant was thus calculated.

The tube, lacking bacteria, lost 1.7 out of 3.2 CH_{50} units. The slightly higher figure could again be partly explained by the depletion of temperature-sensitive components. There could also have been some bacterial components, eg., LPS, in the complement source due to absorption techniques (see 2.4.1), and these would have acted as antigens to trigger either complement pathway.

(b) 1 x 10^4 target cells

The importance of bacterial numbers in such studies was well-illustrated in a similar experiment, that used a much lower number of M206 cells (1 x 10^4 in a reaction volume of 3.0 ml). The same prozoning antiserum but a different batch of HuC was used.

As can be seen in Table 6.4, antiserum dilutions of 1:200 and 1:400 were strongly prozoning whilst further dilutions were bactericidal. The prozoning titre of 1:400 was only two-fold higher than that in the previous experiment, although the complement concentration was doubled and the number of target cells was reduced by a factor of 20,000. The control tube, lacking antiserum, showed complete killing and this could be attributed to the presence of a relatively high level of bactericidal antibody in the dilution of HuC used.

The subsequent haemolytic titrations revealed that approximately one third of the available haemolytic complement had been lost in all tubes, during the bactericidal assay. There was no significant difference between any of the tubes. The results indicated that, although complement was consumed in the killing situation, and

TABLE 6.4

Haemolytic complement consumption in prozoning and killing situations with 1×10^4 *S. typhimurium* M206 cells

Final antiserum dilution	% survival of M206 bacteria [a]	CH50 units of complement consumed during the bactericidal assay [b]
Nil	0	2.03
1:200 [c]	-	2.25
1:200 [d]	269	-
1:200	338	2.21
1:400	364	2.29
1:800	1	2.07
1:1600	0	2.01
1:3200	0	2.03

[a] The mean value of bacteria added per tube = 8.1×10^3

[b] 6.0 - CH50 units remaining in the supernatant

[c] control tube minus M206 cells

[d] control tube minus complement

Doubling dilutions of P-5 antiserum (1:200 to 1:3200) were first tested in a bactericidal assay with 10^4 log-phase M206 cells and 6.0 CH50 units of HuC-12, in a total volume of 3.0 ml.

At 60 minutes, the tubes were centrifuged at 4° (10,000 g for 20 minutes). The supernatants were diluted in cold VBS and a haemolytic titration was performed on each. The number of CH50 units of complement remaining in each supernatant was thus calculated.

probably in the prozone situation also, the level involved was small, due to the low number of M206 cells used. The majority of the consumed complement appeared to be lost because of physical factors in the experiment eg., variations in temperature. It had been shown repeatedly that 0.5 - 1.0 CH_{50} units of HuC were required to kill 5×10^3 M206 cells in a normal bactericidal assay; sufficient complement therefore remained in the tubes, showing prozoning in this experiment, to effect killing of the target cells.

C. Examination of possible steps, in the complement cascade, whose modification results in the Neisser-Wechsberg phenomenon

Section B established that complement was activated and consumed during the prozone phenomenon. The cytolytic pathway, though initially activated, was not going to completion. The following studies sought to pinpoint the actual step that was being modified to cause the phenomenon.

6.2.3 Evidence for the fixation of the first three components of the classical pathway in the prozone phenomenon

Log-phase *S. typhimurium* M206 cells were first incubated with a prozoning and a killing dilution of a hyperimmune rabbit antiserum, and undiluted GpC at 37^{o} or its C142 preparation (see 2.4.5) at 4^{o}.

A standard bactericidal assay was performed on the mixtures containing whole GpC. % survival values were 248% and 0% with final antiserum dilutions of 1:50 and 1:5000, respectively. This initial assay proved that a prozoning

and a bactericidal dilution of antiserum was used to sensitize the target cells.

The mixtures containing GpC-142 were incubated at 4° for 20 minutes. The bacteria were centrifuged, diluted and added to bactericidal assay mixtures containing two dilutions of the hyperimmune antiserum and GpC reagents - whole GpC, GpC-EDTA (see 2.4.3) and C-EGTA (see 2.4.4). Standard bactericidal assays were then performed.

Control tubes, lacking additional antiserum, but containing GpC, GpC-EDTA or GpC-EGTA all showed killing (see Table 6.5); only those with GpC-EDTA showed incomplete killing. The results indicated that at least C1, C4 and C2 of the classical pathway of complement had bound to the M206 cells, initially incubated with the prozoning dilution of antiserum and GpC, or GpC-142. GpC-EDTA provides the components C3 to C9 whilst GpC-EGTA provides C2 to C9. Thus, C142 must have bound and been activated on the cells, initially incubated with the prozoning (1:50) and killing (1:5000) dilutions of antiserum. An excess of at least C3 to C9 components, provided by all three GpC reagents, was sufficient to abrogate the prozone phenomenon. GpC-EGTA and whole GpC were more efficient in this regard, possibly because the alternative pathway was also activated and aided the killing.

The assay mixtures containing additional antiserum all showed prozoning at the lower dilution (1:250) and killing at the higher dilution (1:5000). The % survival values were significantly higher in the prozoning situations, where the cells had been preincubated with a prozoning level of antiserum. These results had

TABLE 6.5

Fixation of C1, C4 and C2
complement components by prozoning
S. typhimurium M206 cells

1.0 ml volumes of bactericidal assay mixtures were first made up, on ice. They consisted of 2.5×10^6 log-phase M206 cells; 1:50 (prozoning) or 1:5000 (bactericidal) dilutions of G antiserum, and; 1:10 dilutions of GpC or GpC-142 (see 2.4.5).

A standard bactericidal assay was performed on those mixtures containing GpC.

The mixtures containing C-142 were incubated at 4^{o} for 20 minutes and then centrifuged at 10,000 g for 10 minutes. The M206 cells were diluted to 5×10^4 per ml in Mg^{2+} saline, and 0.1 ml volumes were added to another set of bactericidal assay mixtures. The final 1.0 ml volumes also consisted of 1:250 and 1:5000 dilutions of G-antiserum and various GpC reagents (1:10 dilutions of GpC, GpC-EDTA or GpC-EGTA). A standard bactericidal assay was then performed on these mixtures.

TABLE 6.5

Fixation of C1, C4 and C2 complement components by prozoning *S. typhimurium* M206 cells

Complement source	Final antiserum dilution	% survival of M206 bacteria[a] preincubated with C142 and: 1:50 antiserum dilution[b]	1:5000 antiserum dilution[c]
GpC	Nil	3	0
GpC	1:250	300	78
GpC	1:5000	0	0
GpC-EDTA	Nil	31	21
GpC-EDTA	1:250	195	66
GpC-EDTA	1:5000	0	0
GpC-EGTA	Nil	0	1
GpC-EGTA	1:250	218	75
GpC-EGTA	1:5000	0	2
heated[d] GpC	Nil	123	NT[e]
heated GpC-EDTA	Nil	111	NT
heated GpC-EGTA	Nil	179	NT

[a] The mean value of bacteria added per tube = 6.3×10^3

[b] prozoning

[c] bactericidal

[d] 56^{o} for 30 minutes

[e] not tested

interesting implications:

(a) the presence of a prozoning level of antibody on M206 cells did not protect these cells, on the addition of a bactericidal level of antibody and GpC reagents. Control tubes, lacking antiserum, indicated that the resulting killing was due to the excess C3 to C9 components provided by these reagents,

(b) the presence of a bactericidal level of antibody on M206 cells was not sufficient to kill these cells, on the addition of a prozoning level of antibody and GpC reagents. Control tubes, lacking antiserum, indicated that activated C142 was present on the washed target cells and that only C3 to C9 components were required to kill these cells. There appeared to be a competition between specific antibody and complement components in binding to the washed M206 cells,

(c) the presence of a prozoning level of antibody on the washed cells potentiated the prozone phenomenon, on the addition of further prozoning antiserum and GpC reagents. This again indicated a competition between specific antibody and complement components for the cells.

6.2.4 Immune adherence by "prozoning bacterial cells" as a measure of C3 activation

A range of dilutions of hyperimmune anti-M206 antiserum was first tested in a bactericidal assay with *S. typhimurium* C5 cells (1×10^8 per ml). Tris buffer, pH 7.2, was used as a diluent for this normally serum-resistant strain (Reynolds and Rowley, 1969; Reynolds and Pruul, 1971a). After the assay, the cells were centrifuged, washed and

resuspended in VBS. Doubling dilutions were then tested with human RBCs in an immune adherence assay (see 2.6.2).

The results are shown in Table 6.6. The 1:50 antiserum dilution was the only one to display prozoning; the remaining dilutions showed good killing in the bactericidal assay. Control tubes, lacking complement or antiserum, showed no killing and partial killing, respectively.

Immune adherence was demonstrated to the same degree with the washed bacteria, from tubes containing antiserum dilutions of 1:50 to 1:800 (see Table 6.6). The lowest concentration of prozoning cells to demonstrate immune adherence was 7×10^7 per ml.

The results indicated that C3 bound to the target bacteria and cleaved to form the biologically-active C3b, in both prozoning and killing situations.

A similar experiment to the one just described was then performed, with Tris buffer, EGTA/Tris buffer and EDTA/Tris buffer as diluents in the bactericidal assays.

Table 6.7 shows that immune adherence was again demonstrated in both the prozoning and killing situations, with Tris buffer as diluent. The titre (1.8) was four-fold higher with the prozoning cells. This indicated that significantly more C3 was activated and more C3b was formed on the prozoning bacteria than on the killed ones. The lowest concentration of prozoning cells to show immune adherence was 5×10^7 per ml. This value agreed with that obtained in the previous experiment.

With EGTA/Tris buffer as diluent, prozoning (1:50 antiserum dilution) and killing (1:5000 antiserum dilution) situations were again demonstrated, with the target cells

TABLE 6.6

Demonstration of Immune Adherence by prozoning *S. typhimurium* C5 cells

Bactericidal assay			Immune Adherence assay	
CH50 units of HuC-15	Final antiserum dilution	% survival of C5 bacteria[(a)]	Immune adherence	Titre[(b)]
Nil	1:50	122	Negative	ND[(c)]
9.6	Nil	81	Negative	ND
9.6	1:50	101	Positive	1:4
9.6	1:200	12	Positive	1:4
9.6	1:800	7	Positive	1:4
9.6	1:3200	13	Negative	ND

(a) The mean value of bacteria added per tube = 3.7×10^8

(b) The last dilution to display haemagglutination

(c) not detected at 1:1

1:5 to 1:320 dilutions of heat-treated C-8 antiserum were tested in a bactericidal assay with 2×10^8 log-phase C5 cells and 9.6 CH50 units of HuC-15, in a total volume of 2.0 ml. Tris buffer, pH 7.2, was used instead of Mg^{2+} saline. After 60 minutes, the tubes were centrifuged at 10,000 g for 10 minutes at 4°. The cells were washed once and resuspended in 1.0 ml of cold VBS.

50 µl of 1% human RBCs were mixed with 50 µl of doubling dilutions of these suspensions, in microtitration plates. After incubating them for 30 minutes at 37° and then at room temperature, the wells were read for haemagglutination.

TABLE 6.7

1:5 and 1:500 dilutions of heat-treated A-7 antiserum were tested in bactericidal assays with 2×10^8 log phase C5 cells and 9.6 CH50 units of HuC-18, in a total volume of 2.0 ml. Tris buffer, 0.01 M EGTA in Tris buffer and 0.005 M EDTA in Tris buffer (pH 7.2) were used as diluents.

After 60 minutes, the tubes were centrifuged at 10,000 g for 10 minutes at 4°. The cells were washed once and resuspended in 0.5 ml cold VBS. The assay for immune adherence (see 2.6.2) was performed on these suspensions.

TABLE 6.7

Alternative pathway involvement in the immune adherence displayed by prozoning *S. typhimurium* C5 cells

CH50 units of HuC-18	Final antiserum dilution	Log phase C5 cells	% survival of bacteria[(a)]	Immune adherence titre
Tris as diluent:				
Nil	1:50	2×10^8	95	ND[(b)]
9.6	Nil	2×10^8	8	1:2
9.6	1:50	Nil	-	ND
9.6	1:50	2×10^8	153	1:8
9.6	1:5,000	2×10^8	1	1:2
EGTA/Tris as diluent:				
Nil	1:50	2×10^8	68	ND
9.6	Nil	2×10^8	118	1:2
9.6	1:50	Nil	-	ND
9.6	1:50	2×10^8	124	1:2
9.6	1:5,000	2×10^8	3	1:2
EDTA/Tris as diluent:				
Nil	1:50	2×10^8	189	ND
9.6	Nil	2×10^8	103	ND
9.6	1:50	Nil	-	ND
9.6	1:50	2×10^8	124	ND
9.6	1:5,000	2×10^8	83	ND

(a) The mean value of bacteria added per tube = 1.5×10^8

(b) ND = not detected at 1:1

showing immune adherence. The lowest concentration of prozoning cells to show immune adherence was 1 x 10^8 per ml; with Tris buffer as diluent, this value was 5 x 10^7 per ml. This comparison indicated that the Ca^{2+}-independent pathway ie., the alternative pathway, was partly responsible for the immune adherence demonstrated by prozoning cells, with Tris buffer as diluent. C3 was therefore bound and activated on such cells via both pathways of complement. The alternative pathway appeared to participate in both prozoning and bactericidal situations, but the control tube lacking antiserum (with EGTA/Tris buffer) indicated that this participation required the presence of specific antibody.

With EDTA/Tris buffer as diluent, neither killing nor immune adherence were demonstrated throughout the reaction mixtures. These results were expected as EDTA prevents the activation of both classical and alternative pathways of complement.

6.2.5 Deviated lysis by "prozoning bacteria" as a measure of C5 activation

The involvement of C5 and the entire MAC of complement, in the prozone phenomenon, was investigated next. A low and a high dilution of hyperimmune antiserum were tested in parallel bactericidal and deviated lysis assays, with *S. typhimurium* M206 bacteria. These assays were performed with both Mg^{2+} saline and 0.01 M EGTA in Mg^{2+} saline as diluents.

Table 6.8 shows that, with Mg^{2+} saline as diluent, there was complete survival of target cells in the

TABLE 6.8

Bactericidal assay mixtures were first set up with a prozoning dilution (1:2.5) and a killing dilution (1:40) of C-8 antiserum, 2×10^8 log-phase *S. typhimurium* M206 cells and 5.0 CH50 units of HuC-15. The total reaction volume was 2.0 ml, and both Mg^{2+} saline and 0.01 M EGTA in Mg^{2+} saline were used as diluents. Appropriate control tubes, lacking antiserum or bacteria, were also set up.

Aliquots of 1.5 ml were transferred to another set of tubes and a standard bactericidal assay was performed on the remaining 0.5 ml volumes.

The aliquoted 1.5 ml volumes were incubated at 32° for 20 minutes. Two further control tubes were included: (1) containing 5 mg inulin, 3.8 CH50 units of HuC-15 and Mg^{2+} saline to 1.5 ml, and (2) containing heat-treated HuC-15 and Mg^{2+} saline.

All tubes were centrifuged in the cold. EDTA was added to the supernatants to 0.01 M. 0.2 ml of 4% chicken RBCs in 0.01 M EDTA/N-saline was then added to both deposits and supernatants. All tubes were incubated (37°, 60 minutes) with frequent shakings. Cold EDTA/N-saline was added to a final volume of 4.0 ml. After centrifugation in the cold, the OD_{541} values of the supernatants were compared with another control tube representing total haemolysis and containing chicken RBCs, water and a few drops of toluine.

TABLE 6.8

Demonstration of Deviated Lysis by prozoning *S. typhimurium* M206 cells

Final antiserum dilution	Log-phase M206 cells (per ml)	CH50 units of HuC-15 (per ml)	% survival of bacteria[a]	% haemolysis of chicken RBCs by: deposit	supernatant
Mg^{2+} saline as diluent:					
Nil	10^8	2.5	0	47	11
1:25	Nil	2.5	-	-	12
1:25	10^8	2.5	325	31	10
1:400	10^8	2.5	0	59	11
Nil	Nil	2.5[b]	-	40	12
Nil	Nil	2.5[c]	-	-	5
$EGTA/Mg^{2+}$ saline as diluent:					
Nil	10^8	2.5	323	14	7
1:25	10^8	2.5	219	28	7
1:400	10^8	2.5	0	35	10

[a] The mean value of bacteria added per tube = 7.7×10^7 per ml

[b] inulin control tube, containing 5 mg inulin

[c] control tube, containing heat-treated HuC

bactericidal assay with the lower dilution of antiserum, but complete killing with the higher dilution. Thus, prozoning and bactericidal situations were represented. The control tube, lacking antiserum displayed efficient killing, due to the bactericidal antibody present in the complement source. This represented activation of the classical pathway of complement, as a similar control tube, with EGTA/Mg^{2+} saline as diluent, showed complete survival of target cells. Here, the alternative pathway was unable to effect killing.

Prozoning and bactericidal situations were also demonstrated with EGTA/Mg^{2+} saline as diluent, indicating the involvement of the alternative pathway. As the control tube, lacking antiserum, showed complete survival, this pathway only appeared to be bactericidal in the presence of a reasonably high level of specific antibody. This was also borne out in Table 6.7.

All supernatants, assayed for deviated lysis, gave negative results (<12% haemolysis). Because deviated lysis involves a transfer of the MAC ($\overline{C5b-9}$) to an unsensitized cell membrane (Hänsch *et al.*, 1977), there was no generation of $\overline{C5b-9}$ in the fluid-phase of the reaction mixtures.

The deposits from all tubes, showing either prozoning or killing in the bactericidal assay, and from the inulin control tube,displayed significant deviated lysis (28% - 59% haemolysis). The deposits, from those tubes with Mg^{2+} saline and showing killing, demonstrated the highest levels of deviated lysis. In contrast, the deposit from that tube with EGTA/Mg^{2+} saline and showing killing, demonstrated

a significantly lower level. This difference indicated the important contribution of the classical pathway in the generation of activated C5 and $\overline{C5b-9}$ with Mg^{2+} saline as diluent.

The major finding of this experiment was that, in the prozone situation, the level of deviated lysis was similar with either diluent. This indicated that the Ca^{2+}-independent pathway ie., the alternative pathway, was wholly responsible for the deviated lysis that resulted with the prozoning target cells. However, because the MAC was easily transferred from the target M206 cells to the chicken RBCs, the complex appeared to be loosely bound to the M206 cells, at sites different from those where it could effect membrane lesions. The classical pathway of complement did not contribute to the deviated lysis, observed in the prozone situation. Thus, it appeared to be unable to generate MAC, either at bacterial sites where membrane damage could result or loosely bound to other bacterial sites. Both pathways of complement were being modified or deviated from their cytolytic activities at the membrane attack phase, in the prozone phenomenon.

6.2.6 Further investigation of the role of the membrane attack complex of complement by the use of R reagents

(a) Testing of the R reagents RaC6-9 and C6-def RaC

Table 6.9 shows the results of bactericidal assays, designed to prove the functional purity of the R reagents used (see 2.4.2 and 2.4.6).

Note that RaC contained sufficient antibody, specific for the target *S. typhimurium* M206 cells, to efficiently

TABLE 6.9

The performance of rabbit R reagents in a bactericidal assay with *S. typhimurium* M206 cells

Complement source	% survival of bacteria[a]
	No other antibody source:
RaC	0
	Whole antiserum[b]:
RaC	0
1:2 RaC	0
1:4 RaC	0
RaC6-9	205
	IgG fraction[c]:
RaC	2[d]
1:4 RaC	0
C6-def RaC	201
RaC6-9, C6-def RaC	0

[a] The mean value of bacteria added = 5.4×10^3

[b] 1:100 final dilution of heat-treated C-3 antiserum

[c] 1:200 final dilution of the IgG fraction of S-6 antiserum (see 4.2.2)

[d] 14% in the presence of 0.01 M EGTA

A standard bactericidal assay was performed with 5×10^3 log-phase M206 cells; bactericidal levels of whole anti-M206 antiserum, or the IgG fraction, and; final 1:10, or higher, dilutions of rabbit complement reagents. The RaC6-9 reagent was prepared from the freshly collected RaC (see 2.4.6) and C6-def RaC was donated (see 2.4.2).

kill them in the absence of any other antibody source. As a complement source, RaC killed even at a final dilution of 1:40, in the presence of anti-M206 antiserum or the IgG fraction. RaC also efficiently killed the target cells in the presence of bactericidal IgG and EGTA, again demonstrating that the alternative pathway could be bactericidal in this system.

As expected, the RaC6-9 preparation and C6-def RaC could not individually kill the target cells in the presence of a bactericidal level of antibody. In the testing of the C6-def RaC, the IgG fraction of an anti-M206 antiserum, rather than the heat-treated antiserum, was used to provide bactericidal antibody because the antiserum would have retained its C6 activity. The RaC6-9 preparation and C6-def RaC together were able to efficiently kill the M206 cells, in the presence of a bactericidal level of the IgG fraction. C6, missing from C6-def RaC but necessary for killing, was provided by RaC6-9.

(b) The effect of C6-def RaC and RaC6-9 on "prozoning bacterial cells"

In this experiment, log-phase *S. typhimurium* M206 cells were first incubated at 32^{o} with prozoning and bactericidal dilutions of a hyperimmune rabbit antiserum, and with RaC. The M206 cells were then washed and diluted in the cold. Standard bactericidal assays were then performed with these cells and the two R reagents (see Table 6.10).

The bactericidal activities of the antiserum dilutions used were established in an initial bactericidal assay, at the start of the experiment. The 1:50 dilution showed good prozoning activity. The 1:250 and 1:1250 dilutions were

TABLE 6.10

The effect of C6-deficient RaC and RaC6-9 on prozoning *S. typhimurium* M206 cells

Bactericidal assay mixtures (4.0 ml) were set up at 4^{o} with 3 x 10^{8} log-phase M206 cells; 1:50, 1:250 and 1:1250 final dilutions of heat-treated A-8 antiserum, and; a final 1:20 dilution of RaC. Control mixtures (3.0 ml), lacking one or more active reagents, were also included.

1.0 ml was removed from the three test mixtures and a standard bactericidal assay was performed on these samples.

The remaining 3.0 ml mixtures were incubated at 32^{o}C for 30 minutes. The cells were centrifuged at 10,000 g in the cold, washed once in cold N-saline and resuspended in 3.0 ml. Each washed suspension was diluted 1:2000. The diluted suspensions provided the target cells in a second bactericidal assay with additional rabbit complement reagents (1:20 RaC, RaC6-9 and 1:20 C6-def RaC) but with no additional antiserum.

TABLE 6.10 The effect of C6-deficient RaC and RaC6-9 on prozoning *S. typhimurium* M206 cells

Initial bactericidal assay		% survival of M206 cells[b] in the bactericidal assays with added complement reagents:			
Reagents	% survival[a]	RaC	RaC6-9	C6-def RaC	RaC6-9, C6-def RaC[c]
M206 cells	NT[e]	6	139	203	206
M206 cells, 1:50 dilution of antiserum, heated[d] RaC	NT	39	95	252	8
M206 cells, RaC	NT	3	116	121	21
M206 cells, 1:50 dilution of antiserum, RaC	266	3	186	188	2
M206 cells, 1:250 dilution of antiserum, RaC	6	(f)			
M206 cells, 1:1250 dilution of antiserum, RaC	3	(f)			

(a) The mean value of bacteria added per tube = 1.5×10^8

(b) The mean value of bacteria added per tube = 4.0×10^3

(c) 0.01 M EGTA/Mg^{2+} saline as diluent

(d) 56^{o}, 30 minutes

(e) not tested

(f) low colony counts at 0 minutes

bactericidal, even in the mixtures incubated at 32^{o} (the viable counts at 0 minutes were markedly reduced in the subsequent set of assays).

The important results in the subsequent set of assays concerned the target cells in the prozone state. They were killed on the addition of RaC. The addition of RaC6-9 or C6-def RaC alone had no such effect. However, the cells were killed when both R reagents were added in the presence of EGTA.

The killing, only observed on addition of RaC or both R reagents, indicated that an excess of all the components making up the MAC of complement (C5-C9), and not just C6-C9 or C5, C7, C8 and C9, was required to abrogate the prozone phenomenon. The classical pathway of complement was presumably activated during the incubation of cells at 32^{o}, but EGTA would have prevented any further activation of C1 in the subsequent bactericidal assay with RaC6-9 and C6-def RaC. The observed killing could therefore not be explained by further classical pathway activation on the cells. There presumably was activation of C3 to C9 via the alternative pathway because the control tube, containing target cells that had been preincubated with the prozoning dilution of antiserum and heated RaC, showed efficient killing on addition of both R reagents and EGTA.

Note that target cells, preincubated with the prozoning dilution of antiserum and RaC, were efficiently killed by this complement source after they had been washed and diluted. Thus, the prozone phenomenon appeared to be sensitive to the relative concentrations of complement and

sensitized bacterial cells.

The control tubes, containing both R reagents and cells that had been preincubated in the absence of active complement, indicated that the alternative pathway was bactericidal only in the presence of specific antibody.

CHAPTER 6.3 Discussion

The involvement of both complement pathways in the NW phenomenon was examined by the work described in this chapter. The classical pathway was shown to be activated but the MAC was not assembled or, if assembled, was not functional. The alternative pathway was also shown to be activated but the resulting MAC did not bind to sites on the target bacteria where it could cause membrane lesions.

Firstly, the prozoning activity of hyperimmune antiserum was shown to be inversely proportional to the concentration of complement in the bactericidal assay mixture (see Table 6.1). This agreed with previously published reports (Neisser and Wechsberg, 1901; Thomas and Dingle, 1943; Thomas *et al.*, 1943; Joos and Hall, 1968; Muschel *et al.*, 1969) that the prozone phenomenon can be abrogated by higher levels of complement. Chapter 4 established that complement-fixing antibody of the IgG class was the only antibody to demonstrate prozoning activity in this system. It followed that IgG fractions should behave like the whole antiserum in the prozone situation. Table 6.2 showed that the prozoning activity of such a fraction was also reduced by a higher level of complement.

Complement was shown to be consumed and therefore activated, in the prozone situation. Haemolytic complement

titrations were performed on the supernatants of prozoning and killing mixtures, at the end of a bactericidal assay. The consumption of haemolytic complement by prozoning mixtures was considerable; it paralleled the consumption by killing mixtures (see Table 6.3). Because complement consumption had previously been shown not to occur in the fluid-phase of prozoning mixtures (Chapter 3), the consumption, and therefore activation, must have occurred on the prozoning cells.

Muschel *et al.*, (1969) demonstrated haemolytic complement consumption in a similar system, with *Salmonella typhi*, but they did not publish the supportive data. However, another group (Thomas and Dingle, 1943) failed to demonstrate such consumption in the prozone phenomenon with *Neisseria meningitidis* bacteria. This could be explained by the large disparity in the numbers of bacterial cells, used in the initial bactericidal assay, and the RBCs, used in the subsequent haemolytic assay (see Table 6.4). It was also significant that these workers reported that the prozone phenomenon can be abrogated by higher levels of complement.

Several authors, studying different bactericidal systems, ie., with *S. typhimurium* (Eddie *et al.*, 1971), *Br. abortus* Hall *et al.*, 1971) and *N. meningitidis* (Griffiss, 1975), showed that IgA antibody can block the bactericidal activity of IgG or IgM antibody. The IgA antibody in the latter two studies was derived from convalescent and hyperimmune sera, that displayed the prozone phenomenon. If the IgA antibody was the prozoning antibody, as Hall *et al.*, (1971) stated, complement consumption would not have been expected to occur.

This is because IgA is unable to fix complement (Ishizaka *et al.*, 1965; Eddie *et al.*, 1971; Knop *et al.*, 1971; Colten and Bienenstock, 1974; Heddle *et al.*, 1975). Hall *et al.*, (1971) and Griffiss (1975) could not assign the prozoning activity of the whole sera to their IgA fractions; IgA, far in excess of that occurring in the whole sera, was required to block the bactericidal IgG and IgM antibody. Complement consumption in the prozone phenomenon, as demonstrated in this Chapter, supported the role of IgG antibody as the prozoning antibody.

Having established that initial complement activation occurred on prozoning bacterial cells, it was then important to establish at which step the cytolytic pathway was being modified or deviated.

Activation of the first three components (C1, C4 and C2) of the classical pathway was first shown to occur on prozoning M206 cells (see Table 6.5). The cells were sensitized with a prozoning dilution of antiserum and C142 reagent. They were washed, diluted and, on addition of GpC, G-C-EDTA or GpC-EGTA, were killed. This experiment also showed that the prozone phenomenon was sensitive to the relative concentrations of prozoning antibody and complement components, particularly the terminal components. There appeared to be a competition between them for binding sites on the bacterial cells. Muschel *et al.*, (1969) previously reported that prozoning SRBCs can be lysed by C-EDTA, in the immune haemolytic system.

C3 was also shown to be present in its activated form, C3b, on prozoning *S. typhimurium* C5 cells (see Table 6.6). The latter participated in the immune adherence of RBCs,

one of the biological activities of cell-bound C3b (Nishiola and Linscott, 1963; Nelson, 1963). (The M206 strain could not be used in this type of experiment as its cells tend to autoagglutinate under the experimental conditions). The only previous demonstration of immune adherence by prozoning cells (Muschel *et al.*, 1969) had been recorded for the immune haemolytic system.

The role of the alternative pathway in both prozoning and killing situations was clarified by the use of the chelating agents, EGTA and EDTA, in another immune adherence experiment (see Table 6.7). Target cells were killed in the presence of EGTA, but only when specific antibody had been added. This meant that the alternative pathway was bactericidal, as EGTA only blocks the classical pathway (Fine *et al.*, 1972), but it required specific antibody. The question of antibody involvement in the bactericidal activity of the alternative pathway has not been clearly answered (Müller-Eberhard and Schreiber, 1980), although there have been many reports in the affirmative (Michael *et al.*, 1962; Sandberg *et al.*, 1970; Reed and Allbright, 1974; Sirŏták *et al.*, 1976). The present experiment also demonstrated immune adherence by the cells, that were killed in the presence of EGTA. This was consistent with C3b, activated by the alternative pathway, being present on the killed cells.

The other important findings, made in the presence of EGTA, were that the prozone phenomenon was evident at a low antiserum dilution (1:50) and that the surviving cells displayed immune adherence. This was the first indication of alternative pathway involvement in the NW phenomenon. The

level of immune adherence (titre 1:2) was significantly less than that demonstrated in the absence of the chelating agent (titre 1:8). This indicated that C3b, generated by both complement pathways, was present on the prozoning cells.

In the light of such studies, it was reasonable to assume that the MAC ($\overline{C5b-9}$) was not being assembled or that it was unable to bind to sites, where it could effect membrane lesions, in the prozone situation. This assumption became a working hypothesis when it was supported by studies involving the deviated lysis phenomenon (Rother *et al.*, 1974) and rabbit R reagents.

In the first instance, an initial bactericidal assay showed that both complement pathways could lead to prozoning or killing situations (see Table 6.8). Again, the alternative pathway was bactericidal only when specific antibody was present. Deviated lysis activity was not demonstrated in the supernatant of any reaction mixture. This indicated that $\overline{C5b-9}$, the MAC transferred in deviated lysis (Hänsch *et al.*, 1977), was either not formed in the fluid-phase or, once formed, was then quickly converted to an inactive form. Fluid-phase or unbound $\overline{C5b-9}$ decays rapidly (Kolb and Müller-Eberhard, 1973) and this is probably due to the inhibitor, S-protein. This normal component of human serum (Podack and Müller-Eberhard, 1979) inhibits the cytolytic activity of the MAC (Podack *et al.*, 1978) by replacing three C9 molecules in the monomeric form of the complex (Podack *et al.*, 1977). S-protein may therefore have been responsible for the lack of deviated lysis activity in the supernatants of the assay mixtures.

The sedimented cells of all prozoning or killing mixtures displayed deviated lysis. Although it was significantly greater for the killing mixture with Mg^{2+} saline as diluent, deviated lysis was similar for the prozoning mixtures with Mg^{2+} saline or EGTA/Mg^{2+} saline as diluent. These results highlighted the major contribution of the classical pathway in the formation of the MAC in the bactericidal situation. They also indicated that a loosely bound MAC was formed in the prozoning situation, but only by the alternative pathway.

The R reagents, RaC6-9 (Cooper and Müller-Eberhard, 1970) and C6-def RaC (Rother and Rother, 1961), and RaC were used as complement sources in another experiment to investigate the role of the MAC in the NW phenomenon.

M206 cells were first incubated with prozoning antibody and RaC. The cells were then washed, diluted and used as the target cells in a bactericidal assay with additional RaC or R reagents. Excess of the terminal complement components C3 to C9 were required to abrogate the prozone phenomenon, as the target cells were killed by RaC or by a mixture of RaC6-9 and C6-def RaC in the presence of EDTA, but not by either R reagent alone (see Table 6.10). Substantial C3b was previously shown to be deposited on prozoning cells, by the demonstration of immune adherence, and so an excess of components C5 to C9, ie., those comprising the MAC, appeared to be necessary to abrogate the prozone phenomenon.

This experiment afforded other important results. Again, the alternative pathway seemed to require specific antibody to be bactericidal, as target cells were killed

by the mixture of RaC6-9 and C6-def in EGTA/Mg^{2+} saline, only after sensitization with specific antibody.

The previous Chapter established that the prozoning activity of an antiserum, or its IgG fraction, was insensitive to bacterial numbers. However, the experiment with R reagents indicated that the prozone phenomenon was sensitive to bacterial numbers, but in an indirect manner. When M206 cells, sensitized with a level of antiserum known to result in prozoning with a certain dose of RaC, were washed and diluted, they were efficiently killed by a similar dose of RaC. The number of antibody molecules bound per M206 cell remained the same, but the numbers of molecules of complement components made available to each cell were significantly higher. This reinforced the belief that the relative concentrations of specific antibody, particularly cell-bound antibody, and complement components were the major factors determining whether a prozone or bactericidal situation existed. It suggested that there was a competition for proximate cell binding sites between specific antibody and complement components. The prozone phenomenon seemed to represent a situation, whereby the competition favoured the binding of a large number of avid antibody molecules to the target cells, and complement components, particularly the MAC, were excluded from their binding sites.

The results from this Chapter indicated a limited number of possible mechanisms by which the cytolytic pathway of complement was being deviated in the NW phenomenon. All involved the membrane attack stage: (i) C5 was not activated, (ii) C5 was activated but could not assemble the remaining

components of the MAC, or (iii) C5 was activated, the MAC was assembled but it was unable to bind to sites on the bacteria where it could cause lesions. The first possibility seemed unlikely because C5 is structurally very similar to C3 (Müller-Eberhard, 1975) and so should also have been activated in the prozone phenomenon. The studies involving deviated lysis indicated that (loosely) cell-bound MAC was only generated by the alternative pathway. Fluid-phase MAC may have been generated by the classical pathway but this would have been quickly inactivated (Kolb and Müller-Eberhard, 1973), presumably by S-protein (Podack *et al.*, 1978; Podack *et al.*, 1980). It is conceivable that the dense cover of antibody molecules was inhibiting the MAC, generated by both pathways, from binding to sites where it could effect lesions. The inhibition may have been due to steric interactions, as the active form of the MAC is a very large dimer of $C5b5789_6$ (Biesecker *et al.*, 1979). It may also have been due to electrostatic interactions, as the binding of IgG molecules to Gram-negative bacteria confers hydrophobicity to their surfaces Stendahl *et al.*, 1977).

The question of cell saturation by prozoning antibody and the role of steric hindrance in the prozone phenomenon will be taken up further in the next Chapter.

CHAPTER 6.4 SUMMARY

6.4.1 The prozoning activity of an antiserum was inversely proportional to the complement concentration in the bactericidal assay mixtures. The prozoning activity of the IgG fraction also shared this relationship.

6.4.2 The prozone phenomenon was abrogated when bacteria, already sensitized with antibody, were diluted and incubated with a similar dose of complement. This strongly suggested that the ratio of antibody molecules bound per bacterium and the level of complement available per bacterium were deciding factors in the NW phenomenon.

6.4.3 Considerable haemolytic complement was consumed by prozoning mixtures. Fluid-phase interactions were previously shown to be unimportant, and so the consumption must have taken place on the target cells.

6.4.4 Both pathways of complement were activated to at least the C3b component on prozoning bacteria. Only the alternative pathway seemed to generate cell-bound MAC, but this was loosely-bound to sites where it could not cause killing. Inhibition of the formation of MAC or the inhibition of the binding of the formed MAC to bacterial membrane sites were suggested mechanisms for the NW phenomenon.

CHAPTER 7 THE INVOLVEMENT OF STERIC HINDRANCE IN THE NEISSER WECHSBERG PHENOMENON

CHAPTER 7.1 Preamble

Much of the data, presented so far, indicated that hindrance of activated, terminal complement components by cell-bound antibody plays an important role in the Neisser-Wechsberg phenomenon. Competition for different but proximate bacterial binding sites appears to favour the very avid antibody (see 5.2.5), in this situation. Preincubation of target bacteria with this antibody, prior to the addition of complement, enhanced the prozoning activity of the antibody source (see 3.2.2), while the prozoning activity of such an antiserum was reduced by increasing the complement concentration (see 6.2.1). Thus, the competition responsible for the hindrance can apparently be altered.

It is possible that the NW phenomenon occurs due to steric hindrance of this lesion-forming complex, by a cell-saturating level of antibody. However, this explanation did not seem very likely as the phenomenon was found to be relatively insensitive to bacterial numbers (see 5.2.6); a similar observation was reported by Normann *et al.*, (1972). Consequently, we decided to investigate the level of saturation with specific antibody of bacterial cells exhibiting the NW phenomenon; the results of this investigation are discussed in this Chapter.

A direct method of determining the number of antibody molecules - IgG molecules, in particular - bound to bacterial cells would have been ideal. The first technique that came to mind involved the removal of bound antibody and its estimation by a radioimmunoassay, such as that used by Ey *et al.*, (1978). However, the work described in 5.2.5

showed that the bound antibody with prozoning activity could not be readily eluted from the bacterial cells. So, this method was not attempted. Another, involving the binding of ^{125}I-protein A to the sensitized cells, was adopted instead. Apart from a few modifications, this method closely followed that described by Mathews and Minden (1979).

Other experiments, described in this chapter, attempted to further define the extent of steric hindrance operating in the NW phenomenon, but by more indirect methods. The effect of polymyxin, an antibiotic with a similar mode of action on bacteria as complement (Pruul and Reynolds, 1972), was studied on bacteria sensitized with a prozoning level of antiserum. It was also important to establish whether a monospecific antiserum ie., one containing antibody specific for only one antigen of the immunogen, could participate in the prozone phenomenon. Lastly, the cells of an antigenically-different Salmonella species and SRBCs were sensitized with the LPS of *S. typhimurium* C5 to see whether the prozone phenomenon could be demonstrated on these cells.

CHAPTER 7.2 Results

7.2.1 The measurement of cell-bound IgG by ^{125}I-protein A

Recently, ^{125}I-protein A has been used to quantitate cell-bound IgG antibody specific for one of several bacterial species (Christensen *et al.*, 1976; Zelter *et al.*, 1978; Mathews and Minden, 1979). The method, described in the last reference, was adopted in an attempt to gauge the relative levels of IgG antibody that were bound to

S. typhimurium cells in bactericidal through to prozoning situations.

An initial titration, ie., an ^{125}I-protein A binding assay incorporating a low dilution of a hyperimmune (rabbit) anti-M206 antiserum and doubling dilutions of the ^{125}I-protein A preparation (see 2.8.2), revealed that a dilution of the preparation containing over 3×10^4 cpm would ensure efficient binding of ^{125}I-protein A to the sensitized cells.

The same hyperimmune antiserum was subjected to one step protein A-Sepharose chromatography (see 2.9.1(b)). The pH 3 eluant, representing the IgG fraction, was concentrated to twice the original serum volume. Doubling dilutions were tested, in parallel, in a standard bactericidal assay and in a ^{125}I-protein A binding assay with *S. typhimurium* M206 cells. Two concentrations of ^{125}I-protein A ie., those containing 3.7×10^4 cpm and 7.4×10^4 cpm, were used in the binding assay.

The bactericidal assay results were as expected (see Fig. 7.1). Final IgG dilutions of 1:25 and 1:50 showed efficient prozoning activity, the 1:100 dilution showed some prozoning activity while further dilutions showed efficient killing of target cells.

% specific binding and specific binding values, for each dilution of the IgG fraction, are plotted in Figs. 7.1 and 7.2, respectively. When 3.7×10^4 cpm of ^{125}I-protein A was added, both parameters rose with increasing IgG concentration but both plateaued when the three lowest dilutions (1:100, 1:50 and 1:25) of the IgG fraction were tested. The plateau therefore occurred at the same time

FIGURE 7.1

% specific binding of ^{125}I-protein A by target *S. typhimurium* M206 cells preincubated with an IgG fraction of hyperimmune antiserum in dilution

Bactericidal and ^{125}I-protein A binding assays were set up in parallel, with doubling dilutions of the IgG fraction of A-8 antiserum and log-phase M206 cells. The IgG fraction was derived by the one step affinity chromatography of 3.0 ml of antiserum on a protein A-Sepharose column (see 2.9.1(b)). The resulting pH 3 eluant was concentrated to 6 ml.

In the bactericidal assay, 1.2 CH50 units of HuC-18 were added to the reaction mixtures of 1.0 ml final volume. The mean number of bacteria added per tube was 9.1×10^7.

The initial 4.0 ml mixtures in the binding assays were incubated at 37° for 30 minutes and were split into four 0.05 ml fractions after the first set of washings. 3.7×10^4 cpm of ^{125}I-protein A was added to two fractions and 7.4×10^4 cpm, to the remaining two fractions. All mixtures were then incubated at 37° for 60 minutes. The radioactivity of the cell pellets was measured as cpm after another set of washings.

% killing (■) and % specific binding (○, 3.7×10^4 cpm; ●, 7.4×10^4 cpm of added ^{125}I-protein A) values were plotted against the corresponding dilutions of the IgG fraction.

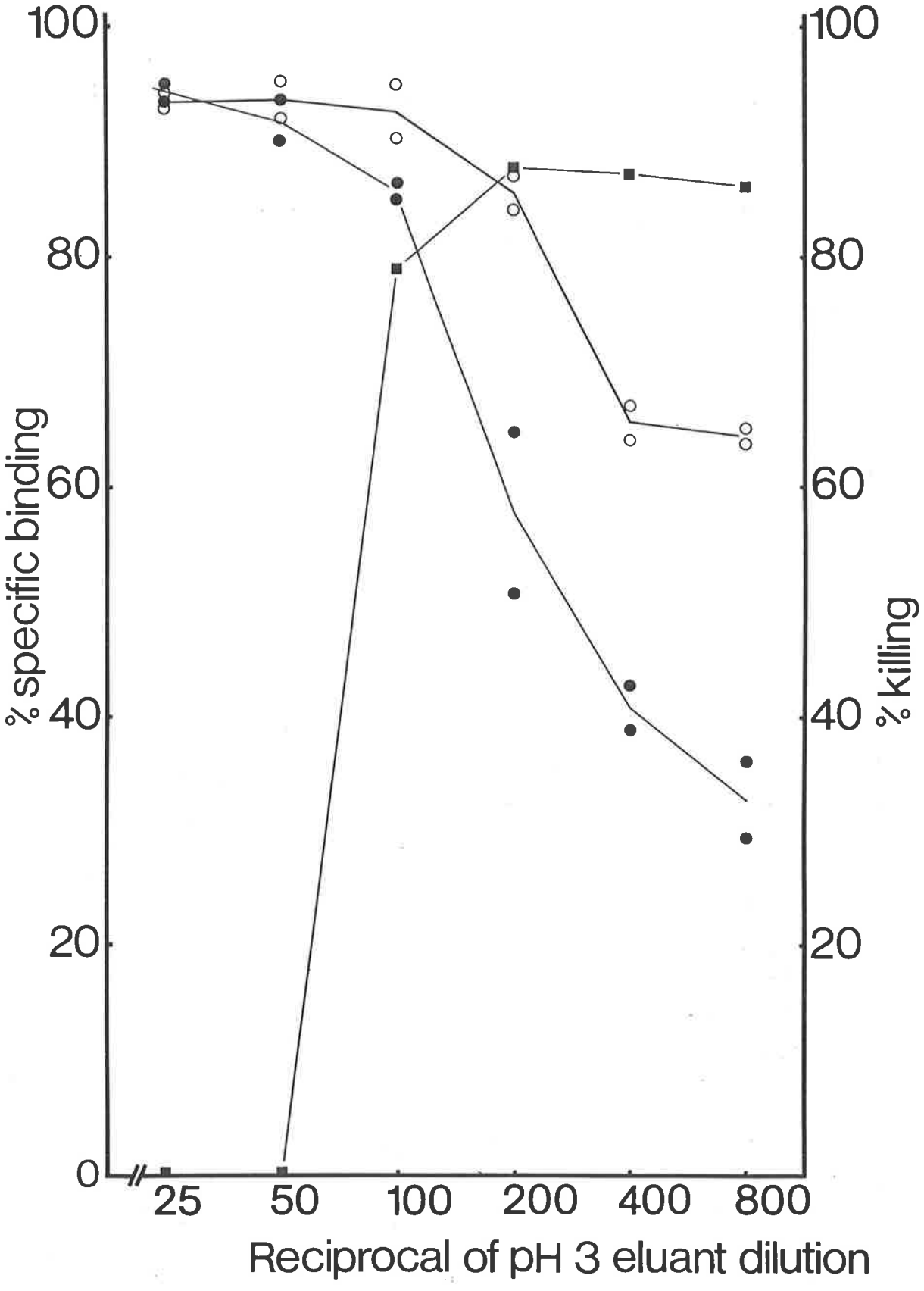
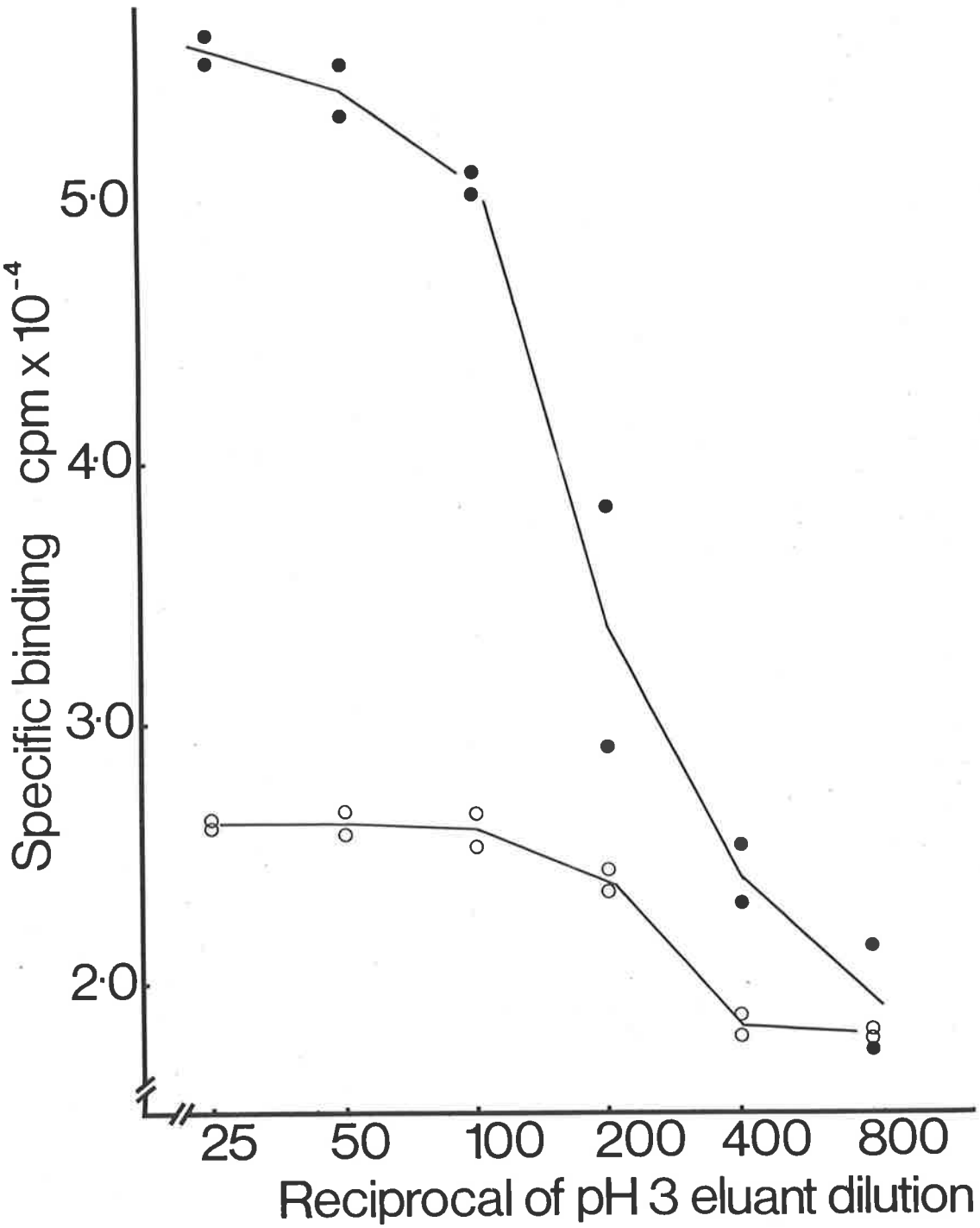

100
80
60
40
20
0
% specific binding
100
80
60
40
20
% killing
25
50
100
200
400
800
Reciprocal of pH 3 eluant dilution

FIGURE 7.2

Specific binding of ^{125}I-protein A by target *S. typhimurium* M206 cells preincubated with an IgG fraction of a hyperimmune antiserum in dilution

Specific binding values (○, 3.7×10^4 cpm; ●, 7.4×10^4 cpm of added ^{125}I-protein A) for the binding assays, described in Fig. 7.1, were plotted against the corresponding dilutions of the IgG fraction of A-8 antiserum.


Specific binding cpm x 10⁻⁴
5·0
4·0
3·0
2·0
25
50
100
200
400
800
Reciprocal of pH 3 eluant dilution

that prozoning activity became evident. When the added ^{125}I-protein A was doubled, however, specific binding values rose steeply with increasing IgG concentration and did not completely plateau out, even when the 1:50 or 1:25 dilutions of the IgG fraction were tested. Specific binding values for any one dilution of the IgG fraction were significantly higher when the higher concentration of ^{125}I-protein A was added; this was especially true for the 1:100, 1:50 and 1:25 dilutions. % specific binding values were around 90% for the two lowest dilutions of the IgG fraction, but were still increasing and so had not completely plateaued.

The results indicated that the plateau of specific binding and % specific binding values, seen with the lower concentration of ^{125}I-protein A, was due to the exhaustion of this reagent rather than a saturation of the target cells by specific IgG antibody. Results with the higher concentration of ^{125}I-protein A indicated that prozoning activity became apparent when a high, but not saturating, level of IgG molecules (in the 1:100 dilution of the IgG fraction) had bound to the target M206 cells. However, a cell-saturating level of IgG molecules seemed to be present in the 1:25 dilution of the IgG fraction.

The TCA control tubes indicated that 97% and 98% of the radioactivity, added in the lower and higher concentrations of ^{125}I-protein A, respectively, was available for binding. 22% and 18% of the added radioactivity non-specifically bound to the M206 cells. These figures were high, compared with those quoted by Mathews and Minden (1979), but they were consistently so in all binding assays

with M206 cells.

A mathematical calculation demonstrated that levels of cell-bound IgG antibody, close to a cell-saturating level, were present in the prozoning situations. The higher concentration of the ^{125}I-protein preparation used contained 11 µg per ml of protein A and so 1.1 µg of ^{125}I-protein A was represented by 72,000 cpm (the TCA control value). The highest dilution of the IgG fraction to show good prozoning activity with 9.1×10^{7} M206 cells per ml was 1:50. The specific binding value was 54,000 cpm in this case and, therefore, 9.1×10^{-9} µg of ^{125}I-protein was estimated to be bound per (prozoning) M206 cell. Using the MW of 42,000 for protein A (Sjoquist *et al.*, 1972) and Avogadro's number (6.023×10^{23}), 130,000 molecules of ^{125}I-protein A were estimated to be bound to each M206 cell. As one protein A molecule can bind to two IgG molecules (Sjoquist *et al.*, 1972), there may have been 260,000 specific IgG molecules bound per M206 cell. This was similar to the number (530,000) of IgG molecules, estimated by Stendahl *et al.*, (1977), required to saturate a smooth *S. typhimurium* cell. However, cells of the M206 strain contain about half the number of O-somatic antigens as a normal smooth strain; so, with the first dilution of the IgG fraction to show good prozoning activity, the target M206 cells seemed to be almost 100% saturated with IgG antibody specific for these antigens.

7.2.2 The bactericidal action of polymyxin in the presence of prozoning levels of antibody

The actions of complement and the antibiotic, polymyxin,

on Gram-negative bacteria have been shown to be synergistic and presumably produce similar lesions on both outer and inner membranes (Pruul and Reynolds, 1972). The study that follows was designed to see whether a bactericidal level of the antibiotic could be inhibited by the presence of prozoning levels of anti-M206 antibody. It was imperative to first establish the minimum concentration of polymyxin to efficiently kill target *S. typhimurium* M206 cells, under standard bactericidal assay conditions.

(a) Minimum killing concentration of polymyxin

Various concentrations of polymyxin B sulphate were added to log-phase M206 cells (5×10^3) and normal bactericidal assay conditions were followed. The results (see Table 7.1) indicated that 250 ng of the antibiotic was necessary to kill over 90% of the target cells. 250 ng per ml was therefore taken to be the minimum killing concentration (MKC).

(b) Effect of prozoning levels of anti-M206 antiserum on the bactericidal action of polymyxin

Doubling dilutions of polymyxin were tested with both a prozoning and a killing dilution of hyperimmune rabbit antiserum, and log-phase M206 cells, in a standard bactericidal assay. To equalize the level of protein binding by the antibiotic in each tube, heat-treated HuC was added.

Table 7.2 shows that the MKC of polymyxin was 250 ng per ml, whether the bactericidal dilution (1:5000) or the prozoning dilution (1:100) of antiserum was present. This meant that a prozoning level of antibody on the M206 cells did not hinder (either sterically or electrostatically) the

TABLE 7.1

Minimum polymyxin concentration to kill log-phase *S. typhimurium* M206 cells

ng of polymyxin added	% survival of M206 bacteria[(a)]
Nil	126
62.5	35
125	58
250	1
500	1
1000	1
2000	0

[(a)] Mean value of bacteria added per tube = 4.2×10^3

A stock solution of polymyxin B sulphate (10 mg per ml) was made up in N-saline. 20 µl of doubling dilutions were added to sterile tubes containing 0.9 ml Mg^{2+} saline. 5×10^3 log-phase M206 cells were added to all tubes. These were incubated at 37^{o} for 60 minutes, with 40 µl volumes being assayed for viable counts at 0 and 60 minutes.

TABLE 7.2

The effect of prozoning levels of anti-M206 antiserum on the minimum killing concentration of polymyxin for *S. typhimurium* M206 cells

ng of polymyxin added	% survival of M206 bacteria[a]	
	1:100 antiserum	1:5000 antiserum
Nil[b]	266	3
Nil	296	253
31	87	235
63	59	30
125	17	17
250	2	2
500	1	2

[a] Mean value of bacteria added per tube = 4.9×10^3

[b] unheated HuC-13 added

Doubling dilutions of polymyxin (20 µl) were tested in a standard bactericidal assay with 1:100 and 1:5000 dilutions of S-6 antiserum, a 1:40 dilution of heat-treated HuC-13 and log-phase M206 cells (5×10^3). Both the antiserum and the complement source were heated at 56° for 30 minutes.

antibiotic from binding to bacterial sites where it could effect lesions.

Control tubes, containing active complement but no polymyxin, confirmed that the lower dilution of antiserum was a prozoning dilution, and that the higher was a bactericidal one. The MKC of polymyxin (250 ng per ml) in this experiment was similar to that calculated in (a). This meant that the presence of serum protein, in the form of heat-treated HuC, did not affect the bactericidal action of this antibiotic. This agreed with Kunin (1967), who stated that the degree of binding of polymyxins to plasma proteins is low.

7.2.3 The prozone phenomenon with *S. minnesota* R595 cells sensitized with the lipopolysaccharide of *S. typhimurium* C5

S. minnesota R595 is a rough strain lacking the somatic antigens (21,26) of the parent strain (Luderitz *et al.*, 1966). The initial aim of this work was to demonstrate killing and perhaps prozoning phases with R595 cells, sensitized with the LPS of *S. typhimurium* C5, and a hyperimmune antiserum specific for *S. typhimurium* M206. It was necessary for this antiserum and the complement source in the bactericidal assays to be free of cross-reacting antibody, specific for the R595 strain. Accordingly, the antiserum was absorbed with acetone-dried R595 cells and the GpC was absorbed twice, with live cells (see 2.4.1).

In the main experiment, log-phase R595 cells were incubated with C5 LPS and used as the target cells, after washing, in a bactericidal assay with various dilutions of

the absorbed anti-M206 antiserum and GpC. Table 7.3 shows that 1:100 and 1:200 dilutions did display prozoning activity whilst 1:500, 1:1000 and 1:50,000 dilutions displayed killing. Note that the latter was incomplete; this was repeatedly observed with R595 cells, sensitized with C5 LPS. The probable reason was that a small percentage of the target cells were not efficiently sensitized with the LPS.

The control tube, lacking anti-M206 antiserum, also showed significant but incomplete killing. This killing was not due to the presence of anti-R595 antibody in the complement source (in a prior bactericidal assay, there was only 21% killing of unsensitized R595 cells by GpC, absorbed only once with R595 cells), but rather the presence of anti-M206 antibody. This had no real bearing on the prozone situation that was demonstrated.

The sensitizing C5 LPS preparation consisted of particles with the MW of 1×10^6 and above (Luderitz *et al.*, 1966). Assuming the MW was 10^6, approximately 10^6 molecules of C5 LPS were incubated per *S. minnesota* cell. This LPS would not have saturated the surface of the target cells, as Smit *et al.*, (1975) estimated that 25×10^6 LPS molecules are normally present on a smooth *S. typhimurium* cell. This argument was supported by the results of a parallel assay, in which the addition of a high dilution of anti-R595 antiserum brought about the efficient killing of target cells whether anti-M206 antiserum was present or not (see Table 7.3). The prozone phenomenon, mediated by the lower dilutions of anti-M206 antiserum, was abrogated by the bactericidal level of anti-R595 antibody. The latter, plus the complement

TABLE 7.3

The bactericidal activity of a hyperimmune anti-M206 antiserum with *S. minnesota* R595 cells sensitized with the lipopolysaccharide of *S. typhimurium* C5

Final dilution of anti-M206 antiserum	% survival of sensitized R595 bacteria[a]	
	without anti-R595 antiserum	with anti-R595 antiserum
1:25	NT[b]	0
1:50	NT	0
1:100	137	0
1:200	68	0
1:500	31	0
1:1000	20	
1:50,000	26	0
Nil	27	0

(a) Mean value of cells added per tube = 8.6×10^3

(b) not tested

Log-phase R595 cells were washed twice in N-saline and then a suspension in Mg^{2+} saline (5×10^8 cells per ml) was incubated at 37° for 30 minutes with C5 LPS (1.0 mg per ml). The cells were then washed twice in Mg^{2+} saline and diluted to about 5×10^4 per ml.

Standard bactericidal assays were performed with the sensitized R595 cells as the target cells. Various dilutions of anti-M206 antiserum, absorbed with acetone-dried R595 cells, were tested in the presence and absence of a 1:5000 dilution of anti-R595 antiserum. The GpC was twice absorbed with live R595 cells before incorporation, as a 1:26 dilution, in this experiment.

components comprising the cytolytic pathway, must have had access to exposed areas on the outer membrane of the target R595 cells. Note that the killing, with even the higher dilutions of anti-M206 antiserum (1:500, 1:1000, 1:50,000), was significantly increased on addition of anti-R595 antiserum. Again, exposed outer membrane was the probable explanation.

The important observations made in these experiments were that: (i) prozoning and bactericidal situations existed for *S. minnesota* cells, sensitised with the LPS of an antigenically unrelated species, and subsequently incubated with a hyperimmune antiserum specific for the unrelated species, and (ii) the prozone situation was abrogated by the addition of anti-R595 antiserum. The results again stressed the importance of the outer membrane LPS and the somatic antigens, in particular, in both prozoning and bactericidal situations.

7.2.4 The prozone phenomenon with sheep red blood cells sensitized with the lipopolysaccharide of *S. typhimurium* C5

SRBCs are significantly larger than bacterial cells and can be easily sensitized with the LPS of Gram-negative bacteria. The following study investigated whether a prozone phenomenon could exist in the passive haemolytic system, with C5 LPS-sensitized SRBCs.

Cells were treated with one of three concentrations of C5 LPS or an optimal sensitizing dose of rabbit haemolysin (see Table 7.4). Each sensitized suspension was then used to test various dilutions of hyperimmune rabbit antiserum,

TABLE 7.4

The effect of high dilutions of antiserum with known prozoning activity on the passive haemolysis of sheep red blood cells sensitized with the lipopolysaccharide of *S. typhimurium* C5

Sensitizing species (μg per ml)	% haemolysis of sensitized SRBCs								OD_{541nm} of control tube[a]
	Anti-M206 antiserum dilutions								
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Nil	
C5 LPS:									
400	77	94	93	81	68	69	67	1	0.85
5	25	42	56	63	66	85	87	1	0.85
2.5	23	26	43	47	57	66	80	1	0.86
Rabbit haemolysin[b]	38	51	62	52	48	48	47	46	0.92

[a] 0.2 ml of sensitized cells and 3.8 ml water

[b] Optimal sensitizing dose

SRBCs were washed in VBS and then sensitized with several levels of C5 LPS at 37° for 90 minutes. The concentration of cells (2×10^9 per ml) was such that when 0.2 ml of the sensitized suspensions were lysed in 3.8 ml of water, the OD_{541nm} was 1.0. Other cells were sensitized for 20 minutes with rabbit haemolysin.

1.0 ml doubling dilutions of a hyperimmune antiserum (rabbit G) specific for *S. typhimurium* M206 were incubated with 0.2 ml of the sensitized cell suspensions and 2.7 ml of VBS at 37° for 20 minutes. 1 CH50 unit of GpC was added and the mixtures were incubated for a further 60 minutes. After centrifugation at 4°C, OD_{541nm} values of the supernatants were measured. The relative haemolysis was expressed as a percentage of the OD shown by a control tube containing sensitized cells and water.

specific for *S. typhimurium*, in a passive haemolytic assay (see 2.6.1(b)).

Table 7.4 shows that the anti-M206 antiserum clearly showed a prozone phenomenon with cells sensitized with the two lower LPS concentrations (5 and 2.5 µg per ml). % haemolysis values progressively rose from around 25% for the 1:4 antiserum dilution to 80% and over for the 1:256 dilution. % haemolysis values of cells, sensitized with the highest concentration of LPS (400 µg per ml), were highest for the 1:8 and 1:16 antiserum dilutions but were still reasonably high for the 1:256 dilution. Only a slight prozone effect was noted for the 1:4 dilution.

The effect of anti-M206 antiserum on the haemolysis of RBCs, sensitized with haemolysin, was slight. The % haemolysis value was 38% for the 1:4 antiserum dilution, 62% for the 1:8 dilution and thereafter, the values plateaued at about 50%. The control tube, lacking anti-M206 antiserum, was the only one of this series to show significant haemolysis (46%). This result was expected, as 1 CH_{50} unit of complement was added and similar control tubes did not contain an antibody source.

It was significant that only low concentrations of sensitizing C5 LPS could mediate the prozone phenomenon. Such sensitizing doses would certainly not saturate the SRBCs, and vast areas of cell membrane were presumably exposed. The prozoning antibody levels were probably sufficient to nearly or completely saturate the cell-bound LPS. Activation of complement must have occurred at the LPS sites but some kind of hindrance appeared to adversely affect the binding of activated components, even though there were vast areas of

exposed RBC membrane. This strongly indicated that an intimate association of bacterial LPS, specific antibody and complement components was necessary for the lysis of the sensitized cells. As the concentration of sensitizing C5 LPS was increased, the high, if not saturating, level of specific antibody could not be achieved at the cell-bound LPS sites, and so activated complement components could bind to such sites to cause haemolysis.

7.2.5 The prozoning activity of monospecific hyperimmune antiserum

All preceding work on the NW phenomenon has involved the participation of whole antiserum or particular immunoglobulin fractions, specific for many bacterial antigens. The following study sought to answer whether an antiserum, specific for only one somatic antigen ie., a monospecific antiserum, could participate in the prozone phenomenon.

Antiserum specific for the Salmonella 5 antigen was first prepared from a hyperimmune rabbit antiserum raised against *S. typhimurium* M206 (see 2.3.3). An initial haem-agglutination assay, using SRBCs sensitized with the LPS of *S. derby*, indicated that antibody specific for somatic antigens 1, 4 and 12 had been efficiently removed.

Various dilutions of the absorbed antiserum were tested in a series of bactericidal assays, with cells of three Salmonella species and one Citrobacter species (see Table 7.5). Only those target cells containing the 5 antigen ie., *S. typhimurium* M206 and *Citrobacter* 396, were efficiently killed. This was further proof of the efficiency of the absorption procedure. In addition, 1:25 and 1:50 dilutions

TABLE 7.5

Prozoning and bactericidal activities of anti-"5" (monospecific) antiserum with several Salmonella and one Citrobacter species

Final dilution of absorbed antiserum	% survival of bacteria			
	S. derby (0:1,4,12)	*S. reading* (0:1,4,12)	*S. typhimurium*[a] M206 (0:1,4,5,12)	*Citrobacter* 396[b]
1:25	>150	>150	100	100
1:50	"	"	100	93
1:100	"	"	0	0
1:500	"	"	0	0
1:1000	"	"	0	0
1:5000	"	"	0	0
Nil	"	"	NT[c]	NT

[a] Mean value of cells added per tube = 6.4×10^3

[b] Mean value of cells added per tube = 7.6×10^3

[c] not tested

Hyperimmune antiserum, specific for *S. typhimurium* M206 (rabbit G), was absorbed with boiled cells of *S. derby* (about 10^{11} cells per ml) for 2 hours at 37°. After removal of the cells by centrifugation and membrane filtration, various dilutions of the absorbed antiserum were tested in standard bactericidal assays. The log-phase cells (5×10^3 per ml) of all three Salmonella species and one Citrobacter species, and a 1:10 dilution of absorbed GpC were used.

of the monospecific antiserum showed good prozoning activity with both types of target cell. This meant that antibody, specific for only one of the several somatic antigens and of the many other antigens on the target cells, was able to effect the prozone phenomenon.

Further, bactericidal assays, in which various dilutions of the monospecific antiserum were tested in the presence and absence of a normally bactericidal dilution of an anti-M206 antiserum, were performed. Table 7.6 shows that the prozoning titre was 1:80, in both instances. The presence of bactericidal levels of antibody, specific for other cell surface antigens as well as the 5 antigen, had no affect on the prozoning activity of the monospecific antiserum.

Antibody, specific for only one of the four somatic antigens of *S. typhimurium* M206 was shown to mediate the NW phenomenon. The antibody probably saturated the 5 antigen on the target cells in this situation, but it probably did not saturate the entire cell surface. Again, activated complement components appeared to be hindered from binding to the LPS antigen-antibody sites, by excess bound antibody. These activated complement components presumably needed to bind to these sites to cause lesions in the outer membrane. The addition of a bactericidal level of anti-M206 antiserum introduced antibody specific for other surface antigens but the prozone phenomenon was not abrogated. It was possible that electrostatic interactions with activated complement components rather than steric hindrance of these components were more important factors operating in this prozone situation.

TABLE 7.6

The effect of a prozoning level of anti-"5" antiserum on the bactericidal activity of an antiserum specific for *S. typhimurium* M206

Final dilution of anti-"5" antiserum	% survival of M206 bacteria[(a)]	
	no other antibody source	1:10,000 anti-M206 antiserum
1:10	100	100
1:20	100	100
1:40	100	100
1:60	100	100
1:80	100	100
1:100	7	23
1:200	4	2
1:10,000	0	0
Nil	68	0

(a) Mean value of cells added per tube = 6.6×10^3

Various dilutions of the anti-5 antiserum (derived from a hyperimmune anti-M206 antiserum) were tested in standard bactericidal assays, in the presence and absence of a high dilution (1:10,000) of an anti-M206 antiserum (rabbit G), collected early in an immunization schedule. Log-phase M206 cells (5×10^3 per ml) and a 1:10 dilution of absorbed GpC were added.

CHAPTER 7.3 Discussion

The NW phenomenon occurs in situations where relatively low dilutions of antiserum, particularly hyperimmune antiserum (Neisser and Wechsberg, 1901), are incubated with target bacteria. The large numbers of specific antibody molecules present may combine with the majority of surface antigens and may even saturate the bacterial cells. The resulting steric (or other) hindrance to complement components may be responsible for the survival of these cells. This type of explanation for the prozone phenomenon has been suggested by Muschel (1965) and it required serious investigation in this thesis. The experimental work, described in this Chapter, was primarily designed to test the degree of antibody saturation on the surface of target cells in the prozone state. It strongly indicated that antibody saturation of surface antigens was not necessary, although a high density of LPS-bound antibody molecules was necessary.

The ^{125}I-protein A binding assay of Mathews and Minden (1979) was adopted to measure the relative amount of IgG antibody bound to *S. typhimurium* M206 cells. In general, % specific binding values obtained with dilutions of the IgG fraction of a hyperimmune rabbit antiserum were high (see Fig. 7.1), compared with those published by Mathews and Minden (1979: Fig. 1(A), (C)). This indicated that the IgG fraction contained a high level of specific IgG antibody.

% specific binding values for prozoning dilutions of the IgG fraction were particularly high (>90%); this indicated that M206 cells in the prozone state were sensitized with a large number of specific IgG molecules. A

saturation level of the latter did not appear to be necessary because, when the concentration of the ^{125}I-protein A reagent was doubled, the specific binding significantly increased for the dilutions of the IgG fraction to first show prozoning activity (see Fig. 7.2). However, specific binding values then did plateau for the lower dilutions of the IgG fraction and a calculation indicated that about 260,000 specific IgG molecules bound per bacterium with such prozoning dilutions. Both facts indicated a near-saturation level present: Stendahl *et al.*, (1979) estimated that 530,000 IgG molecules are required to saturate a *S. typhimurium* cell and the calculated number was in this order, as the target M206 cells contain half the number of O-somatic antigens as a normal smooth strain (Archer and Rowley, 1969).

The surface of Gram-negative bacteria contains phospholipid, structural and enzymatic proteins, and LPS. The latter occupies approximately 45% of the surface area of a smooth strain of *S. typhimurium* (Di Rienzo *et al.*, 1978). Cells of the M206 strain contain half the somatic antigens as those of the C5 strain (Archer and Rowley, 1969). The M206 strain either has more surface phospholipid or, less likely, half the LPS molecules have no side-chains. These facts make it even more unlikely that the cell surface of M206 cells, in the prozone state, was saturated by antibody. The majority of this antibody would be specific for the O-somatic antigens, as the latter are the major immunogens.

The surface phospholipid of M206 cells, sensitized with a prozoning level of antibody, was shown to be accessible to small molecules. The MKC of polymyxin was unaffected

when target M206 cells were incubated with prozoning levels of antiserum (see Table 7.2). The polymyxin molecule, like the MAC of complement, is amphiphilic and is known to attack both (Cerny and Teuber, 1971) the outer (Warren *et al.*, 1957) and inner (Teuber, 1969) membranes of Gram-negative bacteria. The mode of action is believed to be similar to that of complement (Pruul and Reynolds, 1972), although the polymyxin molecule (MW 1200) is many times smaller than the MAC.

Polymyxin molecules were therefore not obstructed by the bound, prozoning levels of antibody from binding to phospholipid sites on the outer and inner bacterial membranes. The failure of the MAC of complement to achieve this in the prozone phenomenon (see Chapter 6) probably relates to the much larger size of this complex. Thus, it was still conceivable that the large, amphiphilic MAC was sterically or electrostatically hindered from attaching to outer membrane sites by high levels of cell-bound antibody.

The importance of the polysaccharide side-chains as antigens, in both the bactericidal reaction and the NW phenomenon, was again demonstrated. LPS of *S. typhimurium* C5 was transferred to the surface of *S. minnesota* R595 cells. These then displayed both prozoning and killing phases with hyperimmune antiserum, raised against the M206 strain but also specific for the C5 strain. The sensitizing dose of C5 LPS indicated that about 10^6 molecules were incubated per R595 cell. 25×10^6 LPS Molecules are normally present per cell of a smooth *S. typhimurium* strain (Smit *et al.*, 1975). This indicated that the surface of the R595 cells was not saturated by the C5 LPS. Experimental evidence was

also provided that antigens, peculiar to the R595 strain, were exposed on the surface of the sensitized cells: the addition of anti-R595 antiserum abrogated the prozone phenomenon. Access of specific antibody and complement components to these antigens - not O-somatic antigens (Luderitz *et al.*, 1966) - was not hindered by the bound, prozoning levels of anti-M206 antibody.

As cell-saturating levels of anti-M206 antibody were not present on the prozoning C5 LPS-sensitized R595 cells, steric or electrostatic hindrance of the MAC of complement was not immediately obvious. However, the MAC has a short half-life (Kolb and Müller-Eberhard, 1973) and could be assumed to bind to an outer membrane site close to the initial antigen-antibody site. Perhaps, steric hindrance of the MAC occurred only around the molecules of C5 LPS and not over the entire R595 cell surface. Electrostatic hindrance of the amphiphilic MAC, by the dense cover of IgG molecules - known to confer hydrophobicity to the surface of Gram-negative bacteria (Stendahl *et al.*, 1977) - over the sensitizing C5 LPS, was also a distinct possibility.

The argument of hindrance to complement components, operating within a defined area of antigen-antibody interaction on a cell surface, was supported by work done with C5 LPS-sensitized SRBCs. These significantly larger cells were sensitized with various levels of the LPS and then used as target cells in a haemolytic assay with various dilutions of hyperimmune antiserum, specific for the M206 strain, and complement. Low dilutions of the antiserum displayed prozoning, particularly when the SRBCs had been sensitized with low levels of the LPS (eg., 1.3 and 2.5 µg in Table 7.4),

implying that the SRBC surface was nowhere near being saturated by the sensitizing LPS. The bound LPS may have been saturated or nearly saturated with specific antibody under prozoning conditions, as higher concentrations of sensitizing LPS (eg., 400 µg in Table 7.4) did not show a prozone phenomenon. Vast areas of exposed cell membrane would still have provided an abundance of binding sites for activated complement components. Again, the MAC could be assumed to bind to a RBC membrane site where LPS molecules had bound and to require interaction with the LPS-antibody complex for its biological activity. Steric or electrostatic hindrance, or both, could prevent this in the prozone situation.

A monospecific antiserum (specific for somatic antigen 5) was prepared from a hyperimmune antiserum raised against the M206 strain. The monospecific antiserum displayed both prozoning and killing phases with M206 cells and complement (see Table 7.5); this prozoning activity was unaffected by the presence of a bactericidal level of anti-M206 antiserum. These results did not exclude the possibility of steric or electrostatic hindrance playing an important role in the prozone phenomenon. There is no information regarding the distribution of the four somatic antigens on *S. typhimurium* M206 cells. If one assumes that they are present in equal numbers and are randomly spaced, high levels of bound anti-"5" antibody could sterically hinder the large MAC of complement. The predominantly IgG antibody would nearly saturate, and possibly completely saturate, sites of somatic antigen 5. The resulting hydrophobic cover (Stendahl *et al.*, 1977) could also repel the amphiphilic MAC (Bhakdi *et al.*, 1978) away from the OM.

CHAPTER 7.4 SUMMARY

7.4.1 An ^{125}I-protein A binding assay was used to measure the relative amount of specific IgG antibody that bound to *S. typhimurium* M206 cells, when they were sensitized with various dilutions of the IgG fraction of a hyperimmune antiserum. The results indicated that near saturation to actual saturation levels of specific IgG molecules were bound to cells in the prozone situation.

7.4.2 The MKC of polymyxin was unchanged with *S. typhimurium* M206 cells, sensitized with antiserum levels that normally showed the prozone phenomenon in the complement-mediated bactericidal reaction. Although, like the MAC of complement, the antibiotic is amphiphilic, it is a much smaller molecule and so may not be hindered from its binding site in this situation.

7.4.3 *S. minnesota* R595 cells, sensitized with the LPS of *S. typhimurium* C5, displayed both prozoning and killing phases in the complement-mediated bactericidal reaction with hyperimmune antiserum specific for the C5 LPS. The prozoning phase was abrogated by the presence of antibody specific for the R595 strain. The interaction of activated complement components with LPS-antibody complexes seemed to be required for killing.

7.4.4 SRBCs, sensitized with low levels of C5 LPS, displayed prozoning and lytic phases in the passive haemolytic assay with hyperimmune antiserum specific for the LPS. Again, interaction of complement and LPS-antibody complexes seemed to be required for killing.

7.4.5 A monospecific antiserum, specific for only somatic antigen 5, was prepared from one raised against *S. typhimurium* M206 and having prozoning activity. The monospecific antiserum also displayed prozoning and killing phases with target M206 cells. This prozoning activity was also unaffected by the addition of bactericidal levels of an antiserum, collected early in an immunization schedule.

CHAPTER 8 DISCUSSION

CHAPTER 8.1 Reiteration of aims

This study concerns the Neisser-Wechsberg phenomenon that commonly occurs in some complement-mediated reactions. It is a prozone phenomenon that results in the deviation of bactericidal complement activity, in the presence of relatively high levels of specific antibody. Thus, in complement-mediated bactericidal reactions, there is an initial phase, where target bacteria are not killed in the presence of low dilutions of antiserum, followed by a killing phase with higher dilutions, and an eventual survival phase with even higher dilutions where specific antibody is no longer present. Although the NW phenomenon is recognized as an *in vitro* phenomenon, evidence (see 3.2.7; Joos and Hall, 1968) suggests that it also occurs *in vivo*, in hyperimmunized animals. As the sera of human patients, chronically infected with or convalescing from infection with Gram-negative bacteria, also display this phenomenon (Hall, 1950; Thomas *et al.*, 1943; Griffiss, 1975; Waisbren and Brown, 1966; Gower *et al.*, 1972; Taylor, 1972), it is possible that humoral factors alone cannot kill the pathogens in these patients. The phenomenon therefore assumes an important clinical role.

The aims of this thesis were the investigation of the important parameters of this phenomenon (ie., the component(s) of antiserum possessing the prozoning activity, and the involvement of target bacterial cells and complement source) and the subsequent explanation of the mechanism(s) involved. To this end, we have generally

studied the bactericidal reaction using the serum-sensitive but smooth M206 strain of *S. typhimurium*, rabbit antiserum specific for this strain, and human or guinea pig complement.

The major parameters of the phenomenon were adequately investigated, although the very high avidity of the specific antibody involved prevented its purification and quantitation. It is proposed that both steric and electrostatic hindrance of terminal complement components by this avid, and therefore firmly, cell-bound antibody of the IgG class is responsible for the NW phenomenon.

This prozone phenomenon is observed with antisera from a number of mammalian species (man, rabbit, goat, dog). It is also observed with a variety of Gram-negative bacteria as target cells: *S. typhi* (Neisser and Wechsberg, 1901; Daguillard and Edsall, 1968; Muschel *et al.*, 1969); *S. typhimurium* (Edebo and Norman, 1969); *Br. abortus* (Hall, 1950); *N. meningitidis* (Thomas *et al.*, 1943; Griffiss, 1975); *E. coli* (Gower *et al.*, 1972), and; *Pr. mirabilis* (Taylor, 1972). The basic findings of the thesis are believed to apply to the phenomenon observed with all Gram-negative bacteria, despite previous evidence to the contrary.

CHAPTER 8.2 Major parameters of the Neisser-Wechsberg phenomenon

The fundamental feature of the prozone phenomenon is that it occurs on the target bacteria, with no contribution from fluid-phase interactions. This has not been stated up to now; in fact, Muschel *et al.*, (1969) proposed that anticomplementary factors present in antiserum,

particularly when the latter is heat-treated, are partly responsible for its prozoning activity. This proposition was based on the equivocal results of a single experiment: the seemingly lower prozoning activity of an unheated antiserum was probably due to the additional complement supplied to the bactericidal assay mixture by the lowest dilution. Results presented here (see 3.2.3 and 3.2.4) indicate that there is no effect on the prozoning activity of an antiserum by exposing it to 56^{o} for a prolonged period (90 minutes) and there is no loss of complement by incubating prozoning dilutions of antiserum with a complement source at 37^{o}.

The NW phenomenon is also not mediated by soluble bacterial antigen in the fluid-phase, as proposed by Coombs (Muschel, 1965). Target bacteria were either extensively diluted or thoroughly washed, before being incorporated into bactericidal assays. However, it is imperative to thoroughly remove bacterial cells and antigenic fragments when absorbing specific antibody from complement sources; otherwise, considerable complement consumption occurs on incubation with prozoning antiserum.

Specific IgG antibody in hyperimmune antiserum is the important mediator of this prozone phenomenon; IgA and IgM antibody species are not involved. The prozoning IgG antibody is able to efficiently fix complement and cannot be assigned to any subclass or subpopulation, with respect to its reactivity with protein A. It is of very high avidity and this feature makes its isolation by adsorption to and elution from bacterial cells or antigens very difficult. It is believed that this type of IgG antibody

also mediates the NW phenomenon in other mammals, including man - the fractionation studies of Taylor (1972) support this.

The central role of specific IgG antibody is one of the controversial findings of this thesis. Serum fractionation studies of Normann (1972) and Normann *et al.*, (1973) are in agreement. However, other workers, using Salmonella and other Gram-negative bacteria, have implicated both IgG and IgM antibody (Daguillard and Edsall, 1968, Muschel *et al.*, 1969) or IgA antibody (Hall *et al.*, 1971; Griffiss, 1975). Serum fractionation procedures in the first three references were either crude or harsh (see 1.4.4 and 4.4) and so fractions were not well characterized or antibody functions were probably impaired. The inhibitory action of IgA antibody on the cytolytic activity of IgG and IgM antibody (Eddie *et al.*, 1971; Hall *et al.*, 1971; Griffiss, 1975; Russell-Jones *et al.*, 1980) is not disputed. However, amounts of IgA antibody, far in excess of that which occurs in whole serum, are required to demonstrate this inhibitory action (Griffiss, 1975) and so cannot explain the prozoning activity of whole antiserum.

The role of specific IgG antibody as prozoning antibody is also reflected in the sequential studies during a vaccination schedule. Although bactericidal activity (initially 2ME-sensitive) appears and peaks early, both prozoning activity and 2ME-resistant activity do so later. This is consistent with the observed early rise in specific IgM antibody followed by a later protracted rise in IgG antibody, on vaccination of laboratory animals with Gram-negative bacteria (Holmgren, 1970; Gupta and Reed, 1971;

Schulkind *et al.*, 1972). The need for a long and intensive vaccination schedule to result in good prozoning activity has also been recognized by other workers (Neisser and Wechsberg, 1901; Joos and Hall, 1968; Normann, 1972). Only Daguillard and Edsall (1968) disagree; however, experimental flaws in this study seem to invalidate their argument.

Strong evidence is presented to suggest that prozoning IgG antibody is very avid. This is expected as prolonged vaccination of laboratory animals with bacteria (Schulkind *et al.*, 1972; Holmgren and Svennerholm, 1972; Ahlstedt *et al.*, 1973) or haptens (Eisen and Siskind, 1964; Makela *et al.*, 1967; Goidl *et al.*, 1968; Werblin *et al.*, 1973a and b) results in antibody of high avidity. Thus, target bacteria can be sensitized with prozoning levels of this antibody and then washed, but still display the prozone phenomenon on addition of complement. This is in agreement with Daguillard *et al.*, (1968), Muschel *et al.*, (1969) and Normann *et al.*, (1972). Target bacteria, sensitized with a bactericidal level of IgM antibody, can then display a prozone phenomenon on the addition of prozoning levels of IgG antibody. Although it is possible that the IgM antibody remains cell-bound in this situation, it is probably displaced from antigenic sites by the large number of avid IgG molecules. Attempts to isolate specific antibody with prozoning activity is difficult, due to its high avidity, in agreement with Normann *et al.*, (1972). Elution of antibody from bacterial cells or the insolubilized LPS proved unsuccessful: prozoning IgG antibody binds to these matrices very efficiently but the antigen-antibody bond cannot then be broken, unless vigorous

techniques, leading to the loss of antibody function (eg., pH <3.0), are used.

The high avidity of prozoning IgG antibody is an important factor, when explaining the mechanism of the NW phenomenon. The hindrance (steric or electrostatic) of terminal complement components from the outer membrane of target cells implies that there is a dense cover of IgG molecules on these cells. The high avidity of these molecules allow them to bind quickly and firmly to surface antigens and so effect this dense cover.

As for the bactericidal reaction (Cundiff and Morgan, 1941; Adler, 1953; Muschel, 1960), the major antigens operating in the NW phenomenon are the polysaccharide O-somatic antigens of the OM. The immunizing bacterium (*S. typhimurium* M206) used in this study, being a smooth strain, has a complete cell wall structure and yet only antibody specific for the polysaccharide side-chains caused the prozone phenomenon. Antibody, specific for other OM components ie., core polysaccharide, lipid A and CWP, was unimportant. Normann *et al.*, (1972) vaccinated rabbits with a rough strain of *S. typhimurium* that lacks the O-somatic antigens and yet the resulting antisera demonstrated prozoning activity. It seems that, in the absence of the O-somatic antigens, on the immunizing bacteria, antibody specific for other OM structures can cause the prozone phenomenon. In the present study, the antisera raised against the smooth strain only showed prozoning activity with target cells possessing the O-somatic antigens (see 5.2.4). Monospecific antiserum, specific for only one such antigen can also cause the prozone

phenomenon and so it is not necessary that all the O-somatic antigen sites of target cells be bound by antibody.

The apparent insensitivity of the NW phenomenon to bacterial cell numbers (Normann *et al.*, 1972) has important consequences and needs to be scrutinized. The prozoning activity of an antiserum and, in particular, the IgG fraction, is relatively independent of the number of target cells in an assay (see 5.2.6). However, the prozoning activity of both are inversely proportional to the complement concentration. It appears that there is a competition between the avid, prozoning IgG antibody and complement components for proximate binding sites on the target bacteria. The ratio of IgG molecules bound per cell and the number of complement components available per cell seems to be the important deciding factor (see 6.2.6(b)). Simply changing the number of target cells does not change this ratio.

There have been relatively few studies done on the involvement of complement in the NW phenomenon, although excess complement has been known to abrogate the phenomenon, since its discovery (Neisser and Wechsberg, 1901; Thomas *et al.*, 1943; Joos and Hall, 1968; Muschel *et al.*, 1969). The present study indicated that considerable consumption of complement occurs on the target bacteria, in this prozone situation. This agrees with the findings of Muschel *et al.*, (1969). Thomas and Dingle (1943) failed to demonstrate a similar significant haemolytic complement consumption; however, they used a much lower number of target bacteria (*N. meningitidis*) in the bactericidal

assay than RBCs in the haemolytic assay (see 6.2.2). The present study also indicated that the alternative pathway, as well as the classical pathway, is activated in the prozone situation. The alternative pathway was shown to be bactericidal but it appeared to require specific antibody for its activation. The question of antibody-involvement in the bactericidal activity of the alternative pathway remains largely unanswered (Müller-Eberhard and Schreiber, 1980).

Both bactericidal pathways of complement are modified at the membrane attack phase (see Table 1.1) in the NW phenomenon. Components C1, C4, C2 and C3 of the classical pathway and C3 of the alternative pathway were shown to be activated and bound to target bacteria in the prozone situation (see 6.2.3 and 6.2.4). Muschel *et al.*, (1969), studying the parallel prozone phenomenon in the immune haemolytic system, demonstrated similar results. The ability of excess of later components in the cytolytic pathways to abrogate the phenomenon indicated that modification of the MAC occurs in the NW phenomenon.

Modification of the membrane attack phase can occur by any or all of three possible mechanisms: (i) C5 is not activated, (ii) activated C5 binds to bacterial sites but cannot assemble the remaining components of the MAC, and (iii) activated C5 assembles the remainder of the MAC about it but the resulting complex cannot bind to bacterial sites, where it can effect lesions. Work with R reagents (see 6.2.6(b)) indicated that either or both of the first two mechanisms can apply to the NW phenomenon. The first mechanism seems unlikely because C5 is structurally very

similar to C3 (Müller-Eberhard, 1975) ie., is of similar size and charge, and so should be activated in the prozone situation, as C3 is. Biologically-active MAC could not be demonstrated in the fluid-phase by deviated lysis; it may have been generated by either pathway but would have been quickly inactivated (Kolb and Müller-Eberhard, 1973), presumably by S-protein (Podack *et al.*, 1978; Podack and Müller-Eberhard, 1980). Cell-bound MAC is generated in the prozone situation, mainly by the alternative pathway. However, it is only loosely bound to sites that cannot effect killing, as it can mediate deviated lysis.

CHAPTER 8.3 Mechanism of the phenomenon

In the NW phenomenon, there exists a competition between avid IgG molecules and complement components (MAC) for different but proximate binding sites on the target bacteria. This competition favours the antibody molecules, since they are the first to interact, and then a dense cover formed by these molecules seems to hinder the MAC from forming and binding.

The MAC is a large aggregate of the five terminal components, bonded by ionic rather than covalent forces (Kolb and Müller-Eberhard, 1973; Podack *et al.*, 1978). The active form seems to be a dimer of $C5b6789_6$ (Biesecker *et al.*, 1979) and seems to assume the shape of a hollow cylinder (Tranum-Jensen *et al.*, 1978; Podack *et al.*, 1980). A major part of the outer surface is hydrophilic but an annular apolar zone seems to be created at one terminus, so conferring an amphiphilic character to the complex (Bhakdi *et al.*, 1978; Podack *et al.*, 1979). In agreement

with the "doughnut hypothesis" (Mayer, 1972), the apolar terminus appears to insert into the phospholipid areas of cell membranes to form a channel across the lipid bilayer. In the NW phenomenon, the bacterial surface is densely covered with IgG molecules that confer to it a strongly hydrophobic character (Stendahl *et al.*, 1977). It is proposed that once C5 has been activated, in this situation, (i) the remainder of the MAC components cannot assemble around it at the cell surface, or (ii) the MAC can be formed distant to the cell surface but cannot bind to phospholipid sites, and so is quickly inactivated by S-protein (Podack *et al.*, 1978; Podack and Müller-Eberhard, 1980). Both steric hindrance and the intense hydrophobic character, imposed on the cell surface by the densely-bound IgG molecules, are thought to be responsible.

Target bacteria, displaying the NW phenomenon, are covered by a dense and almost saturating level of IgG antibody (see 7.2.1). A calculation, based on the number of protein A molecules that bind to *S. typhimurium* M206 cells in the prozone situation, revealed that the number of IgG antibody molecules bound per cell is about 260,000. This number is roughly half that required to saturate a cell with twice the number of O-somatic antigens (Stendahl *et al.*, 1977). Antiserum, specific for only one of several O-somatic antigens, can show good prozoning activity (see 7.2.5). It must also be remembered that the surface of the OM consists of protein and phospholipid, as well as LPS (Osborn *et al.*, 1974; Costerton *et al.*, 1974; Smit *et al.*, 1975), and that the distribution of LPS may be asymmetric (Funahara *et al.*, 1980). Thus, there may be phospholipid

areas on the OM where the large MAC could avoid steric hindrance and so bind to receptor sites, although any such binding may not have a lytic effect. The relatively small polymyxin molecule was not hindered from binding to phospholipid sites on the OM, in the presence of a prozoning dilution of antiserum.

The concept of electrostatic hindrance of the MAC seems to be supported by the work of Normann *et al.*, (1972). These workers found that antiserum loses its prozoning activity on treatment with pepsin. This treatment results in Fc' and $(Fab')_2$ fragments, the latter retaining antigen-binding and complement-fixing (Steele *et al.*, 1977) activities. If steric interactions are the only factor responsible for the NW phenomenon, then prozoning activity should have been retained by the $(Fab')_2$ fragments. The enzyme treatment removes the hydrophobic region of the IgG molecules (Stendahl *et al.*, 1977); the cell-bound $(Fab')_2$ fragments activate complement and then the MAC presumably has no electrostatic constraints to its formation and its membranolytic activity.

SRBCs and *S. minnesota* R595 cells were sensitized with the LPS of *S. typhimurium* C5 and yet displayed prozoning situations with antiserum specific for the M206 strain. Two important observations were made: (i) the sensitizing LPS would have covered only a small part of the surface of the RBCs, and (ii) antibody, specific for the R595 strain, was able to lyse the C5 LPS-R595 cells in the presence of prozoning levels of anti-C5 antibody. Steric and electrostatic hindrances therefore appeared to operate in the immediate vicinity of the sensitizing LPS,

in the prozoning situation ie., MAC formation and activity only need to be prevented in this defined area on target cells. Thus, interaction of activated complement components with LPS-antibody complexes seems to be required for complement-mediated bactericidal action.

CHAPTER 8.4 General conclusions

The NW phenomenon is a consequence of the rapid production of IgG antibody of high avidity in hyperimmunized animals and humans with chronic infection. It appears to be as much an *in vivo* phenomenon as an *in vitro* one. It is responsible for the humoral arm of the immune defense against the immunogen or pathogen being weakened in these animals. However, the dense cover of IgG antibody over the bacteria and the fixation of complement components C1 to C3 in this prozoning situation presumably enhances phagocytosis by white cells, particularly via the activation of specialized cell functions (eg., lymphokine, histamine release). The NW phenomenon does not appear to be the primary cause of the chronic infection state, but can be regarded as an important consequence and even a factor contributing to the persistence of infections.

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