BACTERICIDAL ACTIVITY OF FRESH SERUM FROM DIFFERENT ANIMAL SPECIES.

by

G.E.Schwab. B.Sc. (Adelaide)

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Department of Microbiology,
University of Adelaide.
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CONTENTS.

This thesis contains no material previously submitted for a degree in any University either by the candidate or by any other person, except where due reference is made in the text of the thesis.

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ABSTRACT.

A comparative study was made of the bactericidal activity of seven vertebrate sera for seven species of gram negative bacteria. Each serum was bactericidal towards one or more of the strains but no constant sensitivity of any of the strains to all the sera, or of all the sera to any one strain was found.

Contrary to previous findings, instances were found where smooth bacteria were more sensitive to serum killing than related rough strains.

The optimum, and range of temperatures, over which the sera were bactericidal and haemolytic varied. These temperatures were higher for pig than for toad, fish or lizard.sera.

Sensitisers in human serum for smooth gram negative bacteria were shown by absorption with the homologous strain to be specific, and this was confirmed by inhibition with homologous lipopolysaccharide.

The bactericidal activity of human serum for the rough strain *E. coli* Lilly was also specifically reduced by absorption with the homologous strain, but the absorbing dose required was approximately fifty times greater than that which brought about a similar percentage fall in activity against a smooth strain.

No correlations were found between the bactericidal titres of the various sera for *E. coli* Lilly and the levels of haemolytic complement, or of haemolytic complement and serum lysozyme, which suggested that neither complement, nor complement plus lysozyme, were solely responsible for the death of this bacterium. Lysozyme was not found to enhance the bactericidal activity of any serum.

Yabbie serum was bactericidal for both gram negative and positive bacteria. Limited absorption tests suggested the presence of separate lethal mechanisms for gram positive and negative bacteria, but that within the gram negative group there was no further specificity of the bactericidin.

The injection of living and dead bacteria into yabbies did not lead to the production of demonstrable agglutinins, or to an enhanced bactericidal activity of the serum.

The lethal factor for gram negative bacteria was purified by density gradient centrifugation and by Sephadex chromatography.

The bactericidal activity for the gram negative bacteria was associated with less than 2% of the total serum proteins; the major protein (haemocyanin) was devoid of any demonstrable activity.

The purified active component consisted of at least one protein and five polypeptides.

The significance of these findings were discussed in relation to the background of current knowledge of the mechanisms of serum killing in vertebrate and invertebrate sera.

CHAPTER 1.

HISTORICAL INTRODUCTION.

Normal serum killing is a phenomenon which has attracted the attention of immunologists for over seventy years. In the first part of this review various findings relating to the killing of gram negative bacteria by normal vertebrate sera will be discussed, while in the second invertebrate tissue fluid bactericidal factors will be considered. Brief reference will also be made to the immunological reactivity of several of these animals.

Bactericidal Properties of Vertebrate Sera.

Late in the nineteenth century, serum from normal animals was found to kill some gram negative bacteria and to lyse foreign erythrocytes. Subsequently it was shown that these two activities had a common mediator in complement, and that immunisation increased the magnitude of both activities.

Nuttall (1888) is usually considered to have pioneered the discovery of bactericidal elements in shed blood, although Ledingham (1931) traces all later developments from observations made by Lister in 1880-1. Lister (1880-1) found ox blood taken aseptically from an animal did not readily undergo putrefaction, nor did growth occur

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Nuttall (1888) is usually considered to have pioneered the discovery of bactericidal elements in shed blood, although Ledingham (1931) traces all later developments from observations made by Lister in 1880-1. Lister (1880-1) found ox blood taken aseptically from an animal did not readily undergo putrefaction, nor did growth occur if the blood was seeded with small quantities of tap water or large amounts of dust. In this way serum differed from milk where growth occurred after the introduction of a single bacterium.

The bactericidal property of blood was rediscovered eight years later by Nuttall (1888) who found that _in vitro,_ whole blood destroyed bacteria of the anthrax-subtilis group. The following year Buchner (1889) demonstrated that serum free of leucocytes was also bactericidal for certain organisms, and further, that this property of serum was thermolabile, being lost after heating at 55° for half an hour. Bordet (1895) showed that in addition to the heat labile factor, a heat stable one was involved, and that during immunisation only the heat stable factor was increased in quantity. The heat labile factor we now know as complement, the heat stable as antibody. Bordet (1898-1899) also commented on the similarity of the serum components required for the lysis of sensitised erythrocytes and for killing of bacteria. He showed that when rabbit blood was injected into guinea pigs, their sera became markedly haemolytic towards the rabbit corpuscles, and that this haemolytic activity of serum (like the bactericidal) was heat sensitive. He was able to restore the haemolytic activity to a heated serum by adding fresh normal serum.

Technically it is very much easier to measure the quantitative aspects of the haemolytic than of the bactericidal reaction, and it is from the study of the former reaction that we owe much of our knowledge of the mechanism of complement action. The elucidation of the mechanism, and the components necessary for the bactericidal activity of serum, have in many instances followed prior discoveries in the haemolytic system.

The heat labile component of serum has been shown to be not a single entity, but to be composed of a number of fractions each identified by a specific technique for inactivation. The activity of the whole complex depending upon the interaction of five, and possibly six components. The kinetics and interaction of these components have been investigated only in the haemolytic reaction. All the components are required for serum killing of gram negative bacteria, but their sequence of action can only be assumed by analogy with prior findings for haemolysis.

The multicomponent nature of complement was discovered early this century. When complement containing serum was dialysed (Ferrata, 1907), treated with hydrochloric acid, (Sachs and Altmann, 1909), or with carbonic acid gas after adding water, (Liefmann, 1909), it was split into two components, a fluid portion, and a precipitate. Neither fraction alone was active in the lysis of sensitised erythrocytes, but activity was restored if the two fractions were mixed in their original proportions immediately after preparation.

The insoluble fraction was called the globulin fraction or "mid-piece", and the soluble fraction, albumin or "end-piece." The lytic action of serum was also destroyed when treated with cobra venom, (Ritz, 1912), yeast cells, (Coca, 1914) or zymosan (Whitehead, Gordon and Wormall, 1925). The complement fraction inactivated by these treatments had properties of neither mid-piece nor end-piece. Haemolytic activity was restored with heated whole serum, mid-piece, or end-piece. The complement fraction inactivated by treatment with cobra venom, yeast cells or zymosan was called third component.

The haemolytic activity of complement was also destroyed by treatment of serum with ammonia at pH 8.5 to 10. (Wormall, Whitehead and Gordon, 1925; Gordon, Whitehead and Wormall, 1926). Methylamine and ethylamine inactivated complement in a similar way to ammonia, but other substances containing the amino group were without effect, e.g. glycine, alanine, urea. The lytic activity of the ammonia treated serum could be restored by zymosan treated or heated serum, showing the presence of a previously unidentified component. This fraction of complement became known as the fourth component.

The selective inactivation of complement by amino compounds was reinvestigated by Pillemer, Seifter, and Ecker, (1941). They also found that primary amines and hydrazine were effective but secondary and tertiary amines were inactive.

Pillemer and Ecker (1941) proposed the designation of the complement components as C'1, C'2, C'3, and C'4, corresponding to the old mid-piece, end-piece, third and fourth components respectively. This designation has now found universal acceptance.

During the last decade C'3 has been shown to consist of two, possibly three components, (Rapp, 1958). In addition, a divalent cation requirement for both the haemolytic and bactericidal activities has been shown, (Levine, Cowan, Osler and Mayer, 1953; Landy, Trapani, and Roser, 1960).

The sequence of action of the complement components in the haemolytic reaction has been represented diagrammatically in the following way. (Borsos, Rapp, and Mayer, 1961).

$$S \xrightarrow{2A} SA_2 \xrightarrow[Ca^{++}]{C'1} SA_2C'1 \xrightarrow{C'4} SA_2C'1,4, \xrightarrow[Mg^{++}]{C'2} SA_2C'1,4,2.$$

$$\xrightarrow[\text{factors}]{C'3} S^*$$

Where S = the antigenic site on a red cell surface

and SA = a sensitised site on a red cell surface.

S* = a damaged site.

The technical difficulties involved in kinetic measurements of the bactericidal reaction have prevented an assessment being made of the kinetics, and sequence of action, of the complement components in this reaction.

The Bactericidal Activity of Normal Vertebrate Sera.

Normal sera from all vertebrate species examined, with the exception of the mouse, have the ability to kill some species of gram positive and negative bacteria. Different serum components mediate in the killing of the two groups of bacteria. The former are killed by the ill-defined group of substances referred to as β-lysins, the latter by the "natural antibody"-complement system, with the possible participation of lysozyme (Skarnes and Watson, 1957). This review of the literature is concerned only with the bactericidal action of serum towards gram negative bacteria and so no further consideration will be given to the β-lysins.

Late last century two basic observations relating to serum killing of bacteria were made. The first that the activity was destroyed by heating at 56° for half an hour (Buchner, 1889), the second that a heat stable component was also required (Bordet, 1895). Bordet further showed that during immunisation the second component was increased in quantity, the level of the first remaining unchanged. These components we now know as complement and antibody respectively. The finding that antibody, in addition to complement, participated in the bactericidal activity of immune serum raised the question whether a similar dual mechanism was responsible for the bactericidal action of normal serum. Many investigators subsequently showed this to be so. The early literature was reviewed by Muir (1931) and only those pre-1931 papers will be discussed which are particularly relevant to subsequent work.

In the early years of this century, there was uncertainty on which serum factors mediated the specificity of normal serum killing. The three possibilities considered were, (a) The specificity of normal like immune serum was due to a sensitiser, (b) the sensitiser was non-specific, complement determining specificity, (c) complement alone was bactericidal, no sensitiser being required.

The latter suggestion was put forward by Wright and Windsor (1902) following their investigations into the specificity of the bactericidal activity of normal human serum for Salmonella typhi, and the cholera vibrio. They found absorption with either strain depleted the bactericidal activity of the serum equally for both strains. Filtrates from old smooth cultures also removed bactericidal activity, but filtrates from young cultures had no such effect. These findings, they claimed, provided no evidence for the participation of an immune factor in the bactericidal action of normal serum.

The view that complement alone was bactericidal was not held universally, most workers favouring the theory of a dual mechanism as in the immune bactericidal reaction. (Bordet, 1920). The question still remained, however, whether sensitiser or complement determined specificity. Muir and Browning (1909) claimed specificity was determined by the ability of the bacteria to react with bacteriophilic complement. They found that when a serum was absorbed with increasing amounts of a dead bacterial suspension, the bactericidal activity was first lost for the absorbing strain, then to heterologous strains, and finally haemolytic complement was depleted. Ten times as much heterologous as homologous absorbing suspension was required to bring about a similar depletion of bactericidal activity. They believed that during these absorptions complement of varying degrees of affinity for bacteria was removed, not antibody. However, following absorption bactericidal activity could be restored by the addition of immune antibody. This they did not interpret as a replacement of sensitiser, but rather that the immune factor could react with the remaining complement which had only a weak affinity for bacteria. They were aware that their results could equally well be explained in terms of natural immune bodies, but they considered that specificity could be adequately explained by assuming the presence of a series of bacteriophilic complements.

A criticism of this and many subsequent papers in which bacterial absorptions of serum have been made, is the lack of precision in defining the size of the absorbing dose. This makes both the comparison of one worker's results with anothers, and the interpretation of data difficult, as it has now been clearly shown that the size of the absorbing dose is all important to demonstrate the specificity of serum bactericidal antibodies.

By 1931, Muir had conceded that with some bacteria, specificity of serum killing was clearly due to natural sensitisers, but he claimed this could not justifiably be inferred to hold universally. He still claimed some bacteria could adsorb complement directly, and this combination sometimes had a bactericidal effect.

No further claims that the specificity of serum bactericidal activity was determined by complement appear to have since been made, except for the recent suggestion by Sterzl, Kostka and Lanc,(1962), that complement acts alone on rough gram negative bacteria. The data presented in this thesis, together with that of Muschel and Jackson (1963), casts doubt on the validity of this claim.

Although early in this century there was general agreement that complement did not determine the specificity of serum killing, there were, however, conflicting views on the specificity of the natural sensitising bodies. Evidence was provided which was claimed to indicate non-specificity of these bodies, while equally convincing evidence was put forward to show a high degree of specificity. Much of the confusion can now be explained by sharing of antigenic groups between different bacteria, and the demonstration that specificity can only be shown if the absorbing dose of bacteria is sufficiently small. The evidence for and against the natural sensitisers being highly specific will now be considered in some detail.

Investigations into the mechanism of the bactericidal activity of serum were virtually neglected following the work of Muir and Browning (1909) until the late nineteen twenties. Dunlop (1928), when investigating the fixation of complement from normal guinea pig serum by S. typhi also made a study of the natural sensitisers participating in this reaction. He found the natural sensitisers to be quite distinct from complement either of which could be removed from serum independently of the other. He claimed that the natural sensitisers had some degree of specificity, but that they could also be removed by such non-specific substances as charcoal and powdered glass without affecting the haemolytic complement level. Mackie and Finklestein, (1931) also reported the removal of serum bactericidal activity by charcoal absorption, but the treated serum did not kill sensitised bacteria, which was interpreted as a removal of bactericidal complement rather than sensitising body. This result, they claimed, indicated that different complement or complement-moieties were concerned in haemolysis and bactericidal activity, but they did not mention if the treated serum would lyse sensitised erythrocytes.

Experimental evidence now implicates the same complement complex in both the haemolytic and bactericidal activities of serum. This conclusion has been possible largely from a consideration of the specific inactivation and reactivation of the complement complex. One of the first attempts to clarify the identity of bactericidal and haemolytic complement was that of Gordon and Wormall (1928). These workers critically reviewed data relating to the identity, or otherwise, of bactericidal and haemolytic complement, as well as the techniques used for inactivation. They showed that the inactivation of any of the known complement components in normal guinea pig serum produced an inactive system for the haemolysis of sensitised erythrocytes, and for the killing of *Shigella dysenteriae*. Addition of the missing component reactivated both the haemolytic and bactericidal activities of the serum. Gordon and Wormall (1928) were unable to obtain a serum which was bactericidal but would not lyse sensitised erythrocytes. In addition, they found that when the bactericidal activity of a serum was removed by absorption with dead bacteria, it was still haemolytic for sensitised erythrocytes. This finding could only be explained by the removal of a sensitising body. These workers also showed that the size of the absorbing dose was important in demonstrating the specificity of natural sensitisers. When small doses of bacteria were used for absorption, the bactericidal activity was restored with heated serum, but when the absorbing dose was increased, heated serum did not restore the bactericidal activity. The haemolytic activity of the serum towards sensitised erythrocytes was also lost when absorptions were made with massive doses of bacteria. These results, they realised, did not preclude the existence of separate complements for bactericidal and haemolytic activities of serum, but strongly suggested their similarity.

A recent report by Miyama, Plescia, Braun, and Björklund (1962) suggested that either a basic difference exists between the components of complement involved in the haemolytic and bactericidal reactions of serum, or that there is a difference in the relative importance of identical components. Different electrophoretic fractions of normal human serum inhibited bactericidal activity towards Escherichia coli and the lysis of sensitised sheep red cells. The degree of inhibition/mg. nitrogen also differed in the two systems. They claimed these inhibitions showed differences in the complement components since the cells had been exposed to specific antibody. Confirmation, or otherwise, of these observations has yet to be made.

Following the work of Gordon and Wormall (1928) there was little doubt that complement was non-specific in its action, but there was still uncertainty as to the degree of specificity shown by the natural serum sensitisers.

Two techniques are applicable for showing the presence of natural serum sensitisers, (1) serum bactericidal activity, (2) fixation of complement by bacterial suspensions, as shown by a fall in haemolytic complement activity. The specificity of the sensitising bodies participating in both reactions can be gauged by absorption studies.

Mackie and Finklestein (1930), using the technique of complement fixation, found that complement was fixed when normal animal sera were mixed with bacterial suspensions. If the sera were absorbed with one bacterial strain at 0°, then activity was specifically removed for that strain only, full complement fixing activity remaining for unrelated strains. The following year they carried out a similar investigation of the serum bactericidal reaction and once again demonstrated quite clearly that sensitisers were specific.

This view was not supported by Gordon and Carter, (1932) for they found that when normal rabbit and guinea pig sera were absorbed with "heavy" suspensions of organisms, the bactericidal activity was depleted for both the homologous and heterologous strains. Absorptions did not remove complement, since bactericidal activity was restored with heated serum. They believed that the same serum component mediated in the killing of all serum sensitive bacteria, and that the observed variations in sensitivity were a reflection of differences in the bacteria themselves. During absorption these non-specific factors were removed and the killing of the least serum sensitive bacteria were effected first, presumably because more of the factor was required.

Later Gordon was to change this view and suggest that the sensitisers were in fact specific. Again using "heavy" suspensions he showed that bactericidal activity was depleted for both homologous and heterologous strains, but that it was greatest for the absorbing strain. If the dose was further increased, then complement was also depleted. (Gordon and Johnstone, 1940).

The question of the degree of specificity shown by the sensitisers mediating normal serum bactericidal activity has been clarified by investigations during the last ten years. Adler (1953) investigated the specificity of the sensitisers for S. typhi in different pools of heated guinea pig serum. He provided complement in excess as absorbed guinea pig serum, and sensitiser as heated guinea pig serum. The concentration of reagents in this system were so arranged that any variations in the bactericidal titre were a reflection of differences in the heated serum. The bactericidal titres of the different pools of guinea pig sera for S. typhi, showed considerable variation indicating that the level of sensitisers in the different sera were not constant. He was able to show that absorption of normal rabbit, or guinea pig serum, with suspensions of S. typhi specifically removed bactericidal activity for the absorbing strain. Some fall in killing was recorded for strains which shared common antigens, but there was no effect on unrelated bacteria.

Similar findings were reported by Muschel, Chamberlin and Osawa (1958). They absorbed human serum with strains of S. typhi and found the sensitisers to be markedly specific. Absorption with either smooth or rough strains resulted in a loss of bactericidal activity only for those strains which were serologically related. The sensitisers for strains of E. coli in human serum were also specific. (Muschel, 1960).

Landy and his colleagues, using absorption and inhibition techniques, provided valuable evidence for the existence of strain specific sensitisers in mouse and human serum. When normal human serum was absorbed with one strain of gram negative bacteria, the bactericidal activity was lost for that strain, but not for serologically unrelated strains. Absorption of human serum with either E. coli or S. typhi removed the bactericidal activity only for the homologous strain, since species from six other genera were killed equally well by the absorbed and unabsorbed sera.

The relationship between the quantity of absorbing suspension and the removal of sensitiser was studied. Mouse serum was used as the source of sensitiser, and absorbed human serum as complement. A concentration of 0.1% of bacterial suspension in serum (v/v) gave specific removal of sensitisers. The concentration had to be increased 500 fold before there was a loss of bactericidal activity to unrelated strains. They added graded amounts of lipopolysaccharide to mouse serum and determined what effect this treatment had on the bactericidal activity of the serum extracted. Bactericidal activity was reduced only for the homologous organism, activity for heterologous strains being unaffected. One to 10 µg. of lipopolysaccharide/ml. of serum removed the bactericidal activity for the homologous strain, as much as 100 µg. had no effect on heterologous bacteria. (Michael, Whitby and Landy, 1962).

From the above studies there now seems little doubt that the normal serum sensitisers, like immune antibody, are species, or even strain, specific. There is little doubt, also, that the controversy over their specificity arose because the absorbing doses of bacteria used in attempts to define this specificity were often too large.

Having discussed the work which led to the conclusion that a specific natural sensitiser mediated in normal serum killing, attention will now be turned to a consideration of the nature of the antigenic sites on the bacterial cell towards which the specificity of the sensitisers are directed.

The Sites on the Bacterial Cell towards which the Bactericidal Sensitisers are Directed.

Bordet (1895) established that immune antibody increased the bactericidal activity of a serum but there was no knowledge of the antigens on the bacterial cell towards which this specificity was directed. Felix and Olitzki (1926), using normal rabbit serum found different strains of S. typhi varied greatly in their susceptibility to its lethal action, a difference which must have been due to variations in some component of the bacterial cell. Using immune antibody, normal rabbit serum, and a strain of S. typhi resistant to killing by normal rabbit serum, they showed a parallel between the strength of the bactericidal reaction and the content of "small flaking agglutinins", or as we now know them, (O somatic antigens), as shown by the agglutination test. When these agglutinins were removed by absorption, the bactericidal activity was also lost. They further showed antibodies against the "large flaking agglutinins", (H antigens), exerted no bactericidal effect.

The extent of serum killing of other members of the typhoid-paratyphoid group were also found to be determined by antibodies directed against the smooth O somatic antigens.

The development of techniques for the extraction, and purification, of the somatic antigens provided a means whereby the specificity of serum killing could be investigated by inhibition tests. Thibault (1939) determined the inhibitory effect of the glyco-lipid antigen of Shigella shiga on the bactericidal activity of normal rabbit serum for this bacillus. Thirty three µg. of the glyco-lipid isolated from a smooth strain of gram negative bacteria completely inhibited the killing of the homologous strain, but had no effect against rough strains, or against other smooth gram negative bacteria. Inhibition occurred only with the intact antigen, the polysaccharide alone being ineffective. That the extracted antigen was responsible for the observed inhibition was clearly shown by the reversal of the inhibition by anti-glyco-lipid serum. Since there was no fall in the level of haemolytic complement, the diminution in killing could not be explained by complement destruction.

A similar specificity for inhibition of bactericidal activity was found by Cundiff and Morgan (1941) with the Boiven type antigen. Antigens prepared from S. typhi, S. enteritidis and S. paratyphi B. all inhibited the capacity of anti-typhoid serum to destroy S. typhi, but less antigen was required for homologous than heterologous inhibition. The inhibition of killing of heterologous strains occurred only with those strains which were antigenically related as shown in the Kauffmann-White scheme. An antigen prepared from the unrelated Friedlander's bacillus was without effect, even when used in high concentration. They found the Boiven type antigen extracted from S. typhi was antigenic in the rabbit, the antiserum produced having a bactericidal activity equivalent to that of antiserum prepared against the whole bacterial cell.

These inhibition studies suggested that sensitivity to serum might be explained at least in part by the surface structure of the bacterial cell. If so, then an alteration in the surface structure of the cell might be expected to alter the serum sensitivity. Adler (1950) adsorbed trichloroacetic acid (T.C.A.) extracts of S. typhi and E. coli onto sheep erythrocytes which were then lysed by high dilutions of immune serum to the adsorbed antigen in the presence of complement. Normal cells were not lysed under these conditions. Later he showed that the sensitivity of bacteria to serum killing could also be changed by adsorption of heterologous antigens to the cell surface. He adsorbed the T.C.A. extracts of E. coli onto various Salmonellae which were then killed by anti-E. coli serum and complement; a system to which the untreated cells were resistant. Sensitivity always followed the adsorbed antigen, e.g. adsorption of S. typhi antigens onto various unrelated gram negative bacteria always led to their becoming sensitive to killing by S. typhi specific antibodies. When the antigen from a rough strain was adsorbed to a smooth bacterium of the same species, it then became more susceptible to serum killing. However, foreign antigens could not be adsorbed onto all bacteria, e.g. a mucoid strain of Klebsiella pneumoniae was completely refractory. (Adler, 1952, 1953a).

Adler's observations were confirmed and extended by Michael and Landy (1961a) using both the Boiven type antigen and the lipopolysaccharides (endotoxin) of gram negative bacteria. E. coli and Sh. dysenteriae were cultured in broth containing S. typhi endotoxin and assayed for any alteration in susceptibility to normal human serum. The serum susceptibility of the treated bacteria varied with the time of contact with endotoxin; contact for one hour increased the serum susceptibility of E. coli fourfold, and Sh. dysenteriae sixfold. After eighteen hours the effect was reversed, the endotoxin-treated bacteria being resistant to serum killing. Using immune antibody to the adsorbed lipopolysaccharide, they demonstrated that the alteration of serum sensitivity was a function of the foreign antigen. They were unable to explain the increase in resistance to serum killing when the time of exposure to lipopolysaccharide was increased to eighteen hours.

The following year Michael, Whitby and Landy (1962) showed that as little as one µg. of E. coli endotoxin, or 10 µg. of S. typhi endotoxin, inhibited serum killing of the homologous strain, but amounts of 100 µg. had no effect on the killing of heterologous bacteria from five other genera. These studies leave little doubt that the O somatic antigen is the site on the bacterial cell towards which the antibodies mediating serum killing are directed. No theory has yet been proposed, however, which adequately explains why organisms of apparently similar antigenic composition, and content of lipopolysaccharide, should show such a wide variation in sensitivity to serum killing.

The Effect of the Smooth to Rough Change in Colonial Morphology on Sensitivity to Serum Killing.

The smooth to rough change in colonial morphology has been correlated with a decrease in virulence, and with a decrease in resistance to serum killing. Bordet and Renaux, (1932) found that rough strains of E. coli were more sensitive to killing by guinea pig and rabbit serum than were smooth ones. In the same year, Thjøtta and Waaler (1932) found normal human, guinea pig and rabbit sera killed rough strains of Sh. dysenteriae, but not smooth. A similar difference in susceptibility to serum killing of smooth and rough strains of S. paratyphi B. and S. typhi were observed. During the last decade, there has been a renewed interest in the study of the variations in susceptibility to serum killing of strains of bacteria which have shown the colonial morphological change from smooth to rough. Rowley (1956) compared the killing by normal guinea pig serum, of six strains of smooth gram negative bactéria, with the rough mutants derived from them. The rough strains were always more sensitive to serum killing than their smooth counterparts. He suggested that serum sensitivity might be correlated with the content of somatic antigen, the greater the content the more serum resistant. Similar observations were reported by Muschel, Chamberlin and Osawa (1958) when smooth and rough strains of S. typhi were tested for susceptibility to the bactericidal activity of normal human, guinea pig and rabbit sera.

An interesting corollary to this work was provided by Michael and Braun (1958). They found that different colonial types of Sh. dysenteriae picked from a single plate, showed variations in their resistance to killing by normal human serum. Michael and Landy (1961) determined the susceptibility to normal serum killing of different colonial types of Sh. dysenteriae and E. coli. These strains were obtained by plating the parent culture on tryptose agar and selecting colonies for subculturing which varied in density and refractivity when viewed by oblique lighting. These bacteria, although morphologically smooth, varied greatly in their sensitivity to serum killing. The most sensitive strains of Sh. dysenteriae were killed by a serum dilution sixty four times greater than that at which the resistant strains were killed. Similarly, the sensitive strains of E. coli were sixteen times as susceptible as the resistant strains to the bactericidal action of the serum. Contrary to these findings, however, Thibault (1939) could detect little difference in the sensitivity to normal rabbit serum of smooth and rough strains of Sh. shiga.

Michael and Landy (1961) attempted to correlate the sensitivity of their strains of Sh. dysenteriae and E. coli to killing by normal human serum with the potency of the endotoxin, judged on the ability to sensitise human erythrocytes, and to elicit an antibody response in rabbits to a single intravenous injection. They found the serum-sensitive strains were a less potent source of somatic antigen than the resistant ones. The latter were also more lethal for mice and had a greater capacity to evoke tumour damage. However, certain strains which had similar levels of endotoxic potency varied in their resistance to serum killing, and these bacteria were shown to differ in their capsular or other antigenic constituents. The amount of endotoxin thus would appear to be only one of several cell constituents which determine the susceptibility of bacteria to serum killing.

There is some evidence to suggest that some bacteria are resistant to serum killing in the presence of antibody and complement. Muschel and Treffers (1956) found Paracolobactrum ballerup to be resistant to killing by guinea pig complement and rabbit immune antibody, a system which was highly efficient in the killing of S. typhi. This lack of killing in the presence of immune antibody was also found by Michael and Landy (1961) for their serum resistant strains. As these resistant strains had a similar antigenic composition to some of the sensitive ones, it was at first thought that the lack of killing might be a reflection of an unfavourable ratio of antigen to antibody. They claimed their results obtained using immune antibody showed this not to be so. Changes in susceptibility to serum killing have been observed, however, without any apparent morphological change in the state of the bacteria. Alterations in e.g. type of growth medium, temperature of growth, age of culture, have been shown to affect the susceptibility of bacteria to serum killing. (Maaløe, 1948; Michael and Braun, 1959). Serum resistance thus can no longer be explained purely on a lack of antibody. Resistance for some strains at least, is determined by factors at present unknown.

Range of Animals in which Haemolytic Complement has been Demonstrated.

Haemolytic complement has been detected in the sera of all vertebrate species examined, but until recently there was some doubt as to the presence of complement in the mouse. Mouse is the one animal whose serum has shown no bactericidal activity for gram negative bacteria.(Ali, 1959).

Several workers have now reported the demonstration of haemolytic complement in mouse serum. McGhee (1952) claimed that the usual techniques for assaying haemolytic complement were not sensitive enough for the detection of complement at very low levels, but if the sensitivity of the assay was increased, by reducing the number of erythrocytes, then trace amounts of complement could be detected. Using this technique she demonstrated haemolytic complement activity in mouse serum. Earlier, Brown (1943) investigated the haemolytic activity of serum from five inbred strains of mice, and one wild strain. He could find no haemolytically active complement using the standard sheep red cell-rabbit haemolysin assay system, but the addition of guinea pig C'2 formed a haemolytically active system from which he assumed that the mouse lacked functionally active C'2. Rosenberg and Tachibana (1962) modified the standard haemolytic complement assay system by increasing the concentration of haemolysin relative to the red cells, and demonstrated haemolytic complement in six of eight strains of mice. The haemolytic factor was thermolabile, sensitive to E.D.T.A. and did not lyse unsensitised red cells. Borsos and Cooper (1961) found that by bleeding mice and immediately cooling the blood to 2-4^{o}, all the complement components could be demonstrated by using appropriate intermediates of the complement system. The overall haemolytic complement activity was practically nil.

It appears, then, that mouse serum contains functionally active complement but that it is present only in trace amounts due to some inherent instability of the complex.

Mutant strains of animals naturally deficient in complement have been found. Moore (1919) described a strain of guinea pigs in which haemolytic complement could not be demonstrated. Recently Rother and Rother, (1961) discovered a strain of rabbits which were deficient in haemolytic complement.

Interspecies Differences in the Complement Complex.

The early literature relating to the occurrence and nature of complement in different classes of vertebrates was reviewed by Cushing, (1945 a & b).

Prior to the work of Cushing, complement activity, as demonstrated by the ability of normal serum to lyse foreign erythrocytes, had been found among mammals, birds, reptiles, amphibians and fish, but it was only in guinea pig and human sera that the four components had been demonstrated.

Cushing, (1945 a & b) showed that the optimum temperature for complement activity varied between the mammals, amphibians and fish. Guinea pig complement was more active at 37° than at 16°, and completely inactive at 1-2°. Frog complement gave most rapid lysis at 37°, but the ultimate degree of lysis was greater at 16°, and some activity remained even at 1-2°. Fish (carp) complement lysed erythrocytes more rapidly at 16° than at 37°, and still showed activity at 1-2°. The four complement components of frog and guinea pig complement were found to be similar. Carp complement did not lyse sheep erythrocytes sensitised with rabbit antibody, but lysed these cells when sensitised with its own natural or immune antibody. The complement components resembled those of the guinea pig, but the similarity was not complete. Three of the components were similar, but C'1 and C'2 could not be separated. Cushing's work indicated that complement was widely distributed among vertebrates, with species differences in the optimum temperature for maximum activity, the range of temperatures over which the complex was haemolytically active, and in the interchangeability of some components. It is now clear that for normal serum killing to occur complement and specific sensitiser are required. The position with several other serum factors for which a role in serum killing has been proposed is not so clear. These factors will now be discussed.

The Properdin System, Lysozyme, and Inorganic Ions.

Inorganic ions. The inorganic ions calcium and magnesium have been shown to be necessary for normal serum killing. (Landy, et al., 1960). Their precise role in relation to complement fixation to the bacterial cell have not been investigated. Magnesium is also necessary for the biological activity of properdin.

Properdin. Pillemer, Blum, Pensky and Lepow, (1953) and Pillemer, Lepow and Blum, (1953) found that in order to inhibit the inactivation of complement with zymosan, the serum had to be absorbed under specific conditions of temperature, pH, ionic strength and in the presence of magnesium ions. Pillemer et al., (1954) claimed that since the interaction of antigen and antibody was independent of these factors, zymosan was not interacting with an antibody. They called the factor with which zymosan reacted properdin. Properdin was purified and shown to be a euglobulin constituting less than 0.3% of the total serum proteins.

In addition to its participation in the inactivation of C'3, properdin was shown to participate in the serum killing of certain gram negative bacteria, neutralization of some viruses, and the lysis of certain abnormal erythrocytes.

An early difficulty in assigning a role to properdin in immune phenomena was to explain its apparent non-specificity of action. Properdin adsorbed to such diverse and apparently unrelated substances as bacterial cell walls, bacterial endotoxins and lipopolysaccharides, certain dextrans and levans, and even to lipopolysaccharides and mucins obtained from mammalian tissues, (Pillemer, 1956). The common component of these substances with which properdin combined was later shown to be a polysaccharide of high molecular weight, (Wedgwood, 1958). Wardlaw and Pillemer (1956) were able to show that susceptibility to the properdin bactericidal system was a strain, rather than a species characteristic. They screened forty-four bacterial strains belonging to seven gram negative genera, and one gram positive genus, for sensitivity to the properdin bactericidal system. Twenty strains were killed by fresh serum, but not by serum from which properdin had been removed; fourteen strains were not killed by normal serum, and ten were killed by serum from which the properdin had been removed. In the first group of bacteria which were killed by normal serum but not by serum from which properdin had been removed, the bactericidal activity was restored by the addition of properdin.

The validity of Pillemer's interpretation of his results were questioned by Skarnes and Watson (1957), and by Nelson (1958). They disagreed with the claims of Pillemer and his school that the observed results could not be explained in terms of natural antibody.

Skarnes and Watson (1957) could find no evidence to divorce the activities ascribed to properdin from those of natural antibody. They recognised that the difficulty with such an interpretation was to account for properdin's apparent non-specificity, and the relative specificity of natural antibodies. They believed properdin to be a specific natural antibody to a carbohydrate of wide distribution in nature.

Nelson (1958) made a similar claim and provided experimental evidence which he believed refuted Pillemer's identity of properdin as a new serum protein, rather than a natural antibody. He believed the combination of properdin with zymosan leading to the inactivation of complement, was in fact a complement fixation reaction of zymosan, its antibody, and all components of complement.

Wardlaw and Pillemer (1956) proposed that properdin reacted directly with susceptible bacteria without the mediation of antibody. However, Muschel (1960a) found that serum absorbed with a properdin sensitive organism no longer killed the absorbing strain, but was still equally effective against other properdin sensitive strains. This clearly demonstrated the requirement for a specific sensitiser, and Muschel suggested the activating effect described for properdin might, in fact, be due to a serum enzyme. This theory had already been proposed by Inai, Kishimoto, Hirao and Takahashi (1958). They found that during the preparation of a properdin free serum (R P) there was, together with a depletion of properdin, a fall in serum lysozyme which closely paralleled that of properdin, even to the greater removal at temperatures below 37°. They did not determine if the bactericidal activity of the serum could be restored by the addition of lysozyme. Osawa and Muschel (1960) investigated the effect of adding properdin and egg white lysozyme to an RP. Although lacking in detectable properdin an RP still retained 35-50% of its bactericidal activity, even though the haemolytic complement had fallen by 50%. A 50% fall in the haemolytic complement activity, they considered, could account for the reduced bactericidal activity; a fact which was often not taken into account when assessing the fall in bactericidal activity of an RP. The addition of properdin to this serum did not increase the killing, but the fact was not overlooked that this may have been due to insufficient complement being present to allow any stimulation. In the presence of excess complement a stimulation of bactericidal activity of normal serum, or an RP, by properdin did occur but the possibility that lysozyme or natural sensitiser in the properdin preparation was responsible for the increased activity could not be excluded. The bactericidal activity of an RP could also be partially but not totally restored by egg white lysozyme. Egg white lysozyme did not replace properdin in the inactivation of C'3 (Hook, Carey and Muschel, 1960).

In a recent review of some current concepts of the properdin system, Lepow (1960) suggested the participation of a non-specific properdin with a specific sensitiser. The following observations were put forward to support this hypothesis. When serum was absorbed with bacterial cell walls, there was a fall in activity towards the homologous organism which could be restored by specific antibody but not by properdin. With an RP, however, the bactericidal activity was restored only by properdin, not by specific antibody. Further, after absorption at 0° by cell walls, inactivation of C'3 occurred at 37° by zymosan, but not by the homologous cell walls and conversely, if the absorption was done with zymosan. He conceived that the properdin system was serologically specific, the specificity being determined by an antibody-like principle, while properdin and other serum constituents acted as non-specific accessory factors. Properdin was considered to be distinct from antibody, the "Properdin system" being conceived as a group of non-specific factors acting in a manner analogous to the participation of complement in certain interactions of antigen and antibody.

Rowley (1963) pointed out that properdin was a beta-macroglobulin and he suggested that the possibility should be considered that " by this single word we include a whole special class of antibody-natural macro-globulin antibody." He then posed the question " is it not conceivable that there are many macro-globulins of 19S or greater, each with some specific antibody reactivity, but possessing by virtue of their very large surface area non-specific adsorptive affinity for zymosan and other colloidal particles."

Retention of the concept of properdin as an entity seems to have little value; only future work will determine the precise nature of the serum factors which participated in the various activities ascribed to properdin, but it does seem that natural antibody will be found to have played a major part.

Lysozyme. Fleming (1922) discovered a substance in various secretions and tissues of the body which was capable of dissolving certain species of micro-organisms, particularly *Micrococcus lysodeikticus* and some enterococci, but without activity towards bacteria of the coli-typhoid group. This substance he named lysozyme.

There was no suggestion for any action of lysozyme on gram negative bacteria until Amano, et al., (1954) found that *in vitro* leucocyte extracts, in the presence of complement, accelerated immune lysis of *Vibrio tyrogenes.* Egg white lysozyme in high dilution had a similar accelerating effect, as did other tissue extracts containing lysozyme. Lysozyme was active only in the presence of immune antibody and complement. Further investigations showed lysozyme was involved in the terminal stage of the lytic reaction, antibody and complement first having to react with the cell surface, presumably exposing the lysozyme substrate. Lysozyme, however, was not essential for serum killing, only for lysis. Lysozyme under suitable conditions also lysed some gram negative bacteria in the absence of antibody and complement, but in their presence, lysozyme was effective at much lower concentrations. (Amano, et al., 1955, 1955a; Inai, Kishimoto, Hirao and Takahashi, 1958; Inoue, Tanigawa, Takubo, Satani and Amano, 1959).

The Japanese workers used immune antibody throughout their investigations, but lysis also occurs with normal serum. Michael and Braun, (1959) found bacteria were completely protected against lysis in a normally bactericidal dilution of serum by 20% sucrose. (Brenner, et al., (1958) showed that a concentration of 20% sucrose protected bacteria from lysis). Both natural antibody and complement were required for this lysis. They suggested the lysis was enzymic in nature, but the active principle was not identified. Muschel, Carey and Baron, (1959) identified this principle as serum lysozyme. They were able to convert the rough strains _E. coli_ B and _S. typhi_ strain Mrs. S. to sphaeroplasts by fresh normal guinea pig and human sera in the presence of sucrose and magnesium sulphate. Heat inactivated serum was ineffective. Under these conditions smooth strains of _S. typhi_ were not converted to sphaeroplasts. They further showed complement (free of detectable natural antibody, properdin and lysozyme) produced sphaeroplasts of a smooth strain of _S. typhi_ when O antibody, properdin, magnesium and sucrose were added, but none were formed in the presence of flagella antibody. Since egg white lysozyme would replace properdin, the suggestion was made that the active principle in properdin was lysozyme. Muschel, Carey and Baron, (1959) suggested, as had Inoue, et al., (1959), that serum lysis was a two stage reaction. Firstly, antibody and complement brought about the death of the bacteria, and this was followed by the second stage of lysis of the cells, or the formation of sphaeroplasts if conditions were suitable.

The actual serum lysozyme level apparently did not influence the extent of killing, as a patient with an abnormally high serum lysozyme did not show an increased bactericidal titre. (Osawa and Muschel, 1960).

It seems clear from these observations that the serum killing of gram negative bacteria occurs independently of the presence of lysozyme. Lysozyme may later bring about the lysis of the dead cells, but presumably only if during the process of killing, sufficient of the lysozyme substrate has been exposed.

Having discussed the various factors pertaining to normal serum killing in vertebrates, consideration will now be given to a discussion of antibacterial factors in invertebrates.

The Bactericidal Properties of Invertebrate Sera.

While there is a mass of information relating to the complement mediated bactericidal system in the vertebrates, very little information is available relating to the possible existence of serum killing mechanisms in that large group of animals, the invertebrates.

The early literature relating to immune phenomena in all invertebrates was comprehensively reviewed by Huff, (1940), while Bisset, (1947) and Wagner, (1961) covered the insects only. Insects have been used extensively in these studies largely because of their economic importance, and the ease with which they can be handled in the laboratory. Some of the data from these reviews, relevant to the present discussion, will be mentioned before going on to a consideration of some more recent observations.

Much of our information relating to immunity in invertebrates stems from work in France during the period 1920-35. Wagner, (1961) was somewhat critical of much of this work, largely because adequate controls were not included in many of the experiments. Wagner, (1961) also claimed that the quantitative aspects of this work left much to be desired, principally because the work was done prior to the development of many of the currently used quantitative techniques. However, not all papers fall into this category, as many were purely descriptive, largely referring to the uptake of particles by phagocytic cells. Some of these observations relating to the phagocytic activity of urn cells in a sipunculid worm were recently verified by Bang and Bang, (1962).

The basic conception on which the study of immunity in invertebrates has tended to develop, has been to demonstrate the presence, in these animals, of mechanisms which contribute to immunity in vertebrates. Many attempts have been, and are still being made, to determine whether during infection invertebrates produce substances analogous to the immune antibodies of vertebrates. The reviewers mentioned earlier were able to cite many instances where insects in particular had resisted bacterial infection, but were unable to find any conclusive evidence for antibody production. In their view, the evidence pointed towards the resistance to infection involving cellular rather than humoral factors.

In addition to a species variation in resistance to bacterial infection, insects also show individual variation in resistance to disease. Pasteur (1870) found individual silkworms differed in their susceptibility to the infective agent causing pébrine. He was able to show that this variation in susceptibility had a genetic origin. Huff (1940) cited further examples where individual variation in resistance to infection had been demonstrated.

In addition to the examples quoted of increased resistance to infection being transmitted to the offspring as a stable genetic character, Wagner (1961) cited examples where increased resistance had been produced by the injection of a homologous vaccine. Resistance in general developed within twenty four hours and persisted for a period of four to five days. Immunised animals were resistant to only several lethal doses, larger doses being uniformly fatal.

With few exceptions, these studies were terminated within four to seven days. Chorine (1931), however, followed the changes in resistance to infection of a phasmid, Carausius morosus, after two injections of Vibrio comma vaccine. Enhanced resistance persisted for twenty days, then declined by the end of a month. Those insects which survived a challenge by living organisms were resistant for an additional forty five to sixty days. Repeated injections prolonged the period of heightened resistance still further. This observation shows that at least one invertebrate has a mechanism which enables it to store immunological information for an extended period.

Huff (1940) could find no instances where complement had been found in an invertebrate serum, but he referred to investigations in which natural haemolysins had been demonstrated, e.g. the serum of Eupagurus prideauxii haemolysed normal sheep red blood cells. The observation was also made that the action of rabbit complement on a sheep haemolytic system was retarded by the serum of Maia (spider crab) vaccinated against the red cells. This inhibitary substance was called "opposing antibody". In addition, Huff (1940) cited instances where non-specific thermolabile natural antibodies capable of lysis, precipitation and agglutination had been demonstrated in invertebrate sera.

Huff (1940) also mentioned several instances where highly bactericidal substances were found in the gut of some worms and arthropods. These substances differed from the vertebrate complement system in resisting drying, tryptic digestion, temperatures up to 120°, and alcohol or acetone precipitation.

Frings, Goldberg and Arentzen (1948) described a bactericidal factor with similar properties in the blood of the large milkweed bug, (Oncopeltus fasciatus). The blood was bactericidal for Staph. aureus and one strain of B. subtilus, but inactive towards nine other strains of gram positive and negative bacteria. The serum could be diluted 1/10,000 and still inhibit the growth of sensitive bacteria in the plate assay system used. The substance was water soluble . Most of the activity was lost by boiling for thirty minutes, or autoclaving for ten minutes at 20 lbs. pressure. They showed that the active substance was not one of the proteins precipitated by boiling, since removal of the precipitated proteins after boiling a saline extract did not reduce the activity. They suggested the substance had the properties of an antibiotic, but its chemical identity was not ascertained.

Work during the last two decades, relative to the present review, has centred mainly on investigations of body fluids of several species of invertebrates for specific natural substances, and attempted production of specific active substances in response to the injection of antigens.

Tyler and Scheer (1945), and Tyler and Metz (1945), found at least ten heteroagglutinins for sperm and blood cells of various animal species in the serum of the spiny lobster Panulirus interruptus. The heteroagglutinins were specific. Electrophoresis showed these specific substances, which were most probably protein, to be distinct from haemocyanin.

Triplett, Cushing and Durall (1958) found natural agglutinins for various species of red blood cells in the coelomic fluid of the sipunculid worm Dendostrum zostericolum. The level of these agglutinins were subject to variation in the absence of any known experimental manipulation. They investigated the ability of the sipunculid to respond to foreign tissues. Pieces of skin or tentacle from the same, or different worms, were placed in the coelomic cavity of the sipunculid and the subsequent reaction of the host followed. No difference was found in the ability of the haemocytes to encapsulate homo- or auto-transplants. When the worms were given a second transplant five days after the first, encapsulation was slower. This they interpreted as an inability to rapidly replace those cells used for encapsulating the primary transplant, rather than an immune response. Material from a foreign species e.g. anemone tentacles were encapsulated in a similar manner.

Recent attempts to induce a demonstrable antibody response in several species of invertebrates have not been successful. Bernheimer, Caspari and Kaiser, (1952) were unable to detect antibodies in the blood of Samia cecrophia larvae following injections of heat killed E. coli coliphage T 2 and streptolysin O. Antibodies were not produced to human group O erythrocytes or crystalline egg albumen in Citheronia regalis larvae. These workers determined the time required for the Samia cecrophia larvae to remove the phage from their circulation. Ninety % or more of the injected phage disappeared from the hemolymph between the second and the tenth hours after injection. Although the phage was removed from the circulation, repeated injections did not lead to the production of any neutralising antibodies.

Phillips (1960) injected bovine serum albumen V into Anthopleura elegantissima, (the marine intertidal anemone), and subsequently determined the ability of this "immune" serum to block the reaction between the antigen and rabbit antibody. The anemones received two injections at weekly intervals, and twenty days later were macerated in a Waring blender, and the homogenised material extracted. The extract had a highly specific blocking action giving no cross reaction with human plasma albumen. There was no direct precipitation of antigen. From preliminary observations he suggested a similar material might be produced by a mollusc and a sipunculid.

Recently the claim was made that a heat labile lytic substance was produced in the blood of a sipunculid following the injection of a ciliate. Bang (1962), and Bang and Bang (1962) investigated the response of the marine worm Sipunculas nudas and the spider crab Maia squinado to a marine protozoan parasite, Anophyrs sarcophaga. The ciliates were introduced into the coelomic cavity of the worms and the subsequent reaction of the blood to the ciliates studied in vitro. The ciliates were not affected by this treatment, but if introduced into the sipunculid in blood from the infected crabs, twenty four to forty eight hours later a heat labile substance was produced, which in the in vitro tests, immobilised the parasites in one to two minutes, and brought about complete lysis in ten to fifteen minutes. The capacity to destroy the ciliates was also provoked in the worm by injections of normal crab blood and of bacterial suspensions. The origin of this substance was unknown. The high immobilising activity was retained for five to eight days. Reinjection provoked an accelerated response, but repeated injections did not raise the activity. A small proportion (14%) of the spider crabs had no resistance to the ciliates, succumbing to a rapidly fatal infection.

The coelomic fluid of the marine worm Goldfingia gouldii also destroyed injected bacteria of several unidentified species in vivo. In vitro the lethal factor was present in both serum and cells, and was as active at 0^{o} as at room temperature.

The results of an extensive investigation into the ability of Galleria mellonella larvae to respond immunologically to the injection of several bacterial antigens was recently published. (Stephens, 1959; 1962 a and b; Stephens and Marshall, 1962). They found that normal wax moth larval serum was bactericidal for non-pathogenic bacteria, but had no activity against the larval pathogens e.g. Ps. aeruginosa. Twelve to twenty four hours after immunisation with a heat killed Ps. aeruginosa vaccine, the larvae showed an increased resistance to challenge with homologous living organisms. The resistance developed was specific but lasted less than three days, and throughout this period antigen could be detected in the blood. The immune blood did not contain any demonstrable agglutinins by tube tests, nor did the injection of sonic disrupted cells give rise to precipitins.

The increased bactericidal activity of sera from immunised larvae was specific for the bacterial strain used for injection, and could not be provoked by non-specific agents such as saline, nutrient broth or glucose. The activity could, however, be removed from immune serum by absorption with zymosan.

Normal and immune blood differed in ability to melanize on exposure to air, and immune blood also had a lower oxidation-reduction potential. Normal blood melanized rapidly on exposure to air (1 hour), but there was no melanization of the blood following immunisation with Ps.

aeruginosa vaccine. There appeared to be an association between the ability to inhibit melanization and pathogenicity, since immunisation with a non-pathogen did not lead to inhibition of melanization. Salt (1956) had found a similar inhibition of melanization with pathogens for the stick insect Carausius morosus.

Inhibition of melanization was suggested by Stephens and Marshall (1962) to be due to a depression of tyrosinase activity which also led to an increased ability to withstand infection. They fractionated the blood from immune moths by ethanol precipitation and resin adsorption. The purified bactericidal material showed anti-tyrosinase activity and gave in vivo protection when injected with the organism used to elicit its production.

As the material from whole blood passed through a dialysis sac when dialysed against distilled water, they suggested that the substance was of low molecular weight. The substance was not inactivated by trypsin, and was stable to heating at 100° at neutral pH for five minutes. These properties, they suggested, indicated the substance to have a relatively small molecular weight and to be non-protein.

SUMMARY.

Haemolytic complement activity has been found among diverse species of vertebrates taken from each of the five classes.

The range of animals whose sera have been examined for bactericidal activity has been restricted almost entirely to mammals, but all those which have been examined, with the exception of the mouse, have shown some bactericidal activity. The lack of bactericidal activity in this animal's serum stems from an instability of the complement complex. Although the ultimate result of both the haemolytic and bactericidal activities of the various animal sera is the same, the individual components making up the complement complex appear to differ. There is a species variation in optimum temperatures, range of temperatures over which biological activity occurs and in the mutual interchangeability of individual components. Haemolytic complement activity has not been reported in invertebrate sera. Bactericidal activity has been reported in several instances, but it does not appear to be mediated via antibody and complement.

AIMS OF THE PRESENT STUDY.

Our knowledge of the mechanism of the complement mediated bactericidal system in normal serum has been gained almost exclusively from a study of this phenomenon in the sera of man, rabbits and guinea pigs. Although haemolytic complement has been demonstrated in the sera of many other species of vertebrates, little is known of the distribution of complement mediated bactericidal activity among these species.

This study was initiated to further our knowledge of the distribution of the complement mediated bactericidal system in nature. The animals whose sera were first selected for study were representatives of each of the major lines of vertebrate evolution. A comparison was made of the relative bactericidal activities of the sera and in addition the nature and specificity of some of the components mediating the killing were studied.

The finding that all the vertebrate sera had a complement mediated bactericidal system prompted an investigation being made for the possible existence of similar bactericidal mechanisms in invertebrate sera. The serum of a crustacean was found to possess bactericidal activity and the nature and specificity of this mechanism was then studied.

CHAPTER 2.

MATERIALS AND METHODS.

Bacterial Strains and their Maintenance.

Bacterial Strains.

The bacterial strains used throughout this investigation together with their sources, are listed in Table 1. The bacterial strains which were screened for sensitivity to killing by normal yabbie serum were obtained from the stock collection of micro-organisms in the Microbiology Department, University of Adelaide, from the routine bacteriology laboratory, and the Salmonella Reference Laboratory, both of the Institute of Medical and Veterinary Science, Adelaide, and from the Water and Sewage Laboratories, Glenelg, South Australia.

Maintenance of Strains.

When received, the strains were freeze dried from a suspension in sterile skim milk, of the centrifuged deposit from an overnight broth culture, using a centrifugal freeze dryer, (W.E.Edwards and Co.Ltd., London - Model No. 5 PS.). When received, the smooth and rough strains of S. paratyphi B (designated B.I.S. and B.I.R. respectively)

Table 1.

SOURCE OF BACTERIAL STRAINS USED FOR INVESTIGATING SERUM BACTERICIDAL ACTIVITY.

Bacterial strain.	Antigenic structure.	Reference.	Remarks.
E. coli Lilly.	----	Wardlaw (1962)	Rough strain.
E. coli BV.	0111; B4; H12.	Rowley (1954)	Referred to as strain E2206AV Smooth strain.
S. typhimurium C5	I, IV, V, XII: i, -1,2,3.	Furness and Rowley (1956)	Mouse virulent.
S. typhimurium M206	I, IV, V, XII: i, -1,2,3.	Maaløe (1948)	Strain 206 Maaløe Avirulent for mice.
S. gallinarum 9240	I, IX, XII; - -	Smith (1956)	Referred to as strain 9.ex. N.C.T.C. 9240.
S. paratyphi BIS	I, IV, V, XII: b, -1,2.	Kröger (1953)	The smooth strain was derived from the rough.
S. paratyphi BIR	----		

---- Antigenic structure not known.

were subcultured onto 25% horse serum agar plates, and a typical smooth and rough colony, as viewed by oblique lighting, was used for inoculating the broth cultures for freeze drying. The freeze dried cultures were kept at 4°. When required the freeze dried cultures were reconstituted in 0.5 ml. of nutrient broth and from this suspension six agar slopes and an agar plate were inoculated. If the culture on the plate was pure, the slopes were used for routine subculturing, if not, fresh slopes were subcultured from a single colony on the plate. The agar slope cultures after overnight incubation at 37° were stored at 4° for subsequent use. Ten subcultures were taken from each slope, after which it was discarded and a fresh slope opened.

Media.

Nutrient Broth and Agar.

The nutrient broth and agar used throughout this investigation were prepared from Oxoid dehydrated culture media, reconstituted as specified by the manufacturer. The manufacturer's identification of these preparations were Nutrient Broth No.2, code number CM 67, and Blood Agar Base, code number CM 55. The reconstituted media were sterilised by autoclaving at 120° for 10 minutes.

Minimal Medium.

The minimal medium described by Davis and Mingioli

(1950) was used as diluent for the bactericidal tests. The salts were dissolved at ten times the required concentration, dispensed in 100 ml. quantities, and autoclaved. The composition of this concentrate was:-

KH_2PO_4	30 gm.
K_2HPO_4	70 gm.
$MgSO_4.7H_2O$	1 gm.
$(NH_4)_2SO_4$	10 gm.
Sodium citrate. $3H_2O$	5 gm.
Distilled water	1000 ml.

The pH of the solution was adjusted to 7.2. The medium was sterilised by autoclaving at 120° for 10 minutes. This solution was diluted 1 in 10 in sterile distilled water before use. For tests with E. coli Lilly a 50% (w/v) glucose solution was autoclaved separately, and added aseptically to a final concentration of 1%.

Fraser and Jerrel's Medium.

This was the medium developed by Fraser and Jerrel (1953) for the preparation of high titre stocks of phage ø X174.

Na_2HPO_4	10.5 gm.
KH_2PO_4	4.5 gm.
NH_4Cl	1.0 gm.
$MgSO_4$	0.3 gm.
M $CaCl_2$	0.3 ml.
Gelatin 1% solution.	1.0 ml.
Casamino acids.	15.0 gm.
Glycerol.	30.0 gm.
Distilled water.	1000 ml.

The medium was sterilised by autoclaving at 120° for 10 minutes. One ml. of a sterile 10% solution of magnesium sulphate was added with the phage inoculum.

Preparation of Normal and Immune Sera.

Human. Blood was obtained from persons of blood group O bled at the Adelaide Red Cross Blood Bank. Each pool consisted of the serum from three donors.

Pig. Blood was collected from the excised jugular veins of ten to twenty animals slaughtered at the abattoirs.

Embryo piglets. These were obtained from sows slaughtered at the abattoirs. The placenta was removed from the sows, the embryos detached and bled by cardiac puncture. Twenty embryos were bled for each serum pool.

Kangaroo (Macropus rufus). These animals were shot on Manunda Station, via Yunta, in the northeast of South Australia. The animals were bled from the excised jugular veins. Twenty animals were bled for each serum pool.

Fowl. An inbred line of white leghorn fowls were bled at the South Australian Poultry Station, Parafield, South Australia. The blood was obtained with a syringe from the wing vein. Each serum pool was obtained from approximately fifty birds.

Lizard (Tiliqua rugosa - "sleepy"lizard). The animals were captured in the sandhills near Port Adelaide or along the River Murray in South Australia. The animals were bled by cardiac puncture. Twenty or more animals were bled for each serum pool.

Toad (Bufo marinus). The animals were obtained from the sugar cane growing areas of Queensland. The animals were pithed, a median incision made and the blood drawn from the exposed heart. Sixty animals were bled for each serum pool.

Teleosts (Tinca tinca - "tench"). These fish were caught in the lower reaches of the River Murray, South Australia. They were bled by cardiac puncture. Ten fish were bled for each serum pool.

Elasmobranchs. (Emissola antartica - "gummy"shark). The sharks were caught in Saint Vincent's Gulf, South Australia. The tail was severed and the blood was collected into a bucket. Ten or more sharks were bled for each serum pool.

Yabbies (Parachaeraps bicarinatus - Gray). These animals were captured either in Lake Alexandrena, South Australia or in isolated ponds in the Mount Lofty Ranges, South Australia. The animals were bled by cutting off the tip of a large cheliped and collecting the blood draining out into centrifuge tubes. Twenty or more yabbies were bled for each serum pool.

Method for Obtaining Serum from the Whole Blood.

The blood from the vertebrate species, except kangaroo and fish, was clotted for one hour at 37°, after which the serum was separated by centrifugation at 1,000 g. for 30 minutes. The fish and kangaroo bloods were not incubated at 37° prior to the serum being separated, as these facilities were not available in the field.

The yabbie blood was allowed to clot for one hour at room temperature, and the serum then separated by centrifugation at 4,500 g. for 30 minutes.

Storage of Serum. The pooled sera were stored at -20°. When required, an aliquot was thawed and used the same day. The serum was not refrozen for future use, except in those instances where it was necessary to dispense a large volume of serum into smaller aliquots.

Preparation of Anti-Bacterial Immune Sera.

Rabbits were immunised by thirteen successive injections at three day intervals. The first injection of 5×10^7 steam killed bacteria was given intraperitoneally, followed by four intravenous injections with doses progressively increasing to 5×10^8 bacteria. They were then given three successive intravenous injections of 5×10^7, 10^8, and 5×10^8 alcohol killed bacteria, followed by a similar course of cells which had been disrupted for three minutes in a Mullard ultrasonic disintegrator with a power output of 150 watts and operating at 11 Kc./sec. Finally, three injections of living cells were given, the first intraperitoneally.

The animals were bled by cardiac puncture seven days after the last injection. The blood was incubated for one hour at 37°, after which the clot was allowed to retract in the cold overnight. The serum was separated by centrifuging at 1000 g. for 30 minutes. The serum was stored at -20°.

Basic Technical Procedures.

Preparation of Absorbing Suspensions.

The bacteria were grown in nutrient broth, either with shaking at 37° overnight, or for the water isolates in stationary culture at 30°. The bacteria were removed by centrifugation, resuspended in saline and killed either by heating to 60° for 1 hour, or by suspending in 70% alcohol for six hours. The killed bacteria were washed three times in saline and finally resuspended as a dense suspension in saline. The dry weight of the suspension was estimated gravimetrically after allowing for the quantity of saline present.

Serum Absorptions.

Sufficient bacterial suspension to give the desired dry weight of bacteria per ml. of undiluted serum was pipetted into a plastic centrifuge tube, the bacteria sedimented by centrifugation, and the supernatant discarded. The bacteria were resuspended in serum and absorption allowed to proceed overnight in the cold, after which the bacteria were again removed by centrifugation.

Preparation of Phage ø X174 Suspensions.

One hundred ml. of Fraser and Jerrel's medium, in a 250 ml. Erlenmeyer flask, was inoculated with 0.2 ml. of an overnight shaken broth culture of the propagating strain, E. coli C. When the culture became just visibly turbid, phage was added to a final concentration of 10^5 particles/ml. of culture fluid. The culture was shaken until clearing occurred (about four hours). After centrifugation of the lysed culture, any bacteria remaining in the supernatant were removed by passage through a millipore filter membrane. (0.45 μ pore size).

Preparation of Sheep Red Cell Stroma.

These were prepared as suggested by Kabat and Mayer (1961).

Preparation of Lipopolysaccharides.

Lipopolysaccharides were prepared using the phenol/water extraction method of Westphal, Lüderitz and Bister (1952). The ultra violet absorption of the lipopolysaccharide, dissolved in distilled water and dialysed overnight in the cold against distilled water, was determined from 230-300 mμ. There was no increased absorption between 260 and 280 mμ, which indicated no gross contamination with nucleic acid or protein.

Preparation of Standard Bovine Serum Albumin. (B.S.A.).

Crystalline bovine serum albumin (Commonwealth Serum Laboratories, Melbourne) was reconstituted in saline, and dialysed overnight in the cold against saline. An aliquot of the retentate and of saline were dried to constant weight in a vacuum oven at 60°. The difference between these two weighings gave the amount of B.S.A. present in the solution. This stock solution of B.S.A. was dispensed in ampoules and stored at -20°.

Preparation of Inoculum for the Bactericidal Tests.

Strains other than those isolated from water were grown overnight at 37° with shaking in 25% horse serum broth, after which the cultures were diluted 1:100 into fresh serum broth and incubated for four hours. The inoculum for the serum dilutions was prepared by diluting this latter culture in basal medium. The inocula for the water isolates were prepared from nutrient broth cultures incubated overnight at 30°.

Serum Bactericidal Assays.

The sera were diluted in minimal medium (1% glucose added for E. coli Lilly) to give a final volume of one ml. The serum dilutions were transferred to a water bath, and after allowing five minutes for temperature equilibration, 0.1 ml. (five drops with an 0.02 ml. dropping pipette) of a dilution of culture, containing approximately 5 x 10^4 bacteria/ml. was added. A diluent control was included for each bacterial strain tested.

At time intervals, 0.02 ml. aliquots from each tube were dropped onto quadrants of a nutrient agar plate. After overnight incubation at 30°, the number of colonies were counted. The percentage of bacteria killed was calculated from the number surviving at 90 minutes over the number introduced at zero time.

Haemolytic Complement Assay.

Diluent. The veronal buffer described by Kabat and Mayer (1961) as "diluent 1" was used throughout.

Preparation of sheep erythrocytes. Sheep red blood cells suspended in sodium citrate were washed three times in the diluted buffer and finally suspended as a 5% (v/v) suspension.

Haemolysin. The haemolysin was a product of the Commonwealth Serum Laboratories, Melbourne.

Preparation of sensitised erythrocytes. An equal volume of 10 minimal haemolytic doses of haemolysin was added to the 5% suspension of sheep erythrocytes. After mixing, the cells were incubated for ten minutes at 37° and stored in the cold. The sensitised cells were used on the day of preparation only.

Complement assay. Complement was assayed in 0.4 ml. or less of serum.

The reagents were added in the following order; 0.4 ml. of sensitised erythrocytes, sufficient buffer to make the final volume to 1.2 ml.,0.4, 0.3, 0.2 and 0.1 ml. respectively of an appropriate serum dilution. The tubes were incubated in a water bath for 30 minutes, after which the residual red cells were removed by low speed centrifugation.

The optical density of the clear lysate, was measured against a diluent blank in a Coleman Junior

Spectrophotometer at 541 mμ. The 50% lytic end point was determined by interpolation from a standard curve prepared from the same batch of sensitised red blood cells. The complement titre was defined as the number of 50% haemolytic units (C'H_{50}) contained in one ml. of undiluted serum.

Assay of Serum Lysozyme. (Wardlaw, 1962).

The egg white lysozyme used was the crystallised B grade product of Calbiochem, California, U.S.A. A suspension of Micrococcus lysodeikticus was prepared as the lytic standard for all the lysozyme assays. The growth from lawn plates of M. lysodeikticus, incubated at 30° for forty eight hours, was harvested and washed three times in saline, and finally resuspended in the test buffer. This suspension, in 2-3 mm. layers, was irradiated with ultraviolet light for fifteen minutes, then dispensed in one ml. aliquots and frozen at -20°. Dilutions of serum, and standard lysozyme, were made in 0.033 M. phosphate buffer (pH 6.5), adjusted to ionic strength 0.1 by the addition of an equal volume of 0.045 M. sodium chloride, and containing 0.05% gelatin. The concentration of the M. lysodeikticus suspension was adjusted so that when 0.1 ml. was added to three ml. of buffer the optical density, read at 540 mμ. on a Unicam SP600 Spectrophotometer using a one cm. cell, was between 0.60 and 0.70.

In the lysis tests, three ml. of serum or standard lysozyme dilution, was warmed to 37° in a water bath and the optical density determined. 0.1 ml. of bacterial suspension was then added and the optical density again read (Time 0 reading). The tubes were then incubated with periodic manual shaking and the degree of lysis at the end of fifteen minutes determined by reading the optical density (Time t reading). The term lysis being used to denote a reduction in the optical density of the suspension.

The per cent. reduction in optical density (O.D.) was calculated by the expression:-

$$\frac{\text{O.D. at time 0 - O.D. at time t.}}{\text{O.D. at time 0 - O.D. of diluent and serum}} \times 100.$$

A standard curve, relating percentage lysis to lysozyme concentration was prepared for each series of assays, using lysozyme levels from 0.25 to two µg./ml. of reaction mixture. Suitable dilutions of serum were tested in parallel and the lysozyme level calculated from the standard curve.

Haemagglutination Assays.

The technique used was essentially that described by Crumpton, Davies and Hutchison, (1958). The veronal buffer used in the assays for haemolytic complement was used as the diluent.

Sheep red cells were washed three times in buffer and finally suspended to a concentration of one %. Blood from other animals was taken directly into the buffer to give approximately a one % suspension.

Serial twofold dilutions (0.2 ml. volumes) of the sera were prepared in perspex haemagglutination trays and to these were added 0.2 ml. of the cell suspensions. After mixing, the trays were incubated for one hour at 37° followed by overnight at 4°.

Assay of Phage ∅ X174.

0.1 ml. of serial tenfold dilutions of phage suspension in nutrient broth, were added to three ml. of molten half-strength nutrient agar (equal amounts of nutrient agar and broth) equilibrated to 44°. Following inoculation with five drops of an overnight broth culture of E. coli C, the phage-bacterium mixture was poured onto the surface of dried nutrient agar plates and allowed to set. In order to keep the plaque size small, incubation of these layered plates was restricted to three to four hours at 37°.

Estimation of Serum Proteins Concentration.

Protein was estimated by the modified Folin-Ciocalteu method (Kabat and Mayer, 1961), and by measurement of the optical density at 280 mµ. in a Shimadzu Model QR-50 spectrophotometer. The assays were made quantitative by constructing a standard curve relating protein (B.S.A.) concentration to light absorption. The concentration of protein in the unknown solution was estimated by interpolation from the standard curve.

Chemical Procedures.

Starch Gel Electrophoresis.

For starch gel electrophoresis the method and borate buffer system (pH 8.6) of Smithies (1959) was used. The gels were subject to electrophoresis at four volts/cm. for eighteen hours in the cold. The gel was sliced along its length, and the exposed face stained with amido black 10B for one minute, to identify the protein bands. The second face of the gel was stained for copper using 0.2% rubeanic acid (Decleir 1961).

The preparative starch gel was run similarly, except that all but one of the wells were united, which permitted the electrophoresis of a larger volume of serum. On completion of electrophoresis, the segment of gel containing the single well was sliced, stained, and used to localise the position of the various bands on the remainder of the gel as a guide to sectioning. The gel segments were emulsified in the cold in a M.S.E. homogeniser, with 1.2 M. ammonium sulphate (pH 6.8). The extracts were clarified by centrifugation at 12,100 g. for ten minutes in a Servall refrigerated centrifuge model RC-2. The gels were re-extracted with 1.2 M. ammonium sulphate, followed by a final extraction with 4M. urea buffered at pH 8.6. The combined extracts were dialysed overnight against 1/25 concentration basal medium, and concentrated against "Carbowax 20M"

(Edward Gurr, Ltd., London) to a final volume of approximately five ml. Each fraction was then assayed for bactericidal activity.

Paper Electrophoresis.

Paper electrophoresis was performed in a Beckman Model R paper electrophoresis cell.(Durrum type). The paper strips, inoculated with 0.02 ml. of serum, were dipped either in 0.07 M. veronal buffer (pH 8), or 0.07 M. glycine-sodium hydroxide buffer (pH 9.5). Electrophoresis at pH 8 was run at 110 volts for 12 hours, and at pH 9.5 at 135 volts for 9 hours. After this time the two end strips were stained for protein with bromo-phenol blue. They were then used as templates for localising the position of the protein bands on the remaining strips. The test strips were dried at 37°, sectioned, and homogenised in a M.S.E. homogeniser in buffer adjusted to one M. by the addition of sodium chloride. After extraction for two hours at room temperature, the fluid was removed by filtration through a sintered glass funnel. The extracts were dialysed overnight against 0.1 M. saline, and concentrated against "Carbowax" to approximately 0.25 ml. The concentrated fractions were tested for bactericidal activity.

Curtain Electrophoresis.

Curtain electrophoresis was performed in a Beckman Model CP continuous flow electrophoresis cell. The serum was eluted with 0.02 M. veronal buffer (pH 8.6). A current of 500 volts was applied with a syphon setting of 5.7 cm. on the left hand side, 6.2 cm. on the right hand side, and a buffer overflow setting of 7.5 cm. Serum was applied at the rate of 0.5 ml. per hour. After overnight equilibration, the position of the fastest moving protein band was determined by touching the tabs with a filter paper strip, and staining for protein. After ascertaining that the fastest moving protein was being eluted from tabs 28-9, the apparatus was equilibrated a further six hours, and the eluates then collected. Each eluate was assayed for protein by measurement of the optical density at 280 mμ., and tested for bactericidal activity.

Pyridine-Acetic Acid-Water Electrophoresis. (Spiro, 1962).

0.1 ml. of serum, or purified serum fraction, was spotted onto the centre of a 34 x 4 cm. strip of Whatman No. 3 filter paper. After allowing the drops to dry in, the strips were transferred to a perspex electrophoresis bath containing pyridine-acetic acid-water in the ratio of 25:1:225 at pH 6.4. The paper strips were subjected to electrophoresis for four hours at six volts/cm., dried at 105° for 10 minutes, then sprayed with ninhydrin.

Localisation of protein on paper strips with ninhydrin.

Electrophoresed paper strips were dried for

30 minutes at 105° and then sprayed with a 0.2% solution of ninhydrin in water-saturated butanol. The sprayed strips were developed at 105° for 10 minutes.

Density Gradient Centrifugation.

The sucrose gradients were prepared and sedimented by the technique developed by Kunkel (1960) and modified by Rowley and Turner (1964). Discontinuous sucrose gradients were made in Lusteroid tubes of the S.W./39 Spinco rotor, by successive layering of one ml. aliquots of 40, 35, 25, and 10% sucrose in molar sodium chloride. After standing for 24 hours at 4°, one ml. of a one in two dilution of serum in saline was carefully layered onto the sucrose gradient and centrifuged for 18 hours at 125,000 g. in a Model L Spinco ultra centrifuge. The base of each tube was then carefully pierced with a twenty five gauge needle, and two drops of fluid taken off either into three ml. of saline for estimation of protein at 280 mμ., or in 0.5 ml. aliquots for estimation of bactericidal activity.

Sephadex Chromatography.

Sephadex G75 and G50 granules,(Pharmacia, Uppsala, Sweden.) were swollen overnight in 0.2 M. tris-hydrochloric acid buffer (pH 8.0), and 0.2 M. acetic acid-sodium acetate buffer (pH 4), respectively. The swollen granules were resuspended in the buffer, and after allowing the larger particles to settle, the finer ones and excess buffer were decanted. The swollen Sephadex was packed into wide bore columns (40 cm. x 3 cm.) constricted to 5 mm. at the base. The Sephadex particles were prevented from passing through the column by a glass wool pad overlaid with glass beads, resting on the shoulder of the constriction. During the initial stages of packing, the outlet of the tube was plugged and only the rapidly sedimenting larger particles were used to form the gel. Slowly sedimenting particles and excess buffer were decanted. When the column had been packed, the plug was removed, and the column equilibrated against a continuous flow of the buffer overnight. A filter paper disc was floated onto the surface of the packed Sephadex, and the serum applied evenly over the surface of the paper, using a syringe and polythene tubing. Once the serum had entered the column, buffer was carefully layered onto the surface, and fractions were collected utilising a Locarte fraction collector operating on a drop counting basis. The optical density at 280 mμ. was measured for each tube and on the basis of the elution curve, aliquots were pooled and the bactericidal activity of each pool was estimated.

CHAPTER 3.

BACTERICIDAL AND HAEMOLYTIC COMPLEMENT LEVELS OF VERTEBRATE SERA.

Introduction.

Our knowledge of the various aspects of serum bactericidal and haemolytic complement activities, has stemmed almost entirely from work with the serum of the common laboratory animals, and man. There are scattered reports in the literature dealing with the presence of haemolytic complement in the sera of other species of vertebrates, and that the serum of some of these animals also has bactericidal activity.

This chapter is concerned with a survey of haemolytic complement levels, and bactericidal activity for seven species of gram negative bacteria, in sera from the pig, man, kangaroo, fowl, toad, lizard and several species of fish.

Selection of Diluent for the Bactericidal Assays.

For the estimation of serum bactericidal activity any killing which occurs in a test system must be due solely to serum factors. The diluting fluid used must not itself be bactericidal, must not support a rapid increase in cell numbers, or be anticomplementary.

Several fluids were tested for their suitability to fulfil these criteria. Some difficulty was experienced in getting reproducible titres using physiological saline (0.85% sodium chloride). This was especially apparent with *E. coli* Lilly for which it was distinctly bactericidal. One per cent. glucose in saline buffered to pH 7 with 0.001 M. phosphate buffer was little better. The basal medium of Davis and Mingioli, or a 1/100 dilution in saline, gave consistently satisfactory results with all strains except *E. coli* Lilly which required, in addition, 1% glucose. Basal medium with or without glucose was used as diluent throughout the investigation.

Bactericidal Titres of the Various Sera.

The bactericidal titres,(dilution of serum which killed 50% of the inoculum in 90 minutes) of each serum against all the strains are given in Table 2. In Table 3 these titres have been expressed as a ratio, using the sensitivity of *S. gallinarum* 9240 to human serum as an arbitary reference value 1. There is no common pattern of killing of the strains by any two sera, nor is there any common ratio of sensitivity of any strain to all the sera.

Of particular interest, since previous investigators (Rowley,(1956); Michael and Landy, (1961) found smooth strains of bacteria were more resistant to serum killing than rough strains, was the observation that with

Table 2.

RANGE OF GRAM NEGATIVE BACTERIA KILLED BY THE VARIOUS NORMAL SERA.

Bacterial strain.	Reciprocal of bactericidal titre.						
	Normal Animal Sera.						
	Human	Kangaroo	Lizard	Fowl	Pig	Shark	Toad
E. coli BV.	10	20	40	20	20	0	2
E. coli Lilly.	300	30	60	5	100	20	40
S. typhimurium M206.	50	20	100	40	40	0	40
S. typhimurium C5.	0	0	8	0	0	0	0
S. gallinarum 9240.	2	0	20	0	0	0	0
S. paratyphi BIS.	2	0	10	0	10	0	2
S. paratyphi BIR.	80	8	10	0	40	0	2

The bactericidal activity of human, kangaroo, lizard, pig and fowl sera were estimated at 37°, toad and shark at 25°.

0 = no killing at a serum dilution of 1/2.

Table 3.

SERUM SENSITIVITY OF THE VARIOUS BACTERIAL STRAINS EXPRESSED AS A RATIO USING THE SENSITIVITY OF S. GALLINARUM 9240 TO HUMAN SERUM AS REFERENCE VALUE 1.

Bacterial strain.	Normal animal sera.						
	Human	Kangaroo	Lizard	Fowl	Pig	Shark	Toad
E. coli BV.	5	10	20	10	10	0	1
E. coli Lilly.	150	15	30	2.5	50	10	20
S. typhimurium M206.	25	10	50	20	20	0	20
S. typhimurium C5.	0	0	4	0	0	0	1
S. gallinarum 9240.	1	0	10	0	0	0	0
S. paratyphi BIS.	1	0	5	0	5	0	1
S. paratyphi BIR.	40	4	5	0	20	0	1

some sera, rough strains were equally, or more, resistant to killing than their smooth counterparts. The titres of human, pig, shark and toad sera for the rough strain E. coli Lilly were much higher than for the smooth strain E. coli B.V. The two strains were equally sensitive to killing by kangaroo and lizard sera, while some pools of fowl serum killed the smooth strain at a much higher dilution than the rough. Lizard, shark, toad and fowl sera were unable to kill the rough strain S. paratyphi BIR any more effectively than the smooth strain BIS, while human, kangaroo and pig sera had a much more effective bactericidal mechanism for the rough strain. Next to E. coli Lilly, S. typhimurium M206 was the strain most susceptible to killing by all the sera.

The two strains of S. typhimurium showed a great disparity in their sensitivity to killing by normal animal sera. The strain C5 was resistant to killing by all sera except lizard, while the strain M206 was highly sensitive to the bactericidal activity of all sera except fish.

There now seems little doubt from the experimental findings discussed in Chapter 1, that serum killing of gram negative bacteria only occurs in the presence of a sensitiser and complement. This conclusion suggested that e.g. pig serum might not kill S. typhimurium C5 because there were no sensitisers for this bacterium in the serum which were capable of fixing complement. Attempts were therefore made to determine if any such sensitisers were present in pig serum. The serum was absorbed overnight in the cold with 8, 4, 2, 1, and 0.5 mg. dry weight of living S. typhimurium C5/ml. of serum, after which the bacteria were removed by centrifugation and the supernatant assayed for haemolytic complement. With absorbing doses in excess of one mg. of bacterial suspension/ml. of serum, all haemolytic complement was removed, with one mg. of bacterial suspension/ml. of serum and less a proportional amount was removed (Table 4). As complement was fixed, (and we have no reason to doubt that sensitiser was necessary for this fixation) this strain must have been resistant to the final stage of the bactericidal mechanism in which complement exerted its lethal effect on the cell.

Comparison of the Bactericidal Properties of Different Fish Sera.

Serum from a fresh water teleost, Tinca tinca, (Tench) was bactericidal only for E. coli Lilly with an end titre of 1/10 at 25°, and 1/5 at 37°.

Serum from the marine elasmobranch, Emissola antartica, (gummy shark), like the other fish serum, killed only E. coli Lilly. Some difficulty was experienced in obtaining preparations of shark serum which had bactericidal activity. Some batches of serum contained a high level of haemolytic complement, in others complement could not be detected. The concentration of urea in the blood

Table 4.

EFFECT OF THE QUANTITY OF ABSORBING SUSPENSION OF S. TYPHIMURIUM C5 ON THE FIXATION OF COMPLEMENT FROM NORMAL PIG SERUM.

Absorbing dose in mg/ml. of serum.	Residual haemolytic complement, $C'H_{50}$/ml. of serum.
0	86
0.5	46
1.0	25
2.0	0
4.0	0
8.0	0

of elasmobranchs is much higher than in teleosts, and it was considered that the lack of haemolytic activity in some preparations of shark sera might be due to the decomposition of the urea to ammonia, which then inactivated the fourth component of complement. To test the validity of this theory, freshly drawn blood was dialysed for several hours against running sea water to reduce the urea level. The serum was then separated from the clot by centrifugation. As a control, blood was taken and allowed to clot without any further treatment. Both preparations of serum had similar bactericidal and haemolytic complement titres. The lack of complement activity in some serum preparations was evidently a reflection of the lability of complement in this serum.

The haemolytic complement level of shark serum decreased comparatively rapidly on storage at -20^{o}, there being a four fold decrease in titre after one month, and after three months complement could no longer be detected. The haemolytic complement titres of sera from the other vertebrate species showed little variation after six to twelve months storage.

Variation in Bactericidal Activity Between Different Pools of Sera from the Same Species.

In a comparative investigation it is desirable to use the same pool of serum throughout in order to obviate possible pool to pool variations in activity. This ideal could not be adhered to, however, either because the complement level fell on prolonged storage, or insufficient serum could be obtained at any one bleeding.

Table 5 shows the variations in bactericidal activity between some of the pools. The bactericidal titres differ slightly from pool to pool, but the same bacteria are, in general, killed by serum from the same species of animal and in general to a similar degree. There were several instances, however, where marked differences in titres were found. The bactericidal activity of the second pool of pig serum for E. coli Lilly was only a tenth of the first, and an eighth of the third.(see Table 5). Two pools of fowl serum had very low bactericidal activity for E. coli Lilly, while the third was four times as active. In the third pool of fowl serum the rough strain of E. coli was equally as sensitive as the smooth, not less sensitive, as had been the case with the previous two serum pools. S. paratyphi BIS was killed by a much higher serum dilution in the second pool of human serum than in the first. These pool to pool variations in bactericidal titre were due to differences in the sera, since the bactericidal titres of control sera were always reproducible.

Haemolytic Complement Levels of the Various Sera.

The haemolytic complement levels of the sera are given in Table 6. Some of these sera had been kept for a

Table 5.

POOL TO POOL VARIATION IN SERUM BACTERICIDAL ACTIVITY.

Bacterial strain.	Reciprocal of bactericidal titre.							
	Normal animal sera.							
	Pig.			Fowl.			Human.	
	Pool 1.	2.	3.	Pool 1.	2.	3.	Pool 1.	2.
E. coli Lilly.	100	10	80	5	5	20	300	400
E. coli BV.	20	10	40	30	20	20	10	40
S. typhimurium M206.	40	40	80	40	40	40	50	200
S. typhimurium C5.	0	0	0	2	0	0	0	0
S. gallinarum 9240.	0	0	0	4	0	0	2	0
S. paratyphi BIS.	10	10	20	2	0	2	2	20
S. paratyphi BIR.	40	20	40	2	0	0	80	80

0 = no killing at a serum dilution of 1/2.

Table 6.

HAEMOLYTIC COMPLEMENT LEVELS OF THE VARIOUS SERA.

Serum.	Temperature at which assayed.	$C'H_{50}$/ml. of serum.
Human.	37	40
Kangaroo.	37	32
Lizard.	37	35
Fowl.	37	18
Pig.	37	25
Shark.	25	100
Toad.	25	150

year at -20^{o} before the comparative assays were made, and consequently the haemolytic complement levels of several of the sera were lower than originally found. Subsequent pools of fowl and pig sera contained 24 $C'H_{50}$/ml. and 44 $C'H_{50}$/ml. respectively. Although the haemolytic complement levels of the new serum pools were higher than those assayed in the originals, the bactericidal activities were comparable.

Both toad and shark sera contained high levels of haemolytic complement, approaching that reported for the guinea pig. Equal haemolytic titres for shark serum were obtained using normal sheep red blood cells and those sensitised with rabbit haemolysin.

Discussion.

The observations reported in this chapter extend the range of vertebrate species whose serum has been titrated for haemolytic complement, and bactericidal activity against gram negative bacteria.

One of the surprising features of this investigation was that contrary to previous findings (Rowley, 1956) smooth bacteria were not invariably more resistant to serum killing than their rough counterparts. The expected greater sensitivity of the rough strain compared with the smooth, was found with human serum, but with the other sera either some or all pools showed equal, or reversed, sensitivities for one or both of the rough strains. Fowl and shark sera had no bactericidal activity at all for the rough strain

S. paratyphi BIR.

It could be inferred from the results presented in Table 2 that shark serum lacked a highly developed bactericidal mechanism, but this apparent lack of activity may well be only a reflection of the bacterial strains used to assay the activity. The shark may never have come into contact with these bacterial species, and hence, may not have produced sensitisers against them. The overall picture may have been quite different had marine bacteria been used. The relative lack of bactericidal activity in the fish sera compared with, for example, lizard serum does not detract from the importance of the observation that these sera possess a bactericidal mechanism.

Summary.

All seven vertebrate sera examined possessed haemolytic complement and were bactericidal towards one or more strains of gram negative bacteria. The range of bacteria killed, and the serum dilution to which they were killed varied from one species of animal to another and in some instances in the same species from one serum pool to another.

Contrary to previous observations rough strains of bacteria were not invariably more sensitive to serum killing than their smooth counterparts.

CHAPTER 4.

THE EFFECT OF TEMPERATURE ON THE BACTERICIDAL AND HAEMOLYTIC COMPLEMENT ACTIVITIES OF SEVERAL VERTEBRATE SERA.

Introduction.

In the previous chapter it was shown that the bactericidal activity of fish serum was greater at 25° than at 37°. Cushing (1945a) reported a variation in the optimum temperatures, and the range of temperatures, over which sera derived from warm and cold blooded animal species were haemolytically active.

No similar investigations appear to have been made to determine what effect variations in temperature have on the bactericidal reaction. This chapter is therefore concerned with a comparative investigation of the effect variations in temperature have on the bactericidal and haemolytic complement activities of several sera.

The Effect of Temperature on the Haemolytic Complement and Bactericidal Activities of Sera.

The haemolytic complement activities of pig, lizard, toad and shark sera were estimated at five degree intervals from five to 50°. All the estimations were made on the one day using the same preparation of sensitised sheep red blood cells. Toad serum was not tested at 5° or shark above 40°.

The bactericidal activities of pig, lizard and toad sera were estimated at five degree intervals from five to 45° with E. coli Lilly as test strain.

The temperature-activity relationships for the haemolytic complement and bactericidal activities of the sera are shown in Figures 1 and 2 respectively. The optimum activity temperatures calculated from these curves are given in Table 7.

These results supported the observations reported by Cushing (1945a) that there is a species variation in the optimum temperature for haemolytic complement activity. For a homeothermic animal (pig) the optimum temperature approximated to the body temperature, for the poikliothermic animals (toad, lizard and shark) the optimum temperature was approximately ten degrees lower.

The sera also differed in the relative rates at which they lysed sensitised erythrocytes at the extremities of the temperature range over which lysis was followed. Sera from the poikliothermic species were more haemolytically active at the lower temperatures than pig serum, and vice versa at the upper end of the temperature range.

The shape of the temperature bactericidal activity curves for toad and lizard sera closely paralleled those

Figure 1.

EFFECT OF TEMPERATURE ON THE HAEMOLYTIC COMPLEMENT ACTIVITIES OF PIG, LIZARD, SHARK AND TOAD SERA.

Curve 1.	Pig serum.
Curve 2.	Lizard serum.
Curve 3.	Toad serum.
Curve 4.	Shark serum.

The titres of each serum are expressed as a % of the maximum titre for that serum.

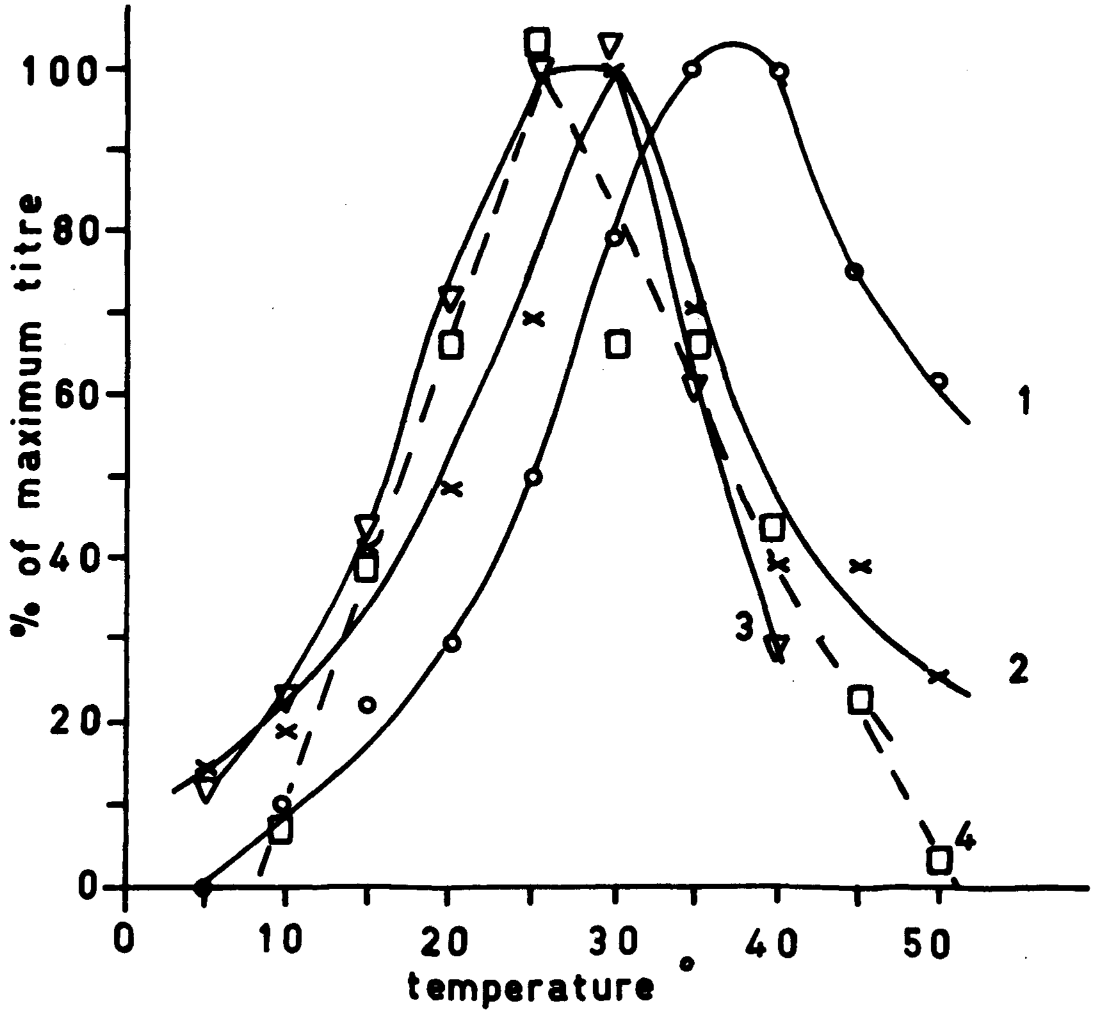

% of maximum titre
100
80
60
40
20
0
0
10
20
30
40
50
temperature °
1
2
3
4

Figure 2.

EFFECT OF TEMPERATURE ON THE BACTERICIDAL ACTIVITIES OF PIG, LIZARD AND TOAD SERA FOR E. COLI LILLY.

Curve 1.	Pig serum.
Curve 2.	Lizard serum.
Curve 3.	Toad serum.

The titres for each serum are expressed as a log % of the maximum titre for that strain.

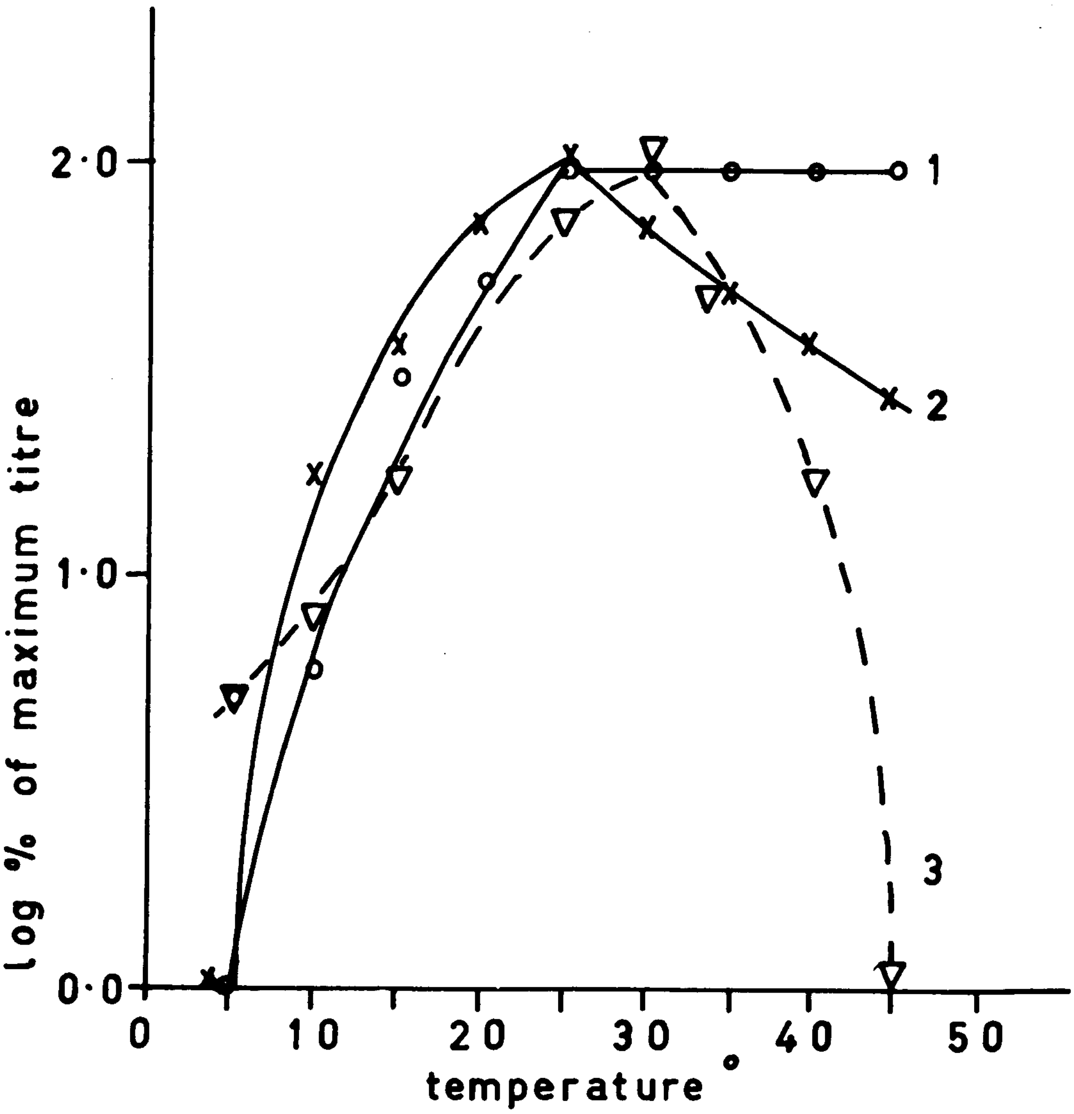

2·0
1·0
0·0
0
10
20
30
40
50
temperature °
Log % of maximum titre
1
2
3

Table 7.

OPTIMUM TEMPERATURES FOR THE HAEMOLYTIC COMPLEMENT AND BACTERICIDAL ACTIVITIES OF SERA FROM DIFFERENT ANIMAL SPECIES.

Serum.	Haemolytic complement.	Bactericidal activity.
Pig.	35-40°	Above 25°
Lizard.	30°	25°
Toad.	25°	30°
Shark.	25-30°	-

- = not done.

for the haemolysis of red blood cells, with the optimum activity occurring over the same temperature range.

These results would suggest that with these sera the same components were rate limiting for both activities.

The relationship between the bactericidal activity of pig serum and temperature differed markedly from that of its haemolytic activity, and from the temperature dependence of the bactericidal activities of the other sera. Maximum killing commenced at 25°, i.e. 10° lower than in the haemolytic reaction, and the level of killing remained steady over the entire upper temperature range.

Determination of the Part Played by Complement Destruction in the Depression of the Haemolytic and Bactericidal Activities of Sera at Temperatures Above the Optimum.

In the previous section it was shown that with the exception of pig serum, both the haemolytic and bactericidal activities of the sera fell as the temperature was raised above the optimum.

In a simple enzyme reaction, the decrease in the overall rate of the reaction at temperatures above the optimum, is the resultant of the interaction between an increase in the rate of the reaction with rising temperature, and destruction of the enzyme. Since the complement complex has enzymic properties it would be expected that complement destruction would be responsible, at least in part, for the decrease in both the lytic and bactericidal activities of the sera at temperatures above the optimum.

The heat lability of toad and lizard serum complement was determined by heating the sera at 40° and 45° for thirty minutes, and then assaying the residual level of haemolytic and bactericidal activity at 25°. These titres were compared with those of normal serum tested in parallel. The results of the haemolytic tests are given in Table 8.

Toad and lizard serum complement differed in heat stability, thirty minutes at 45° led to the loss of all haemolytic complement activity in toad serum; lizard serum treated similarly lost 70% of its activity.

The haemolytic complement titres of the heated sera, when assayed at 25°, were less than those of normal sera assayed at the same temperature, but greater than those of normal sera assayed at the temperature to which the sera were first subjected.

These results indicated that the decrease in haemolytic complement titres at temperatures above the optimum were due partly to complement destruction, and partly to a decrease in the rate of the reaction.

There was a similar diminution in the bactericidal activities of the sera.

Table 8.

EFFECT OF HEAT ON THE HAEMOLYTIC COMPLEMENT TITRES OF TOAD AND LIZARD SERA.

Serum.	Serum treatment.				
	Normal.			Heated for 30 minutes at 40°	45°
	Haemolytic complement activity estimated at				
	25°	40°	45°	25°	
Toad.	100	30	5	70	0
Lizard.	100	65	30	70	30

The haemolytic complement titre of the normal serum at 25° was given the arbitrary value of 100 and the other titres expressed as a percentage of this value.

Summary.

The optimum temperatures for the haemolytic activities of shark, lizard and toad sera were between 25 and 30°, that for the pig between 35 and 40°.

The optimum temperature for the bactericidal activity of toad and lizard sera was 25°, but pig serum reached its maximum bactericidal activity at 25° with no alteration in activity as the temperature was further increased.

Complement destruction was shown to be responsible, at least in part, for the lowered serum bactericidal and haemolytic complement titres at temperatures above the optimum.

CHAPTER 5.

SPECIFICITY OF SENSITISERS MEDIATING IN NORMAL SERUM BACTERICIDAL ACTIVITY.

Introduction.

Serum killing of gram negative bacteria can be mediated either by immune antibody or by natural (normal) antibody. There is no doubt that immune antibody is highly specific, reacting only with the antigen used to elicit its production, or with antigens which are very closely related chemically. There has been no such unanimity on the question of the degree of specificity shown by the natural antibodies. There are many reports in the literature which claim to show that these antibodies are highly specific, others by equally reputable workers show them to be non-specific. The significamce of these observations was discussed in Chapter I.

The term "natural antibody" has become somewhat ill-defined, being used to describe proteins which participate in a number of serological phenomena, often with scant regard to the specificity of the factors. The term "sensitiser", (Muir, 1931) rather than natural antibody, has been used throughout this thesis to describe the serum factors mediating with complement in the killing of gram negative bacteria.

This chapter is concerned with investigations designed to define the specificity of the sensitisers.

Specificity of Serum Sensitisers for Smooth Strains of Gram Negative Bacteria.

The specificity of the natural sensitisers were studied by absorbing the serum with one bacterial serotype, then determining what effect this absorption had on the bactericidal activity towards homologous and heterologous bacterial strains. Normal human serum was absorbed overnight in the cold with heat-killed suspensions of E. coli BV and S. typhimurium M206, at dose levels of 8, 4 and 2 mg. dry weight of bacteria/ml. of undiluted serum. Following the removal of the absorbing bacteria by centrifugation at 4,500 g. the sera were tested for both haemolytic complement and residual bactericidal activity against E. coli BV, S. typhimurium M206 and S. paratyphi BIR (Table 9).

With both absorptions, the bactericidal activity for the homologous strain was reduced by more than 80% without significantly affecting the level against the unrelated ones. Haemolytic complement was reduced following absorption with E. coli BV but this had no apparent effect on the bactericidal activity of the serum for the other strains. The variations in the bactericidal titres of lizard serum following absorption with S. typhimurium C5 were more difficult to interpret.(Table 10). The titre against the homologous strain was reduced by more than 75%

Table 9.

THE SPECIFICITY OF NATURAL SENSITISERS IN HUMAN SERUM.

(1) Following absorption with *E. coli* BV.

Bacterial strain.	Reciprocal of bactericidal titre.			
	Dry weight of absorbing suspension/ml. of serum.			Unabsorbed control.
	8 mg.	4 mg.	2 mg.	
E. coli BV.	2	2	2	10
S. typhimurium M206	50	50	50	50
S. paratyphi BIR	40	80	80	80
E. coli Lilly.	200	200	200	200
$C'H_{50}$/ml. of serum.	25	35	35	35

(2) Following absorption with *S. typhimurium* M206.

Bacterial strain.	Reciprocal of bactericidal titre.			
	Dry weight of absorbing suspension/ml. of serum.			Unabsorbed control.
	8 mg.	4 mg.	2 mg.	
E. coli BV.	10	10	10	10
S. typhimurium M206	5	15	15	60
S. paratyphi BIR	40	40	40	40
E. coli Lilly.	200	200	200	200
$C'H_{50}$/ml. of serum.	35	35	35	35

Table 10.

SPECIFICITY OF THE NATURAL SENSITISERS IN LIZARD SERUM.

Bacterial strain.	Reciprocal of bactericidal titre.		
	Dry weight of absorbing * suspension/ml. of serum.		Control serum.
	8 mg.	2 mg.	
S. typhimurium C5.	2	2	8
S. typhimurium M206	25	25	50
S. gallinarum 9240	10	10	20
S. paratyphi BIS	5	5	10
S. paratyphi BIR	5	5	10
E. coli BV	10	10	20
$C'H_{50}$/ml.	20	25	40

* Serum absorbed with S. typhimurium C5.

following absorption with 2 mg. dry weight of suspension/ml. of serum, and the fall was greater with 8 mg. The titre against S. paratyphi BIS also fell by more than 50%, but since these two strains share the same somatic antigens, (I, IV and XII) this result was not unexpected. The titre against S. typhimurium M206 was not affected to the same extent, even though this strain is identical with S. typhimurium C5, (I, IV, V and XII) as far as can be ascertained by Salmonella antigenic typing techniques. The only known differences between these two strains is in their virulence for the mouse and their phage type.(Furness and Rowley, 1956).

This investigation has shown another manifestation of the differences between these two strains of S. typhimurium, that of a greater susceptibility to serum killing of the strain M206 compared with the strain C5. These differences in susceptibility to serum killing can only be due to some as yet undetected variation in the surface structure of the two cell types.

In these absorptions there was a decrease in the level of haemolytic complement which possibly accounted for the overall reduced bactericidal activity. The picture, however, is one of specific absorption.

In the above experiments the minimum quantity of absorbing suspension required to decrease the bactericidal activity was not ascertained, but with pig serum and E. coli BV this was between 0.1 and 0.5 mg. dry weight of bacterial suspension/ml. of serum. (Figure 3). That the sensitisers in pig serum for E. coli BV were directed against the somatic antigens, (lipopolysaccharide) was shown by inhibition tests following the technique described by Michael, Whitby and Landy, (1962). Graded amounts of lipopolysaccharide were added to undiluted serum and allowed to react for fifteen minutes at room temperature, after which the bactericidal activity of the treated serum was assayed in the usual way. The results of these tests are shown graphically in Figure 4.

Restoration of Bactericidal Activity to an Absorbed Normal Serum with Immune Antibody.

If during absorption, antibody is removed from a serum, then the bactericidal activity should be restored with the appropriate immune antibody. The ability of rabbit immune antibody to restore the bactericidal activity to human serum absorbed with E. coli BV and S. typhimurium M206 was therefore investigated. An equal volume of a dilution of the absorbed serum was added to serial two-fold dilutions of the homologous immune antibody. The bactericidal activity of this reconstituted serum was then assayed. The results of these experiments are shown graphically in Figures 5 and 6. Immune antibody restored the bactericidal activity to the absorbed sera over a limited range of immune serum

Figure 3.

THE EFFECT OF ABSORPTION OF PIG SERUM WITH VARYING LEVELS OF E. COLI BV ON THE BACTERICIDAL TITRE FOR E. COLI BV.

Bactericidal titre plotted as a % of the titre of normal serum.

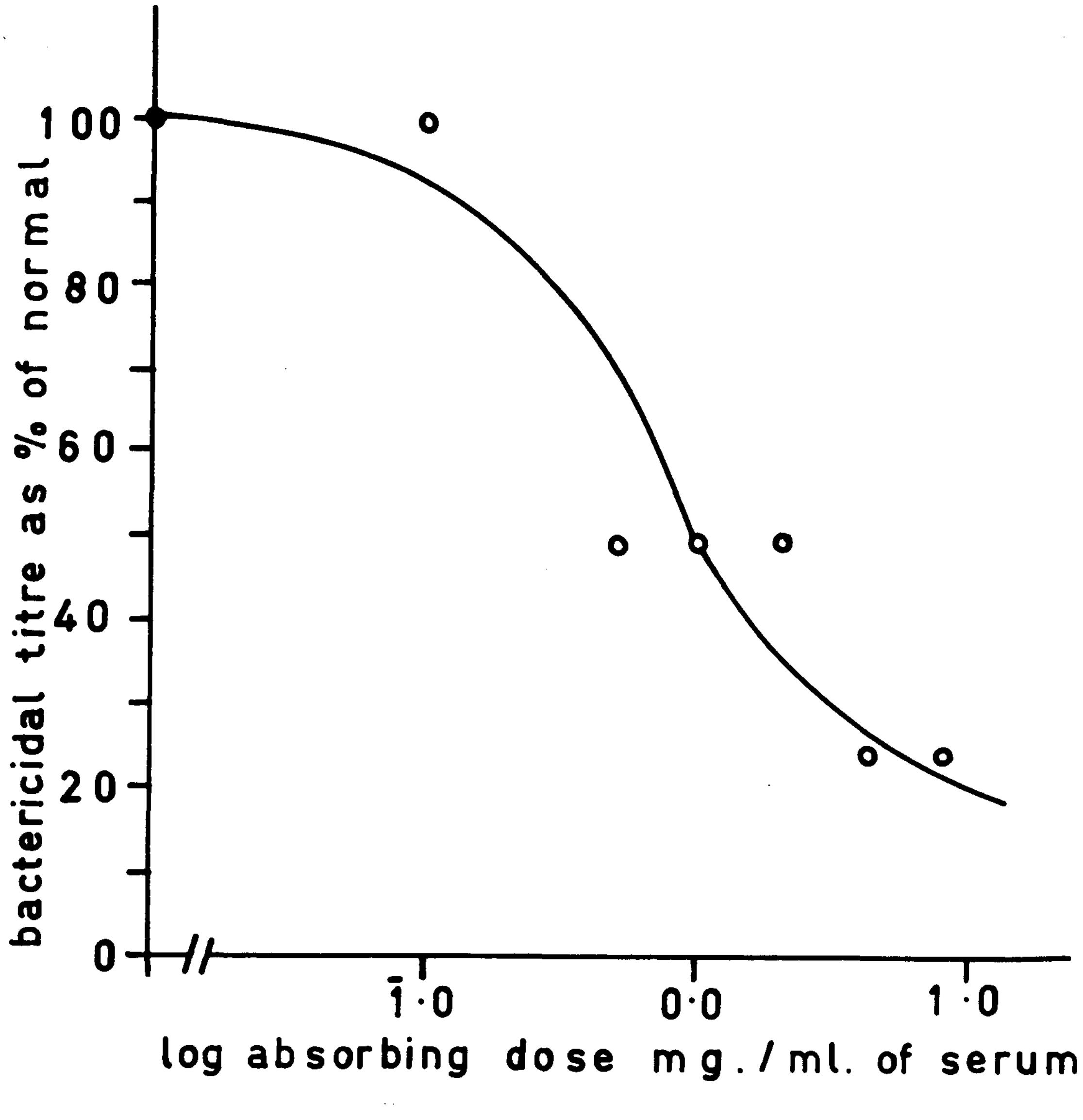

100
80
60
40
20
0
bactericidal titre as % of normal
$\bar{1}\cdot0$
0·0
1·0
log absorbing dose mg./ml. of serum

Figure 4.

VARIATIONS IN THE BACTERICIDAL TITRE OF PIG SERUM FOR E. COLI BV FOLLOWING INCUBATION WITH VARYING QUANTITIES OF E. COLI BV LIPOPOLYSACCHARIDE.

Bactericidal titre plotted as a % of the titre of normal serum.

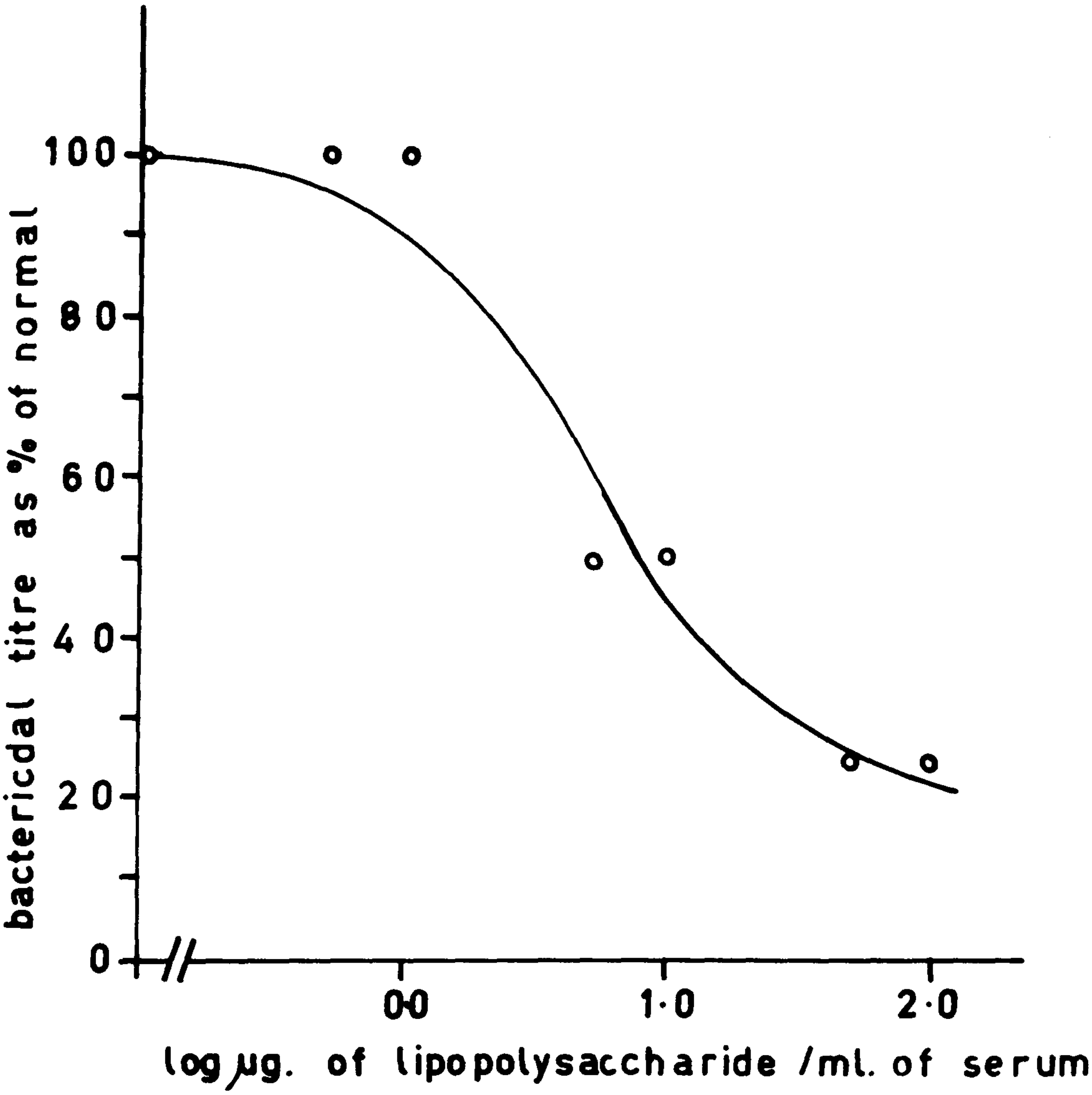

bactericdal titre as % of normal
100
80
60
40
20
0
0·0
1·0
2·0
log µg. of lipopolysaccharide /ml. of serum

Figure 5.

RECONSTITUTION OF THE BACTERICIDAL ACTIVITY OF HUMAN SERUM ABSORBED WITH 4 mg. E. COLI BV/ml. OF SERUM WITH RABBIT IMMUNE SERUM TO E. COLI BV.

Absorbed human serum used at a final dilution of 1/10.

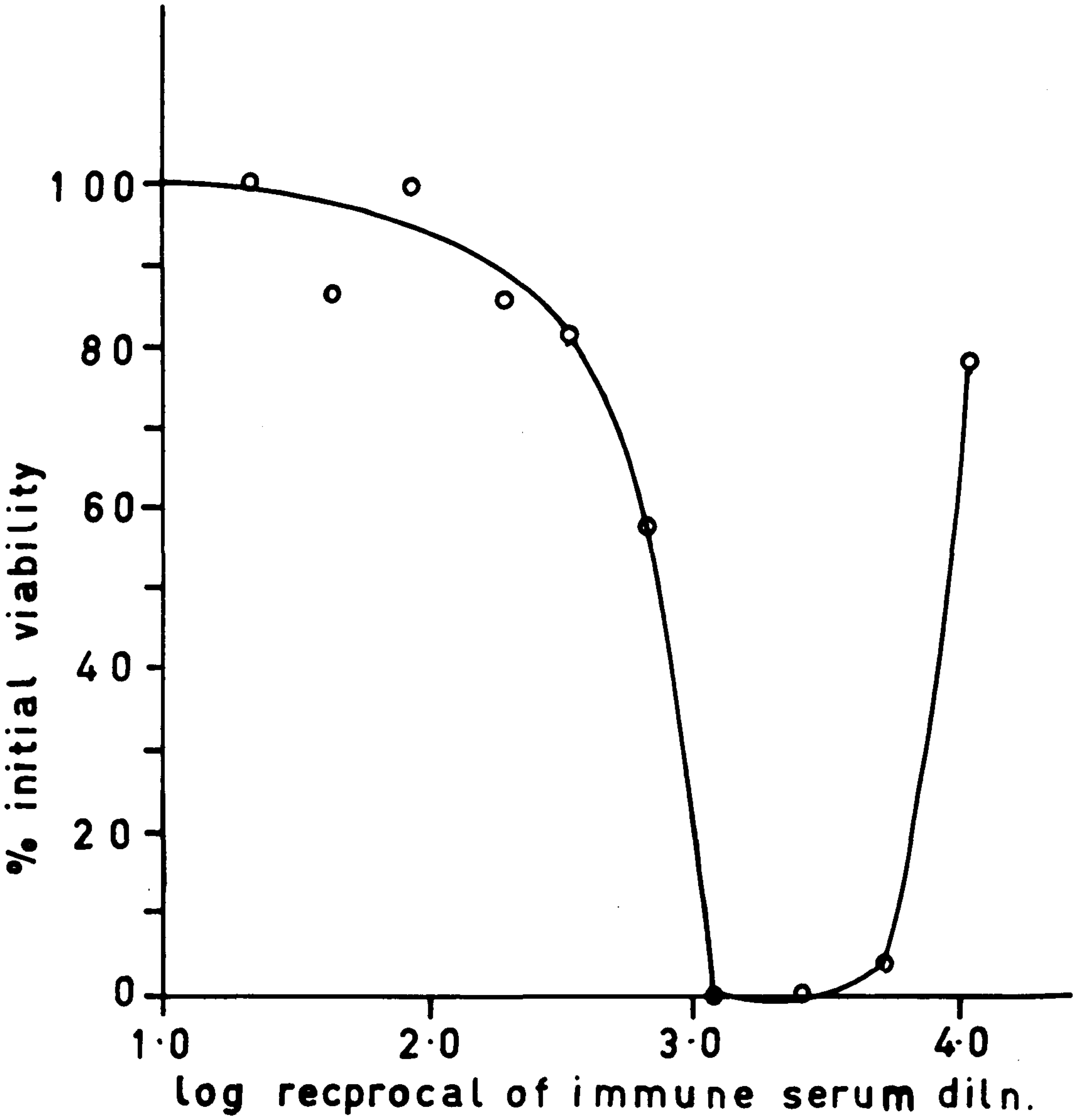
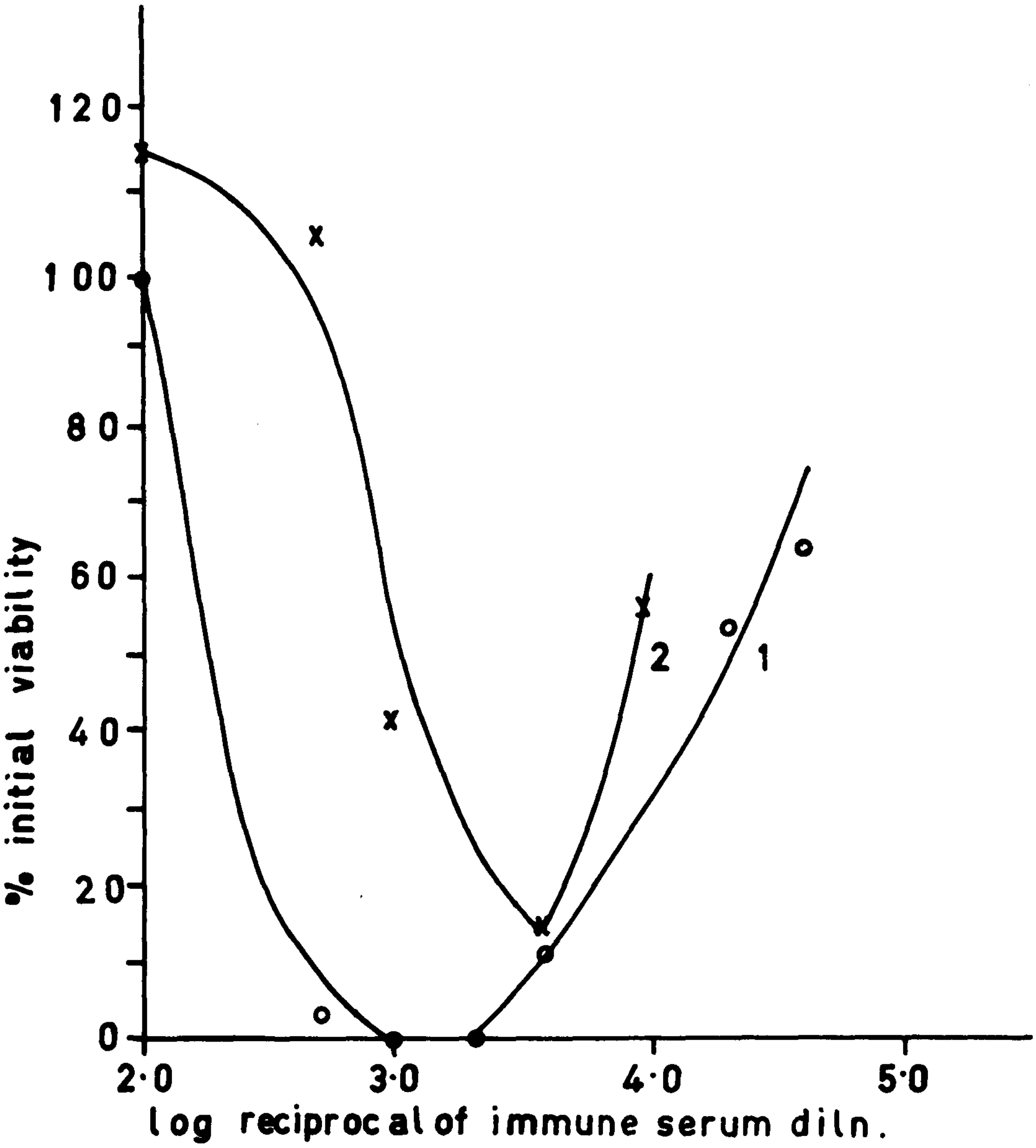

% initial viability
100
80
60
40
20
0
1·0
2·0
3·0
4·0
log recprocal of immune serum diln.

Figure 6.

RECONSTITUTION OF THE BACTERICIDAL ACTIVITY OF HUMAN SERUM ABSORBED WITH S. TYPHIMURIUM M206 WITH RABBIT IMMUNE SERUM TO S. TYPHIMURIUM M206.

1. Human serum absorbed with 8 mg. dry weight of bacteria/ml. of serum and used at a final dilution of 1/40.
2. Human serum absorbed with 4 mg. dry weight of bacteria/ml. of serum and used at a final dilution of 1/80.


120
100
80
60
40
20
0
% initial viability
2·0
3·0
4·0
5·0
log reciprocal of immune serum diln.
2
1

dilutions, killing being inhibited by excess immune antibody, and no killing occurred when the antibody had been diluted to extinction.

Specificity of Serum Sensitisers for Rough Gram Negative Bacilli.

Difficulty was experienced in specifically reducing the bactericidal activity of both human and fowl sera for E. coli Lilly, since with many absorptions, the bactericidal activity was reduced or lost due to the inactivation of complement.

When the pool of human serum used in the early part of this investigation was absorbed with heat killed suspensions of E. coli Lilly and S. paratyphi BIR, all haemolytic complement activity was lost after several minutes contact at 4°. This loss still occurred when the absorbing dose of bacteria was reduced to as low as 0.1 mg. dry weight of bacteria/ml. of serum. The loss of haemolytic complement during absorption was a peculiarity of this human serum pool, since a subsequent pool was absorbed with no loss of haemolytic complement activity. (To be discussed on page 88).

Because of the difficulty of carrying out absorptions of human serum with E. coli Lilly without the complete loss of haemolytic complement, the absorptions were repeated with fowl serum. This serum was selected because of its low bactericidal activity for E. coli Lilly which it was presumed indicated a lower ratio of sensitiser to complement in this serum than in human serum.

Fowl serum was absorbed with 10 mg. dry weight of *E. coli* Lilly/ml. of serum for from 15 minutes to twenty four hours at 4°. The absorbed sera were assayed for bactericidal and haemolytic complement activity. The bactericidal activity of the absorbed sera was not significantly altered, although there was a progressive fall in haemolytic complement as the period of absorption was increased, (Table 11).

As it was not possible to deplete the bactericidal activity of the sera for *E. coli* Lilly without a reduction of the haemolytic complement levels, efforts were made to show that sensitisers for this strain were present in human serum by using complement depleted human serum as a source of sensitiser and fowl serum as a source of complement. The human serum was depleted of complement by absorption with sheep red cell stroma sensitised with haemolysin prepared in the rabbit, such that 0.4 ml. of the treated serum gave no haemolysis in the standard haemolytic assay. To serial two-fold dilutions of this serum was added fowl serum diluted beyond its 50% bactericidal end point for *E. coli* Lilly. This mixture of sera had bactericidal activity for *E. coli* Lilly. When falling dilutions of human serum were added to an excess of fowl complement and the complex assayed for bactericidal activity, the end

Table 11.

THE EFFECT OF ABSORPTION OF FOWL SERUM WITH 10 mg. DRY WEIGHT OF E. COLI LILLY/ml. OF SERUM FOR VARYING TIME INTERVALS, ON BOTH THE HAEMOLYTIC COMPLEMENT LEVEL AND THE BACTERICIDAL ACTIVITY FOR E.COLI LILLY.

Time of absorption in minutes.	$C'H_{50}$/ml. of serum.	Reciprocal of bactericidal titre.
0	27	5
15	20	10
30	13	10
60	12	10
120	12	10
240	12	5
480	9	5
24 (hrs)	6	5

point of the titration occurred with the human serum diluted to the same extent as it would have been had the titration been performed in the presence of its own complement. This suggested that it was some factor in the human serum which was limiting the extent of the reaction. In these reconstitution experiments, fowl complement was three to four times less efficient than the homologous human complement.

The bactericidal activity of the reconstituted sera was not affected when the complement-depleted serum was absorbed overnight with 80 mg. dry weight of E. coli Lilly/ml. of serum.

Levine, Cowan, Osler and Mayer, (1953) showed that the uptake of complement by specific antigen-antibody precipitates could be prevented by complexing the serum calcium and magnesium ions with sodium EDTA. The inclusion of barium ions in the reaction mixture minimised complement destruction. Full haemolytic complement activity was restored, following the removal of the antigen-antibody precipitate, by the addition of calcium and magnesium ions. This technique was used in an attempt to prevent the uptake of complement during absorptions of human serum with E. coli Lilly. Sodium EDTA, barium sulphate, and calcium chloride were made up to a final concentration of 0.1 M, and magnesium sulphate to 0.15 M. Sufficient sodium EDTA was added to the serum to chelate all the serum calcium and magnesium ions together with any which might be present on the bacteria. The barium sulphate was then added, followed by the bacteria. The serum was absorbed overnight in the cold, followed by the removal of the bacteria by centrifugation. Sufficient calcium and magnesium ions were than added back to compensate for those chelated by the versene. Table 12 shows the relation between the size of absorbing dose and the reduction in bactericidal activity. The titre of the serum against E. coli Lilly fell progressively as the size of the absorbing dose was increased. These absorptions did not affect the bactericidal activity of the serum for E. coli BV.

The bactericidal activity of this absorbed serum was not reconstituted with E. coli Lilly rabbit immune antibody.

Serum Lysozyme.

There have been two recent reports which claimed that rough gram negative bacteria were killed by complement acting alone (Sterzl, et al., 1962), and by complement together with serum lysozyme.(Wardlaw, 1962). If either of these reports mirror the true situation, then there should exist a direct relationship between either the haemolytic complement levels of different sera and their bactericidal titres, or between the bactericidal titres and the combined haemolytic complement and lysozyme levels.

The lysozyme level in the serum of each animal

Table 12.

ABSORPTION OF HUMAN SERUM WITH DIFFERENT LEVELS OF E. COLI LILLY IN THE PRESENCE OF SODIUM EDTA AND BARIUM IONS.

Absorbing dose of *E. coli* Lilly. mg/ml. of serum.	Bactericidal titre as % of control.
----	100
8	50
25	25
50	12½
100	12½

---- = not absorbed.

species was assayed using the technique described by Wardlaw, (1962). These levels together with those for haemolytic complement and bactericidal activity against E. coli Lilly are given in Table 13. The data presented in this table shows no correlations between the bactericidal titres for E. coli Lilly of sera from different species and the haemolytic complement levels, or combined complement and lysozyme levels. The following are some examples of this lack of correlation. The haemolytic complement titres of human, pig, lizard and kangaroo sera were all similar, but the bactericidal titre of human serum for E. coli Lilly was eight to fifteen times greater than the titre of any other sera.

Toad serum had more than twice the haemolytic complement of human serum, but only 8% of the bactericidal titre. Haemolytic complement could not be detected in embryo pig serum, although the bactericidal activity for E. coli Lilly was half that of toad serum.

The lack of correlation between the bactericidal titre for E. coli Lilly and the haemolytic complement level in fowl serum was referred to in an earlier section.(page 84). It was shown that absorption with E. coli Lilly reduced the haemolytic complement level without affecting the bactericidal titre. These few examples show that the observed species variations in serum bactericidal titres for E. coli Lilly can not be explained

Table 13.

RELATION BETWEEN THE BACTERICIDAL TITRES OF EACH OF THE SERA FOR E. COLI LILLY AND THE LEVELS OF HAEMOLYTIC COMPLEMENT AND SERUM LYSOZYME.

Serum.	Serum lysozyme µg/ml. of serum	Reciprocal of bactericidal titre.	$C'H_{50}$/ml. of serum.
Human.	9	300	40
Pig.	0.6	40	53
Lizard.	11	20	43
Toad.	<0.25	20	100
Kangaroo.	2	30	32
Embryo pig.	<0.25	10	Not detected.

All figures refer to tests made at 37°.

solely by differences in the levels of haemolytic complement.

A similar situation exists when the bactericidal titres are compared with the combined haemolytic complement and lysozyme levels. Both toad and embryo pig sera contained no detectable amounts of lysozyme, yet they were as bactericidal as lizard serum which had a lysozyme level of 11 µg./ml. The embryo pig and toad sera also showed greater than a hundred-fold variation in haemolytic complement titre. Human and lizard sera had similar levels of both lysozyme and haemolytic complement but, as already mentioned, the bactericidal activity of human serum for E. coli Lilly greatly exceeded that of all other sera tested.

The addition of 10 µg. of egg white lysozyme/ml. of toad and pig sera did not increase their bactericidal activity for E. coli Lilly, although the levels of both lysozyme and haemolytic complement were then equal to, or in excess of, that in human serum.

Alterations in the Bactericidal Titre, Serum Lysozyme and Haemolytic Complement Levels of Human Serum Following Absorption with E. coli Lilly.

Normal human serum was absorbed with E. coli Lilly by the technique described by Wardlaw (1962), i.e. three fifteen minute absorptions at 4^{o} with 50 mg. dry weight of bacteria/ml. of serum. The haemolytic complement, lysozyme and bactericidal activities of these absorbed sera were assayed in parallel with normal serum. The bactericidal titre decreased four-fold with the first absorption, but showed no further decline with the subsequent ones; there was a small overall drop in the haemolytic complement level but the lysozyme level fell progressively with each absorption. There was thus no correlation between the level of serum lysozyme and the degree of killing. The bactericidal activity against E. coli BV was not affected. (Table 14).

The bactericidal activity was not restored to the absorbed sera either by lysozyme or by E. coli Lilly rabbit immune antibody.

The Bactericidal Activity of Embryo Pig Serum.

The evidence on which Sterzl, et al.,(1962) based their claim for the non-participation of a sensitiser in the killing of rough gram negative bacteria, was the inability to deplete the bactericidal activity of embryo pig serum by absorption, and the observations that the serum of the embryo pig had no bactericidal activity for smooth gram negative bacteria unless fed colostrum.

These observations of Sterzl and his colleagues were not confirmed in the present investigation despite the fact that several pools of embryo pig serum were used. Smooth strains as well as rough were killed by the embryo pig serum as shown in Table 15. The titres against all strains were

Table 14.

COMPARISON OF THE LEVELS OF HAEMOLYTIC COMPLEMENT, LYSOZYME AND BACTERICIDAL ACTIVITY AGAINST E. COLI LILLY OF HUMAN SERUM ABSORBED ONE, TWO AND THREE TIMES WITH 50 mg. DRY WEIGHT E. COLI LILLY/ml. OF SERUM.

Serum treatment.	$C'H_{50}$/ml. of serum.	Lysozyme µg/ml. of serum.	Reciprocal of bactericidal titre.
Normal.	130	7.5	160
First absorption.	110	5.8	40
Second "	110	3.8	40
Third "	100	1.0	40

Table 15.

COMPARISON OF THE BACTERICIDAL TITRES OF EMBRYO AND ADULT PIG SERA FOR SEVERAL SPECIES OF GRAM NEGATIVE BACTERIA.

Bacterial strain.	Reciprocal of bactericidal titre.	
	Serum.	
	Embryo pig.	Normal pig.
E. coli Lilly.	10	100
E. coli BV.	2	20
S. typhimurium M206.	10	40
S. typhimurium C5.	0	0
S. gallinarum. 9240.	0	0
S. paratyphi BIS	0	10
S. paratyphi BIR	5	40
$C'H_{50}$/ml. of serum.	0	53

lower than with adult pig serum, but S. paratyphi BIS was the only strain killed by the adult pig serum and not by the embryo serum. There is little likelihood that these results could be explained by contamination of the embryo serum with adult serum since the embryos were separated from the placenta prior to bleeding by cardiac puncture.

Since no one doubts that a sensitiser is required for killing smooth gram negative bacteria, there is no reason to believe that sensitisers were not present in the embryo pig serum for the rough as well as the smooth strains despite the inability of Sterzl and his colleagues to deplete the bactericidal activity of their embryo pig sera for rough strains of gram negative bacteria by absorption.

Summary.

A study was made of the specificity of natural sensitisers in human serum to several gram negative bacteria.

The sensitisers to the smooth strains were specifically removed by absorption, those to rough strains were also specifically removed, but the absorbing doses required were approximately fifty times greater.

The serum lysozyme level in each serum was estimated, and an attempt made to correlate the degree of bactericidal activity against E. coli Lilly with the levels of haemolytic complement and lysozyme. The extent of killing could not be correlated with the level of either complement, or complement and lysozyme.

Summary of Chapters 3, 4 and 5.

Human, pig, lizard, fowl, toad and fish sera were all shown to possess a complement mediated bactericidal system capable of killing some strains of gram negative bacteria. Evidence was obtained which showed that the killing was mediated by complement and a specific sensitiser. No evidence could be found to suggest that lysozyme participated in the serum killing of rough strains of gram negative bacteria.

While each serum was capable of killing some strains of gram negative bacteria and lysing foreign erythrocytes, the nature of the complement complex mediating these activities differed, since the optimum temperatures, and range of temperatures over which the individual sera were active were not the same.

These studies which demonstrated that the complement mediated bactericidal system was ubiquitous in the sera of vertebrate animals prompted an investigation being made to determine if similar bactericidal mechanisms were present in invertebrate sera. The following chapters deal with the demonstration of a bactericidal mechanism in the serum of a crustacean and the subsequent investigations to define the specificity of the bactericidal factor(s) and to determine its chemical nature.

CHAPTER 6.

BACTERICIDAL SPECTRUM OF YABBIE SERUM AND SPECIFICITY OF THE BACTERICIDAL MECHANISM.

Introduction.

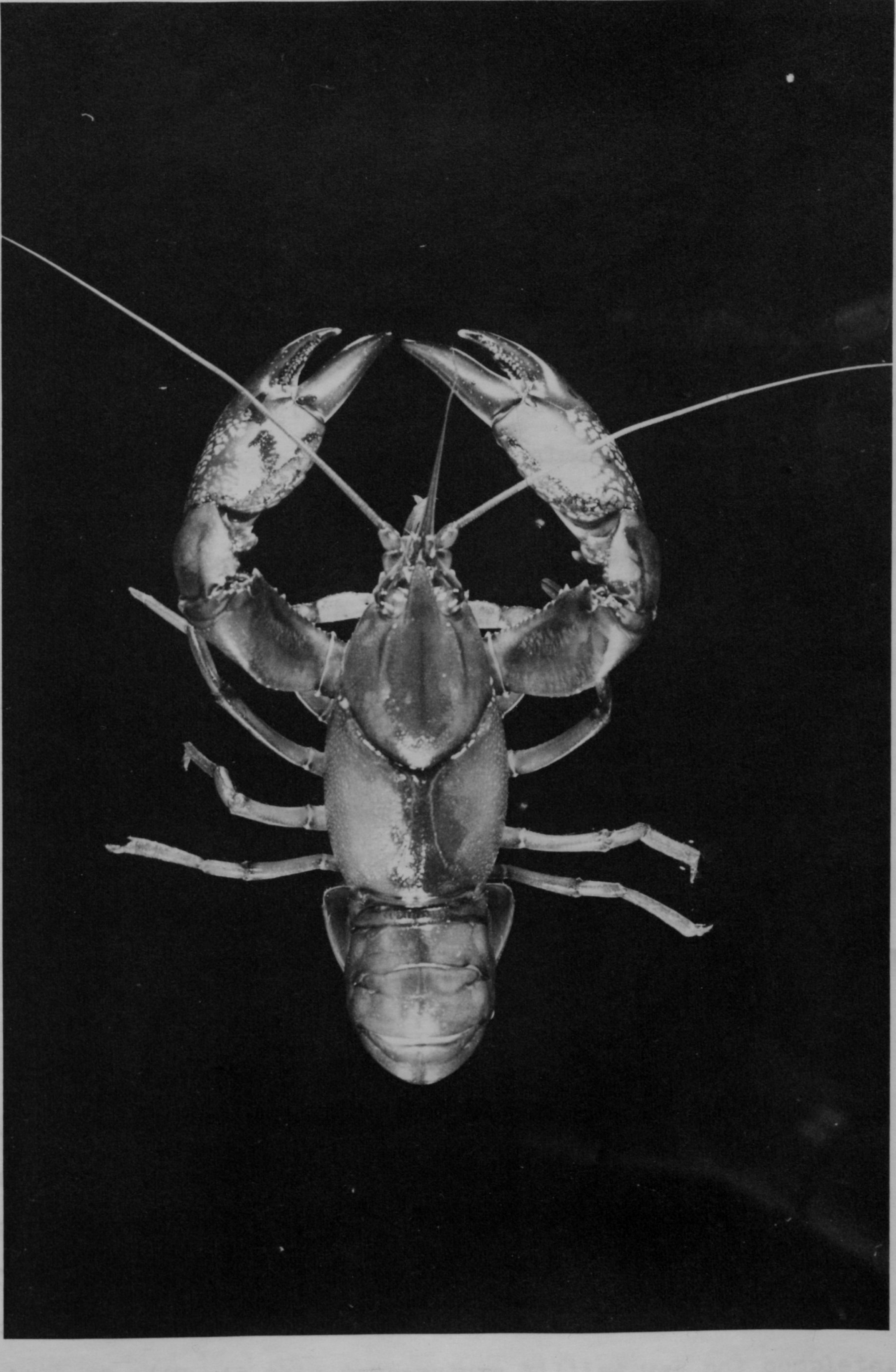
Our knowledge of the occurrence and nature of bactericidal factors in invertebrate sera is very scanty, but where killing has been observed it does not appear to have been mediated by antibody and complement. Haemolytic complement activity has not been demonstrated in the serum of any invertebrate.

To further our knowledge of the phylogenetic development of the antibody-complement bactericidal system, the range of animals under investigation was extended to include an invertebrate, the yabbie, Parachaeraps bicarinatus (Gray), (Figure 7).

The yabbie is a crustacean found in fresh water streams and isolated ponds all over Australia. This animal is very similar to the European fresh water cray-fish. The animals used in this investigation were netted mainly in the shallow lakes at the mouth of the River Murray. A small number of animals were obtained from isolated ponds in the Mount Lofty Ranges. The animals were transported to the laboratory packed in moist weeds gathered from the shores of the lake. Transported in this way, mortality was low, even if the animals remained in the containers overnight. On

Figure 7.

PARACHAERAPS BICARINATUS (Gray) - THE YABBIE.



arrival at the laboratory the yabbies were transferred to plastic baths containing a shallow layer of water, constantly aerated from a compressed air supply. Provided they were not overcrowded, the animals lived under these conditions for many months. The colour of the animals varied from light green to dark blue and black. The larger ones were up to 25 cm. in length and weighed up to 150 gm. The chelipeds of these larger specimens were almost as long as the rest of the body with the protopodite measuring up to five by three cm. The animals were bled as already described by cutting off the tip of a large cheliped; it was from the animals with the largest chelipeds that the most blood was obtained. The animals which were bled weighed from 20 to 150 gm. and gave from 0.5 to 10 ml. of blood. The serum obtained by the technique described approximated to half the total volume of the whole blood. Serum kept at -20° showed no appreciable decrease in bactericidal titre after six months storage.

Bactericidal Spectrum.

The yabbie serum was assayed for bactericidal activity against the seven strains of gram negative bacteria used in the earlier part of this investigation. The first tests were made at 25°, since it was assumed from work with fish sera that this would be the optimum activity temperature. The only strain killed was *S. typhimurium* M206 with a titre of 1/40. At this temperature killing was not complete, even in a 1/2 dilution of the serum; approximately 10% of the inoculum always remained viable.

When the bactericidal activity of the serum for S. typhimurium M206 was assayed at different temperatures, maximum activity occurred at 35°; 25° being nearer the lower end of the activity range(Figure 8). When tested at 35° the titre of the serum was 1/320 and killing was complete in the lower serum dilutions. When strains previously tested at 25° were re-tested at the higher temperature, there was no alteration in their serum susceptibility.

The finding that yabbie serum was bactericidal for one gram negative bacterial strain posed several questions. Was the activity specific? Was it effective against other gram negative bacteria? What serum components mediated in this killing?

To provide an answer to the first two questions, a number of organisms were screened for killing by a 1/10 dilution of the serum. The strains tested and the resultant killing is listed in Table 16. The first strains tested were from our stock collection of micro-organisms and from the Salmonella Reference Laboratory. No killing was observed even though rough strains were included which previously had been found to be sensitive to all vertebrate sera. The three gram positive bacteria - Staph. albus, Staph. aureus and B. cereus, were also resistant to killing by this serum. The bactericidal activity of yabbie serum for various

Figure 8.

TEMPERATURE-BACTERICIDAL ACTIVITY CURVE OF YABBIE SERUM.

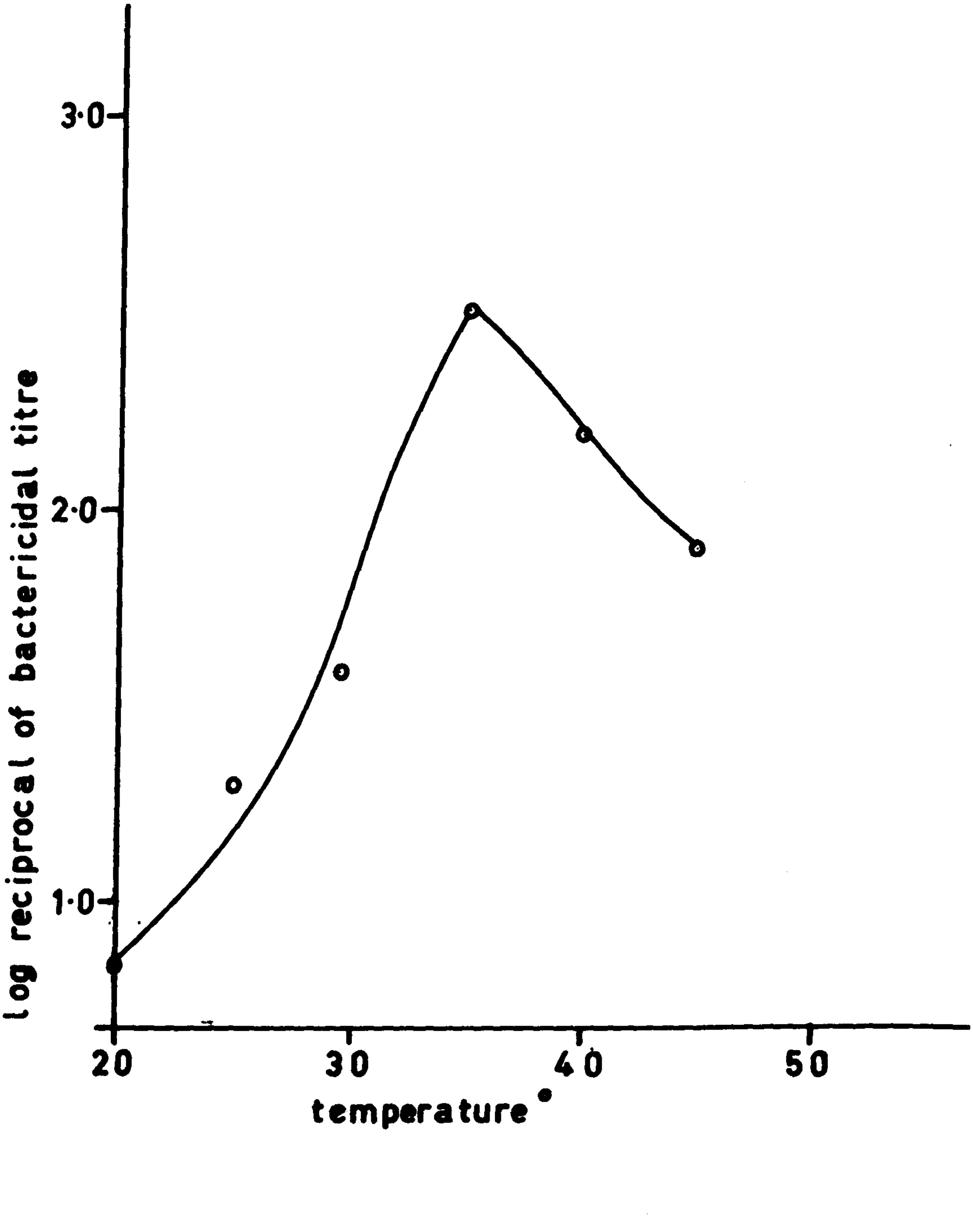

3·0
2·0
1·0
log reciprocal of bactericidal titre
20
30
40
50
temperature°

Table 16.

BACTERICIDAL SPECTRUM OF YABBIE SERUM.

Bacterial species.	No. tested.	Reciprocal of bactericidal titre.
Staph. albus.	1	-
Staph. aureus.	1	-
B. cereus.	1	-
Other gram positive bacilli.	5	-
Bacillus sp. No. 2.	1	80
S. typhimurium M206.	1	160
Other *S. typhimurium.*	7	-
S. saint paul.	2	-
Other *Salmonella* species.	14	-
Sh. sonnei.	1	-
E. coli.	4	-
Ps. aeruginosa.	1	-
Providence sp.	1	-
Citrobacter sp.	1	-
Arizona sp.	2	-
Hafnia sp.	1	-
Achromobacterium sp. No. 12.	1	40
Achromobacterium sp. No. 13.	1	160
Other gram negative bacilli.	7	-

- = no killing by a 1/10 dilution of serum.

strains of bacteria, both gram positive and negative, which had been isolated from water was then investigated. One of six gram positive strains was killed to a serum dilution of 1/80, and two of nine gram negative to a serum dilution of 1/40 and 1/160 respectively. (Table 16).

The three water isolates, sensitive to killing by normal yabbie serum, were kindly identified by Mr. J. Harris, Division of Soils, Commonwealth Scientific and Industrial Research Organisation, Urrbrae, South Australia, as a *Bacillus* sp. (No.2), and as *Achromobacterium* sp. (No. 12 and 13).

After several subcultures on laboratory media, the *Bacillus* sp. became more difficult to grow and maintain in culture and at the same time the bactericidal titre fell from 1/80 to 1/20. This change in sensitivity to serum killing apparently reflected an alteration in the surface structure of the cells.

Specificity of the Serum Bactericidal Activity.

While it has now been firmly established that the natural antibodies of vertebrates are specific, little is known of the specificity of the serum killing factors among the invertebrates.

Early in the investigation when *S. typhimurium* M206 was the only strain known to be sensitive to the bactericidal action of the serum, several attempts were made to define the specificity of the killing mechanism. The serum was absorbed with varying quantities of S. typhimurium M206, and of several of the resistant gram negative strains, and the effect on the killing of S. typhimurium M206 by the absorbed sera was determined.

Yabbie serum when absorbed with S. typhimurium M206 at a dose level of 1 mg. dry weight of cells/ml. of serum, completely lost its bactericidal activity for the absorbing strain. There was a similar loss of bactericidal activity for S. typhimurium M206 when the serum was absorbed with E. coli BV or Ps. aeruginosa. Absorption with a coagulase positive Staph. aureus or with B. cereus at 4 mg. dry weight of cells/ml. of serum did not affect the bactericidal activity for S. typhimurium M206. Absorption with 4% (v/v) sheep red blood cells also had no effect on the bactericidal activity.

The lipopolysaccharides of S. typhimurium M206 and S. paratyphi BIR when added to the serum at 10 µg/ml. of serum completely inhibited bactericidal activity against S. typhimurium M206. One µg. of lipopolysaccharide/ml. of serum had no effect.

Although these absorptions could give no absolute information on the specificity of the bactericidin they did, however, provide two interesting facts. Firstly, the bactericidin adsorbed to gram negative bacteria which it was incapable of killing, and secondly that no adsorption occurred with gram positive bacteria. In addition, the inhibition of killing with lipopolysaccharide suggested that the specificity of the bactericidal substance was directed against the lipopolysaccharide of the bacterial cell.

The isolation of further serum sensitive strains made possible a direct comparison of the effect absorption with one serum sensitive strain had on the bactericidal titre towards itself and other sensitive strains.

The serum was absorbed with varying quantities of an alcohol-killed S. typhimurium M206 suspension, and the titres of the absorbed sera for both S. typhimurium M206 and Achromobacterium sp. No. 13 determined. The experiment was then repeated with Achromobacterium sp. No. 13 as the absorbing strain. The effect of these absorptions on the bactericidal titres for the two strains are shown graphically in Figures 9 and 10. The absorbing strain removed the bactericidal activity towards itself and the heterologous strain. There was, however, a quantitative difference in the amount of absorbing suspension required to give an equivalent depletion of the bactericidal activity against the two strains. A greater dose of both absorbing strains was required to deplete the bactericidal activity against S. typhimurium M206, than against Achromobacterium sp. No. 13. When similar experiments were done comparing the effect of absorbing the serum with S. typhimurium M206

Figure 9.

YABBIE SERUM ABSORBED WITH VARYING QUANTITIES OF AN ALCOHOL KILLED SUSPENSION OF S. TYPHIMURIUM M206.

Residual bactericidal activity assayed for *S. typhimurium M206* and for *Achromobacterium* sp. No. 13.

1. *S. typhimurium* M206.
2. *Achromobacterium* sp. No. 13.

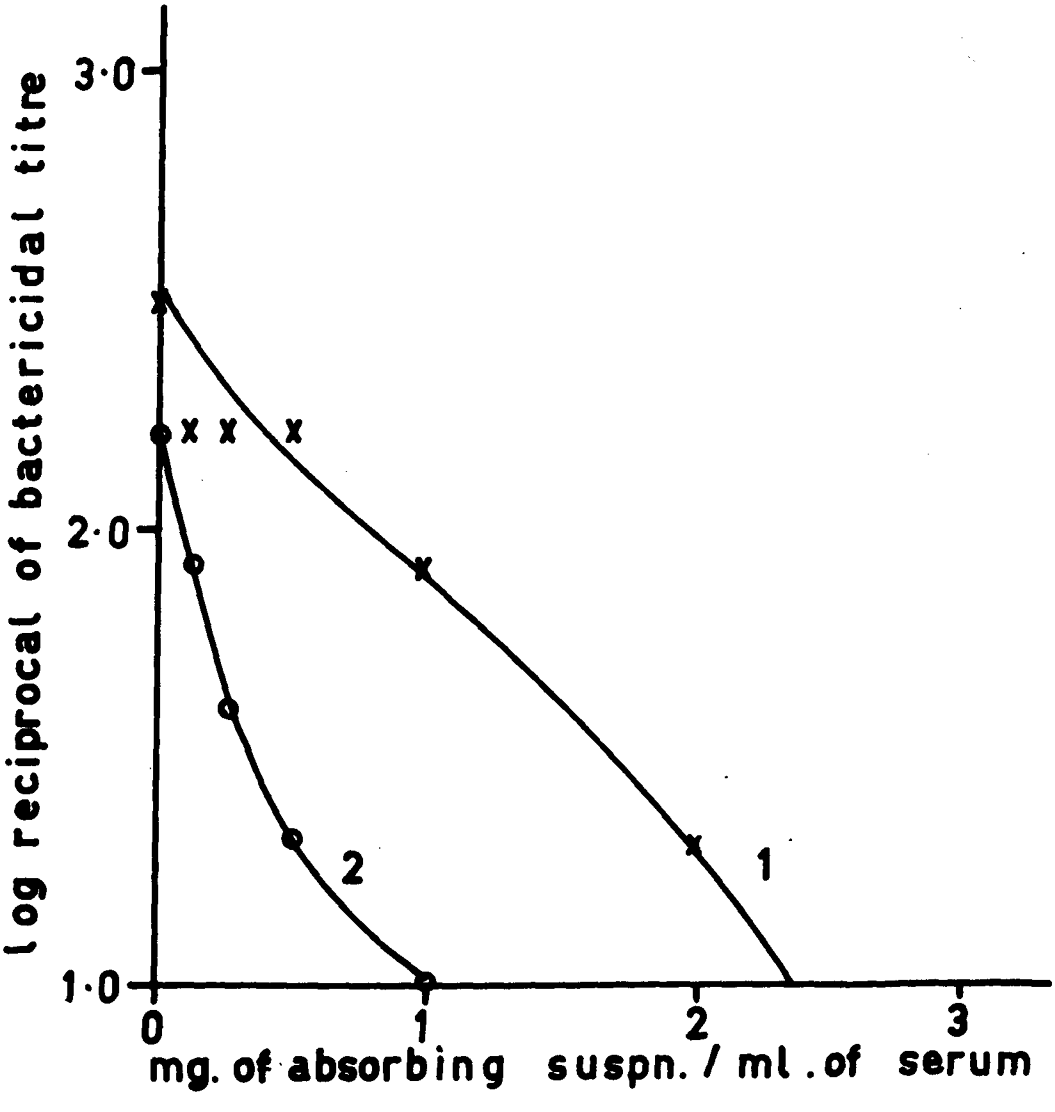
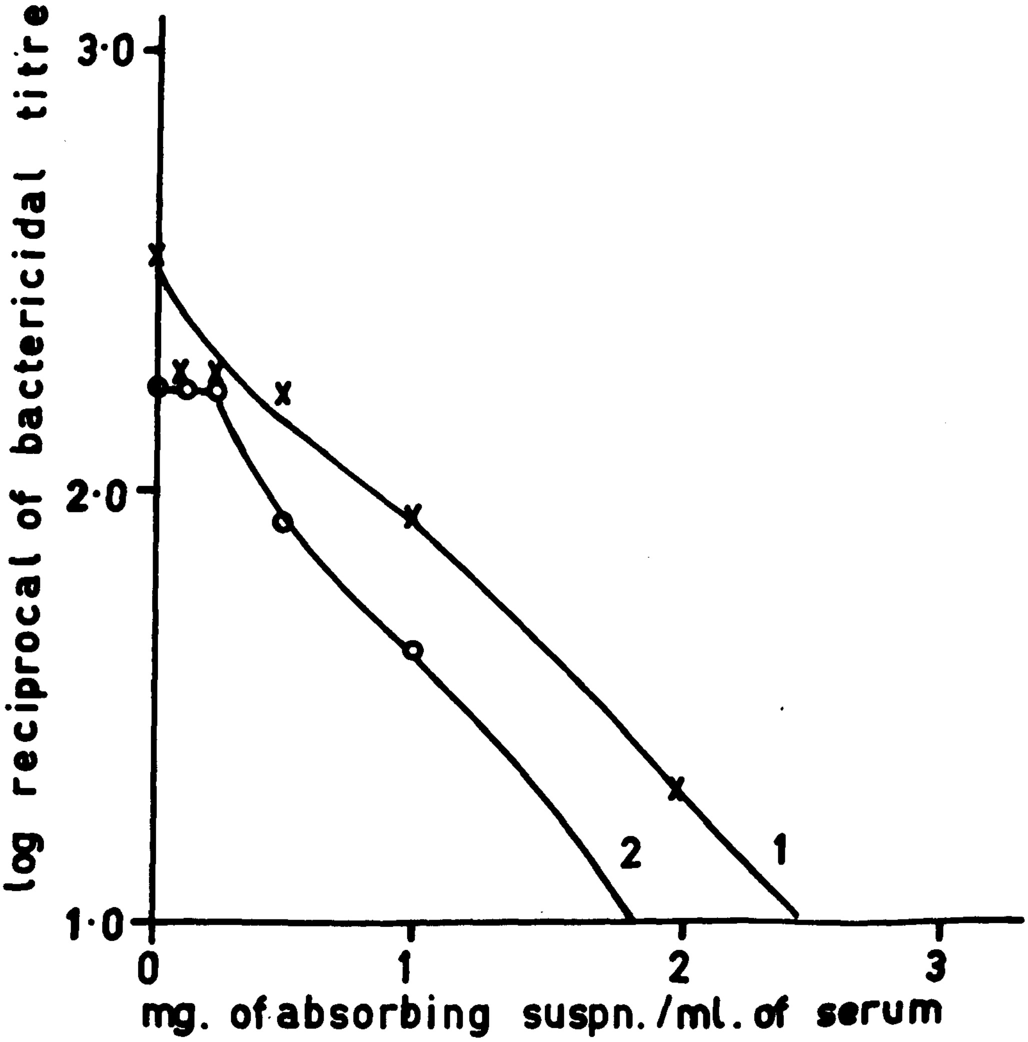

log reciprocal of bactericidal titre
3·0
2·0
1·0
0
1
2
3
mg. of absorbing suspn. / ml. of serum
1
2

Figure 10.

YABBIE SERUM ABSORBED WITH VARYING QUANTITIES OF AN ALCOHOL KILLED SUSPENSION OF *ACHROMOBACTERIUM* sp. No. 13.

Residual bactericidal activity assayed for *S. typhimurium* M206 and for *Achromobacterium* sp. No. 13.

1. *S. typhimurium* M206
2. *Achromobacterium* sp. No. 13.


log reciprocal of bactericidal titre
3·0
2·0
1·0
0
1
2
3
mg. of absorbing suspn./ml. of serum
1
2

and Bacillus sp. No.2, on the bactericidal activity for the two strains, the bactericidal activity was depleted only for the absorbing strain (Figures 11 and 12). While these results have to be interpreted with some caution due to the limited number of strains which have been investigated, they do, however, suggest that yabbie serum contains two separate bactericidal mechanisms, one effective against gram positive, and the other against gram negative bacteria.

Haemolytic Complement.

No evidence could be found for the presence of haemolytic complement in yabbie serum using the standard haemolytic assay system of sheep red blood cells sensitised with rabbit antibody, or with normal unsensitised sheep cells. There was no lysis of sheep, rat, rabbit, mouse or human red cells in the haemagglutination assays to be discussed in a subsequent chapter. Normal unheated vertebrate serum produced lysis of the red cells in these tests.

Effect of EDTA on the Bactericidal Activity of Yabbie Serum.

Calcium and magnesium ions are necessary for the biological activity of the vertebrate antibody-complement bactericidal system. Chelating these ions with EDTA depressed all bactericidal activity.(Chapter 5).

The effect of EDTA on the bactericidal activity of yabbie serum for S. typhimurium M206 was determined.

Figure 11.

YABBIE SERUM ABSORBED WITH VARYING QUANTITIES OF AN ALCOHOL KILLED SUSPENSION OF S. TYPHIMURIUM M206.

Residual bactericidal activity assayed for *S. typhimurium* M206 and for *Bacillus* sp. No. 2.

1. *S. typhimurium* M206.
2. *Bacillus* sp. No. 2.

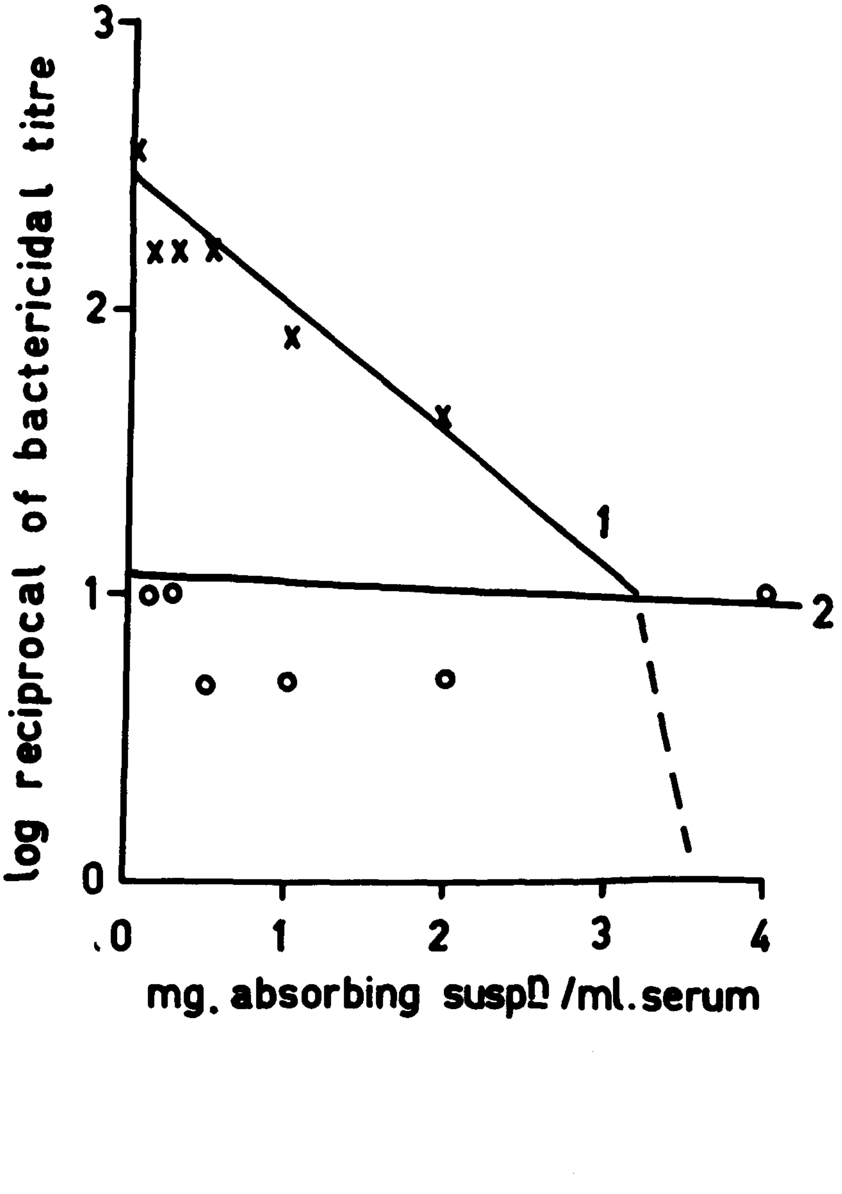
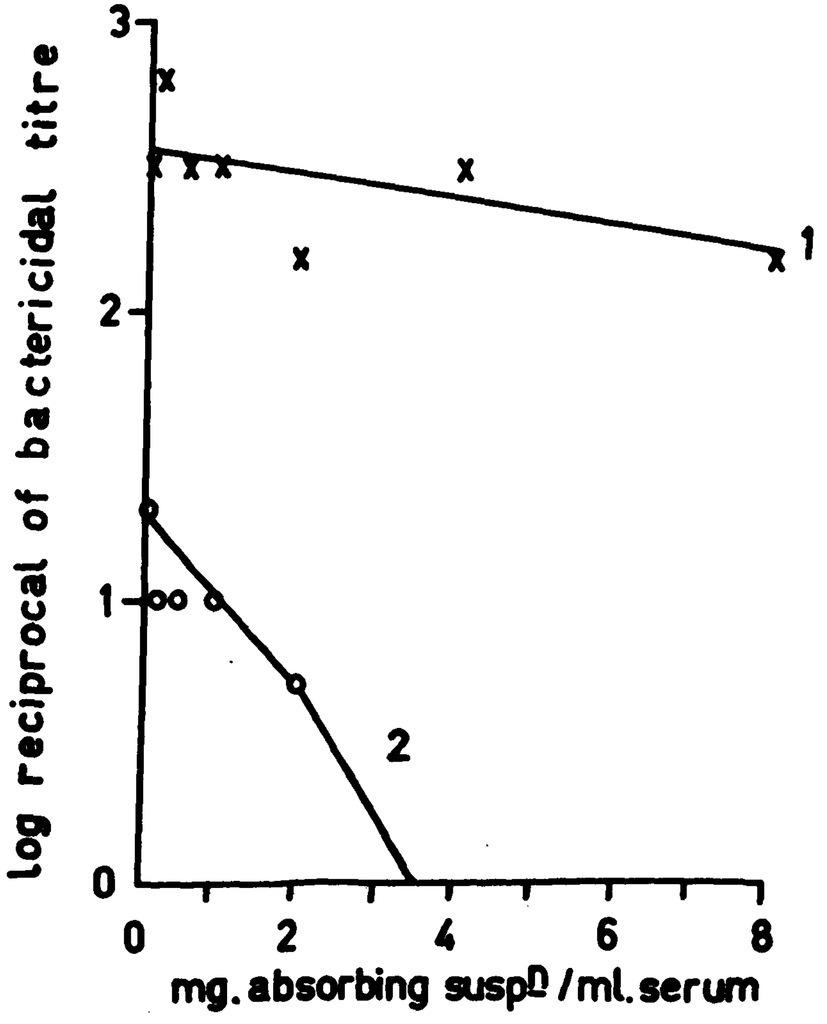

log reciprocal of bactericidal titre
3
2
1
0
.0
1
2
3
4
mg. absorbing suspⁿ/ml.serum
1
2

Figure 12.

YABBIE SERUM ABSORBED WITH VARYING QUANTITIES OF AN ALCOHOL KILLED SUSPENSION OF BACILLUS sp. No. 2.

Residual bactericidal activity assayed for *S. typhimurium* M206 and for *Bacillus* sp. No. 2.

1. *S. typhimurium* M206.
2. *Bacillus* sp. No. 2.


Log reciprocal of bactericidal titre
3
2
1
0
1
2
0
2
4
6
8
mg. absorbing suspⁿ /ml. serum

EDTA was added to the serum to give a final concentration of 0.5 M, which was in excess of that necessary to chelate all the calcium and magnesium ions present. In these tests the minimal medium described in Chapter 2 was used but no magnesium sulphate was included, and in addition EDTA was added to a final concentration of 0.1 M.

The bactericidal activity of the serum was not affected by the addition of EDTA.

Individual Variation in Bactericidal Titre.

All experiments (unless otherwise stated) were done using pooled serum collected from twenty to one hundred individuals. The day to day variation in titre of any one serum pool, or between different pools, did not exceed one two-fold dilution from a mean titre of 1/320. These titres were independent of the locality from which the animals were obtained, and the time of the year when they were captured and bled.

Similar bactericidal titres were obtained for the serum of six animals tested individually.

Plate Assay of Bactericidal Activity.

Experiments were made to determine if the bactericidin was active in a plate assay system. There was no inhibition of growth, when large loopsful of yabbie serum dilutions from neat to 1/1280 were spotted onto the surface of nutrient agar plates seeded with S. typhimurium M206, or when lawn plates were used. Growth was not inhibited if the serum dilutions were allowed to diffuse for one to twenty four hours prior to incubation. The incorporation of 10% yabbie serum in the agar did not inhibit the growth of S. typhimurium M206 spread, or streaked, over the surface of the plate.

Effect of Heat on the Bactericidal Activity of Yabbie Serum.

Yabbie serum heated at 56° for half an hour was not bactericidal when tested at 25°, and the titre was only 25% of normal when tested at 37°. In addition, killing was not complete in any dilution at the higher temperature. The active fraction was more stable to heat under acid conditions than in normal serum. The supernatant, (obtained after precipitation of the serum proteins at pH 3.5) when heated to 80° for thirty minutes still retained 50% of its bactericidal activity for S. typhimurium M206.

Dose Response Curve.

The effect of variations in the size of the bacterial inoculum on the bactericidal titre of yabbie serum for S. typhimurium M206 was determined in order to ascertain if increasing the number of bacteria would bring about a corresponding reduction in the bactericidal titre. The number of bacteria inoculated into each series of tubes varied by ten-fold steps from 6 x 10^3 to 6 x 10^7. The bactericidal titres for each dose level of bacteria are given in Table 17. The fall in bactericidal titre is as one would expect if the bactericidal factor was enzymic in nature.

Discussion.

Yabbie serum was found to be bactericidal for some strains of gram positive and negative bacteria. While only tentative conclusions can be drawn from the absorption tests due to the small number of strains studied, it appears that different factors are involved in the killing of gram positive and negative bacteria. Whether this conclusion is valid or not can only be ascertained by investigating the serum killing of a greater number of strains. The degree of specificity exhibited by the factor responsible for killing the gram positive strain was not ascertained, but from the cross absorption tests the same factor appeared to mediate in the killing of all sensitive gram negative strains. The bactericidin was also removed from the serum by gram negative bacteria for which the yabbie serum had no bactericidal activity. This interesting finding indicated that the killing of gram negative bacteria by yabbie serum was probably a two stage reaction. In the first stage the bactericidin adsorbed to presumably the lipopolysaccharide of the bacterial

TABLE 17.

THE EFFECT OF VARYING THE NUMBER OF BACTERIA IN THE INOCULUM ON THE BACTERICIDAL TITRE OF YABBIE SERUM FOR S. TYPHIMURIUM M206.

Number of bacteria inoculated/tube.	Reciprocal of bactericidal titre.
6×10^6	40
6×10^5	80
6×10^4	160
6×10^3	160

cell wall and in the second stage the bactericidin exerted its lethal action on the cell. The second stage presumably only occurred if the surface structure of the bacterium was in some as yet unknown way amenable to the lethal action of the bactericidin. This situation is similar to that recorded in Chapter 3, where it was shown that pig serum possessed no bactericidal activity for S. typhimurium C5, despite the fact that these cells fixed complement from pig serum.

The yabbie serum bactericidal system responsible for the killing of gram negative bacteria differed from the vertebrate antibody-complement bactericidal system in several major respects. In addition to its non-specificity of action (which has already been discussed) there was mo divalent cation requirement, it was more stable to heat and had a higher optimum activity temperature than would be expected for an antibody-complement system derived from a vertebrate in a similar habitat. The constant occurrence, and level, of the bactericidal factor(s) among the yabbies tested suggested that the factor(s) was constitutional rather than induced. Attempts were therefore made to determine if the level of the bactericidin for S. typhimurium M206 could be increased by injecting the homologous strain into the yabbie, and whether the injection into the yabbie of a strain resistant to the lethal action of its serum would lead to the production of a bactericidin.

The results of these investigations are recorded in the next chapter, together with those of an investigation into the ability of these animals to respond, in an immunologically demonstrable manner to the injection of several other antigens.

Summary.

The ability of yabbie serum to kill fifty-three bacterial strains, both gram positive and negative, was investigated. One of nine gram positive, and three of forty-four gram negative strains were killed by the serum.

Absorption tests indicated that a different serum factor mediated in the killing of gram positive and negative bacteria, and further, that the same factor mediated in the killing of all the susceptible gram negative bacteria. These conclusions must be treated with some reserve, however, due to the small number of strains which were involved.

The yabbie bactericidal system for gram negative bacteria was shown to differ in several ways from the classical vertebrate antibody-complement system.

CHAPTER 7.

THE IMMUNOLOGICAL RESPONSE OF YABBIES TO THE INJECTION OF FOREIGN ANTIGENIC MATERIAL.

Introduction.

Experiments were discussed in the previous chapter which led to the demonstration of a bactericidal mechanism for some gram negative bacteria in yabbie serum. As only a limited number of bacterial strains were killed by the serum, the question was asked, would a serum bactericidal mechanism be induced for these resistant strains of bacteria by their injection into the animal?

If the development of a bactericidal mechanism was subject to antigenic stimulation, then the manner by which the yabbie reacted to any antigenic stimulus might give some indication of the nature of the killing mechanism.

This chapter is concerned with various attempts to elicit a demonstrable immune response in yabbies.

Titration of Yabbie Serum for Natural Haemagglutinins to Several Species of Vertebrate Red Blood Cells.

Because of an interest in the possible immunological origin of the yabbie serum bactericidin, efforts were made to determine if the serum possessed any substances with antibody-like specificity. As the haemagglutination assay system is one of the most sensitive techniques for the detection of antibody, yabbie serum was titrated for haemagglutinins for human, sheep, rat, rabbit and mouse red blood cells. The serum haemagglutinated the red blood cells from all the animal species to a low titre (Table 18). This result clearly indicated that yabbie serum possessed substances with antibody-like reactivity. It was therefore of interest to determine if the level of these factors could be increased by injection of red cell stroma.

Immunological Response of the Yabbie to the Injection of Sheep Red Cell Stroma.

The demonstrable antibody response in poikliothermic vertebrates has been shown to be temperature dependent (Evans, 1963). In these studies with the yabbie it was therefore considered desirable to immunise animals maintained at different temperatures. Five animals, maintained at room temperature, were given five injections of 200 µg. of stroma in 0.1 ml. saline, at five day intervals. All injections were made into the soft tissue of a large cheliped, the needle being inserted at the junction of the dactopodite and protopodite. The animals were bled ten days after the last injection. The haemagglutinating titre of the serum was not raised, nor were haemolysins produced.

Table 18.

LEVEL OF NATURAL HAEMAGGLUTININS IN YABBIE SERUM FOR SEVERAL SPECIES OF VERTEBRATE RED BLOOD CELLS.

Red blood cells.	Haemagglutination units/ml. of serum.
Human.	32
Sheep.	16
Rabbit.	8
Mouse.	32
Rat.	16

A rise in the serum haemagglutinating titre for sheep red blood cells did not occur when inoculated yabbies were maintained at 37°.

The possibility had to be considered, that the lack of an immunological response was due to the antigen being trapped at the site of injection, and never reaching the tissues where antibody might be produced. The efficiency of the injection technique was determined by injecting methylene blue into a cheliped and visually following its distribution through the animal. Almost immediately after injection the gills and tail of the animal took on a bluish tinge, which indicated that the injected material was in fact going into the circulation.

Using the large cheliped as the route of inoculation had the disadvantage that periodically these limbs were shed at the time of, or shortly after the injection had been made. The site of injection was consequently changed and the injections were given into the abdomen at the junction of the first and second segments from the cephalothorax. The suitability of the tail as an injection site was studied using carbon in 1% gelatin. The carbon suspension (0.1 ml.) was injected into the tail and thirty minutes and forty eight hours later animals were sacrificed and the tissues examined macroscopically for traces of carbon. Thirty minutes after injection some carbon remained localised at the site of injection while the gills were considerably darker than normal. Some carbon was also present in the digestive gland. After forty eight hours most of the carbon was in the digestive gland, little remaining in the tail or gills.

Since the injection technique gave a rapid dispersal of the injected material, it would be expected that any particles introduced by this route would reach sites where antibody production might occur.

Immunological Response of the Yabbie to the Injection of S. typhimurium M206.

Normal yabbie serum contained no agglutinins at a 1/4 dilution to S. typhimurium M206 (the tests being incubated at 56° for 18 hours). Five yabbies given five injections, at five day intervals, of 20 µg. of an alcohol-killed suspension of S. typhimurium M206, and bled ten days after the last injection, did not produce any demonstrable agglutinins, or show a rise in bactericidal titre. When the injected dose was increased to 200 µg. all animals died overnight.

Injection of Living Bacteria into Yabbies.

It is well known that with many bacterial diseases of vertebrates a primary infection often leads to heightened resistance to a subsequent challenge by the same organism. As no bacterial pathogens were known for the yabbie several species of living bacteria were injected in an effort to find a pathogenic strain. Yabbies were injected with 10^7 viable E. coli BV and Lilly, S. typhimurium C5 and M206, S. gallinarum 9240, S. paratyphi BIS and BIR, Ps. aeruginosa, Staph. albus and B. cereus, with no apparent injurious effects. Fourteen days after the initial injection the serum was not bactericidal for the injected strain.

Rate of Removal of S. typhimurium C5 from the Circulation of the Yabbie.

Since the animals were resistant to infection by this bacterium, either the bacteria remained in the circulation indefinitely with no apparent ill effect to the animal, or they were removed from the blood to some organ for subsequent disposal.

The time required for the removal of S. typhimurium C5 from the circulation was determined. An overnight broth culture was washed and resuspended in saline so that 0.1 ml. contained approximately 10^7 bacteria. This suspension was injected into the tail and the subsequent rate of removal of the bacteria from the circulation assayed by estimating the number of bacteria in samples of blood obtained by cutting off a leg. A separate leg being used for each count. Two or three drops of blood were taken directly into 1 ml. of broth containing 1 mg. of trypsin. The mixture was incubated for half an hour at 37° after which the number of viable bacteria were estimated by a plate count. The tryptic digestion was necessary to solubilise the small gelatinous clot which formed as the blood was taken. Control tests showed that this concentration of trypsin had no effect on the viability of the bacteria. The weight of the blood was obtained by subtracting the weight of the bottle plus trypsin, from that of the bottle plus blood and trypsin. The density of the blood was assumed to be one. In order to provide a basis for comparison, all counts were adjusted to the number of bacteria which would be expected to be recovered from one ml. of blood.

The curves for the rate of removal of the injected bacteria with time are plotted in Figure 13. Six yabbies were used. The curves drawn represent the average rate of removal, together with those of the yabbies showing the minimum and maximum rates. Less than one % of the injected bacteria were recovered after the first hour, and the numbers which could be recovered thereafter showed a steady decline. Bacteria could not be recovered from a series of twelve animals bled twenty four hours after injection.

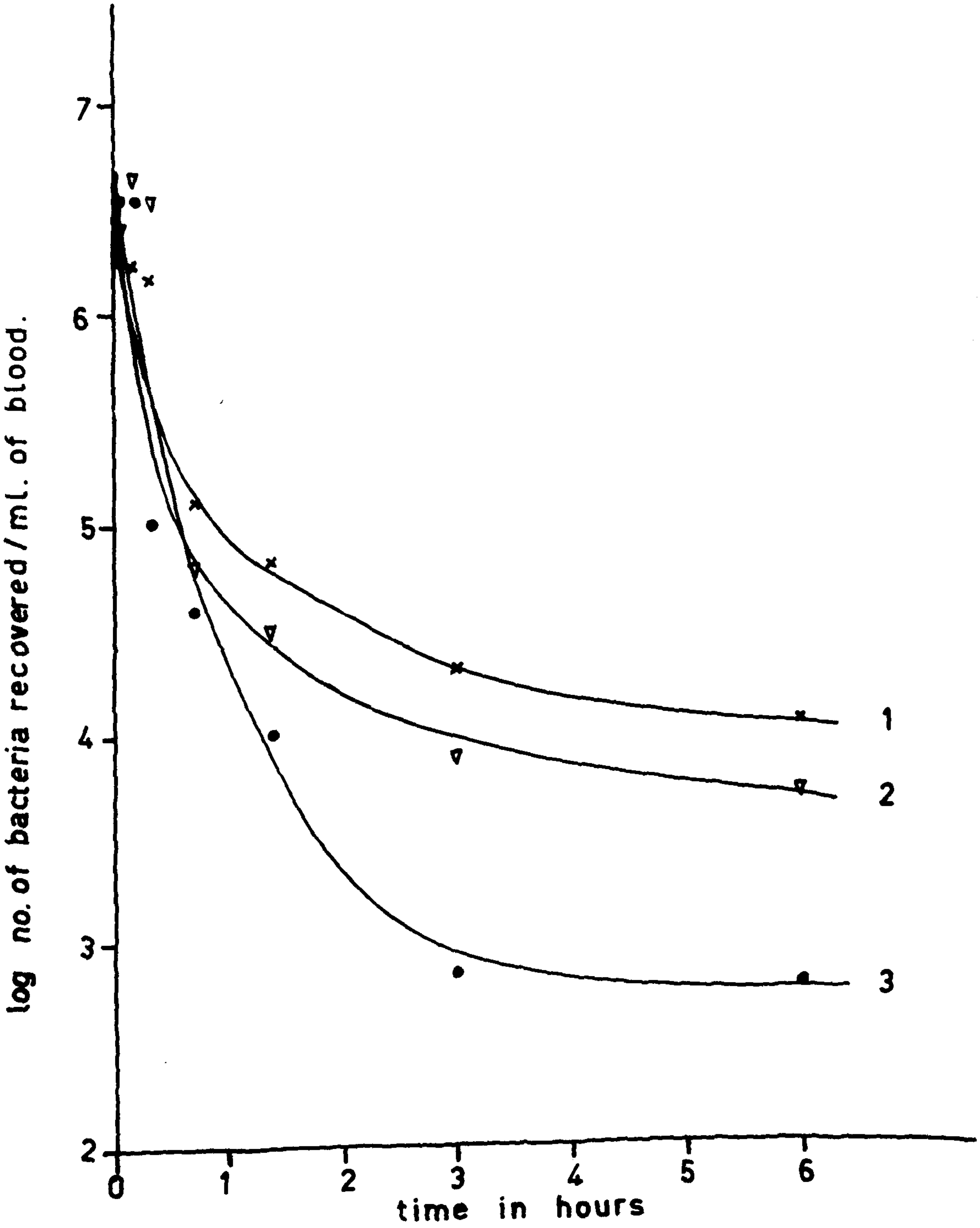
Rate of Removal of Bacteriophage ØX174 from the Circulation of the Yabbie.

The fate of injected bacteriophage was also studied. These particles have been used very successfully in vertebrates to detect antibodies during the early stages of antibody production. The highly sensitive technique of

Figure 13.

RECOVERY WITH TIME OF S. TYPHIMURIUM C5 FROM THE BLOOD OF YABBIES.

Curve 1.	Numbers recovered from the animal showing the minimum rate of clearance.
Curve 2.	Average number recovered from all animals.
Curve 3.	Numbers recovered from the animal showing the maximum rate of clearance.


log no. of bacteria recovered / ml. of blood.
7
6
5
4
3
2
0
1
2
3
4
5
6
time in hours
1
2
3

phage inactivation permitting the demonstration of far fewer antibody molecules than is possible by most other techniques.

The yabbies were injected with approximately 10^7 phage øX174 and bled by the technique previously described for recovering bacteria. The number of phage particles present were estimated by the agar layer technique of Adams (1959) using E. coli C as the indicator strain.

Since there was no significant decline in the number of phage particles which could be recovered from the blood of the yabbies during the first twenty four hours post injection, the fate of phage in the circulation was then followed over an extended period of time.

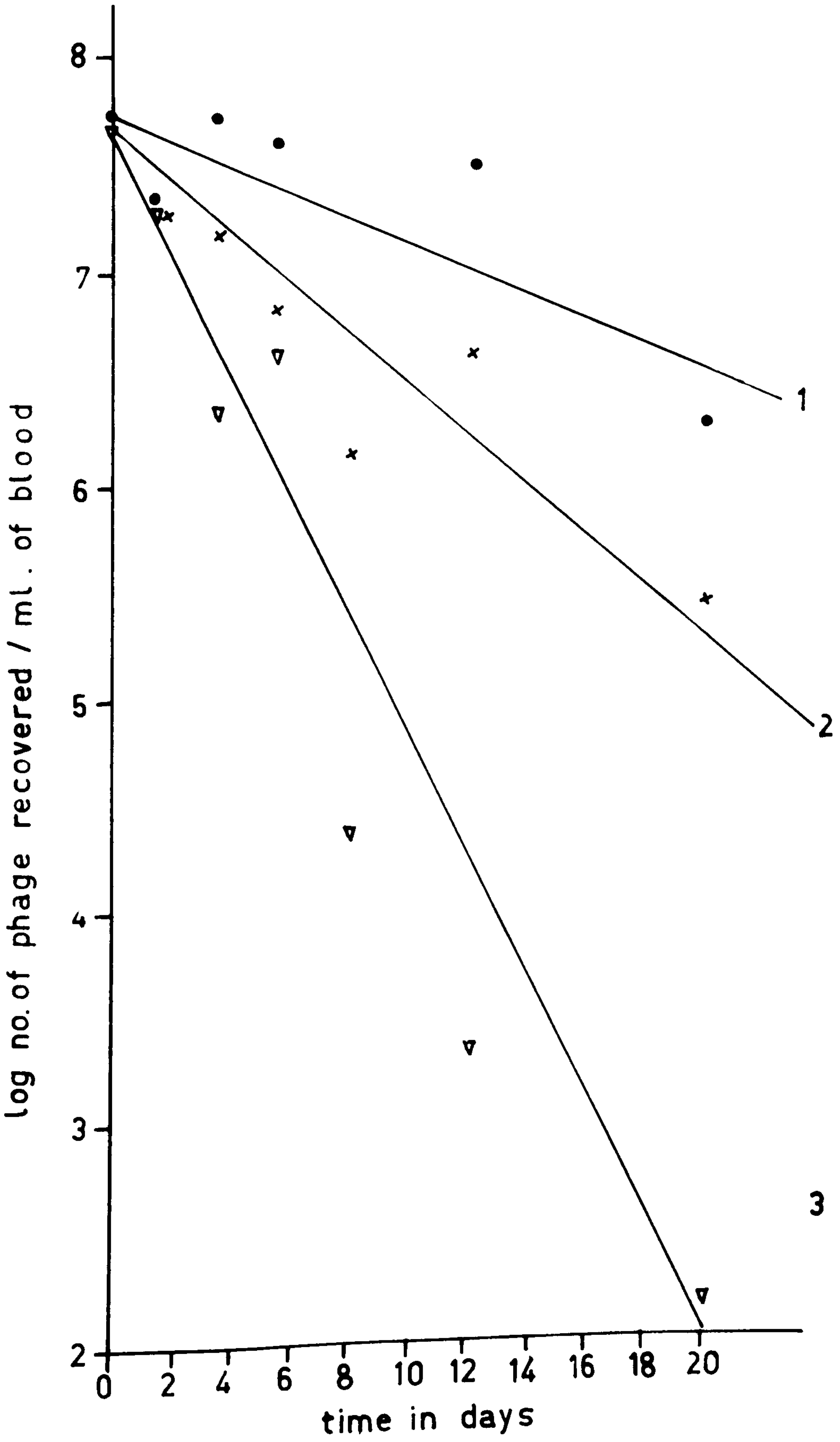
The recovery of phage with time is shown in Figure 14. The average clearance values, as well as the fastest and slowest values, are plotted. Of the twelve animals injected, five survived at the end of three weeks, and phage could still be isolated from their blood. Throughout this period there was a considerable difference between the number of phage particles which were recovered from the animals showing the most rapid, and the slowest rate of removal.

If the primary injection of phage particles into the circulation led to the development of an immune mechanism for their removal, then phage injected subsequently would be expected to be cleared more rapidly. Twelve yabbies were

Figure 14.

RECOVERY WITH TIME OF PHAGE øX174 FROM THE BLOOD OF YABBIES.

Curve 1.	Numbers recovered from the animal showing the minimum rate of clearance.
Curve 2.	Average number recovered from all animals.
Curve 3.	Numbers recovered from the animal showing the maximum rate of clearance.


log no. of phage recovered / ml. of blood
8
7
6
5
4
3
2
0
2
4
6
8
10
12
14
16
18
20
time in days
1
2
3

injected with phage as previously described, and the numbers recoverable from the blood determined several times during the ensuing three weeks; the remaining eight animals were then given a second injection of phage. The animals did not clear these phage particles from their circulation at a significantly greater rate than those from the primary injection (Figure 15). Phage could still be recovered in low numbers, from two of the three remaining animals seventy five days after the primary injection.

There was no indication from these results that the animals developed an immune mechanism for the disposal of these particles. It was not possible to determine if the decline in the number of phage particles recovered was the result of a slow clearance by the animal, or to a gradual fall in the number of circulating phage particles due to death of the phage, independent of the yabbies defence mechanisms.

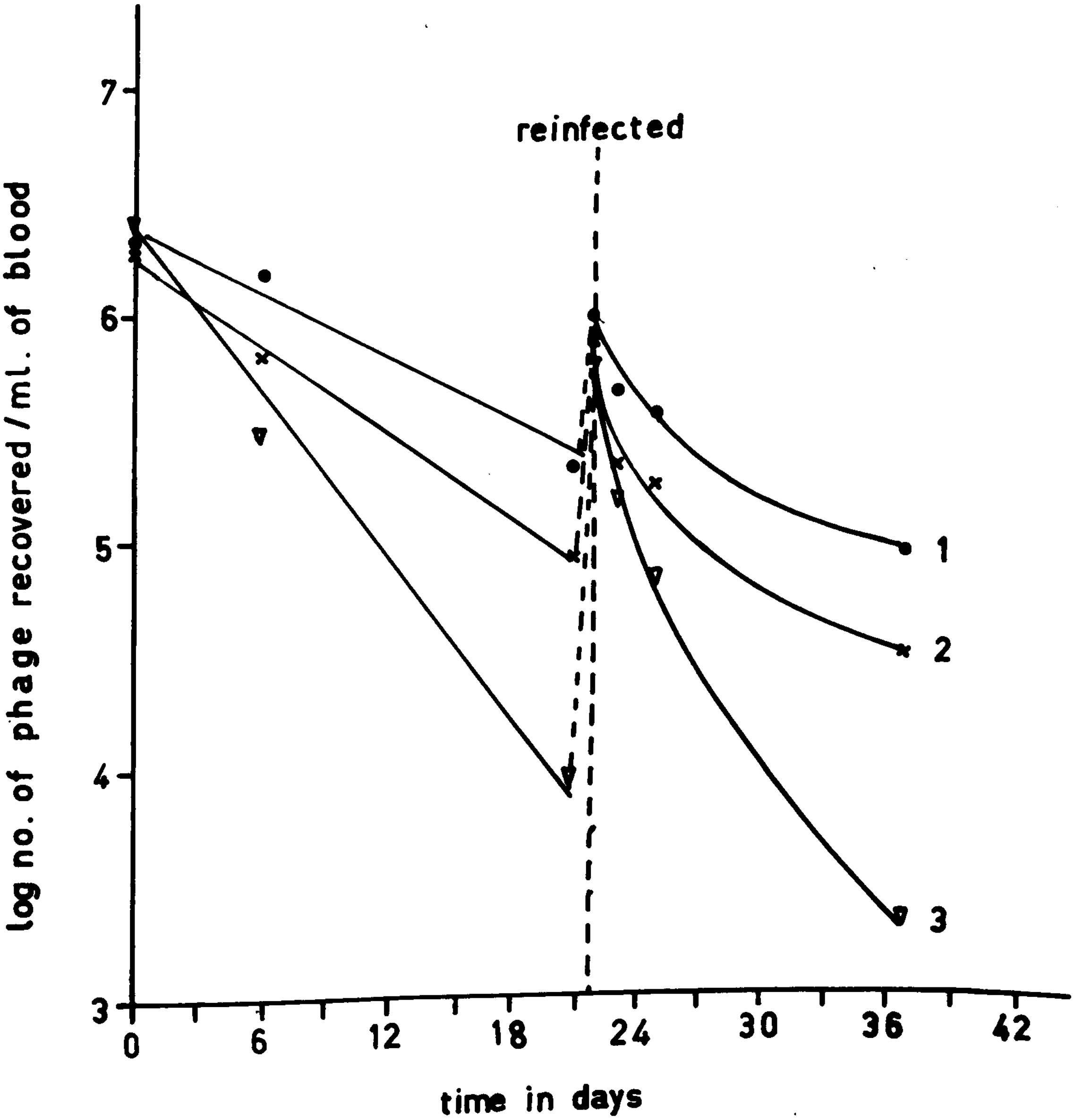
Discussion.

The observations recorded in this chapter show that the yabbie has an efficient mechanism for the clearing of bacteria from its circulation and presumably for their inactivation, since none of the strains of bacteria injected produced any obvious signs of infection. The inability of these bacteria to produce a demonstrable disease would appear to be a reflection of the choice of organisms, since

Figure 15.

RECOVERY WITH TIME OF PHAGE øX174 FROM THE BLOOD OF YABBIES FOLLOWING A PRIMARY AND SECONDARY INJECTION OF øX174.

Curve 1.	Numbers recovered from the animal showing the minimum rate of clearance.
Curve 2.	Average number recovered from all animals.
Curve 3.	Numbers recovered from the animal showing the maximum rate of clearance.


reinfected
log no. of phage recovered / ml. of blood
7
6
5
4
3
0
6
12
18
24
30
36
42
time in days
1
2
3

it is inconceivable from our knowledge of the distribution of bacterial disease among living organisms, that the yabbie should be completely refractory to bacterial infection.

Although all attempts to produce a demonstrable immune response in yabbies were unsuccessful, the experiments with bacteriophage showed that the yabbie has some type of recognition mechanism, since it was able to clear bacteria rapidly from its circulation, but not bacteriophage. No information was obtained on the possible nature of this mechanism.

Another instance has been recorded where an animal was unable to rapidly remove bacteriophage from its circulation. Papermaster, Condie and Good (1962), working with the hagfish (one of the most primitive species of the true vertebrates) found that bacteriophage persisted in the circulation for at least fourteen days after injection. In addition to the inability to rapidly clear bacteriophage from their circulation, both the yabbie and the hagfish lacked serum proteins with the electrophoretic mobility of γ-globulin and in neither species could a demonstrable immune response be elicited. It is premature at present to speculate on any possible interrelationship of these diverse observations.

Summary.

An immune response could not be detected in yabbies following the injection of living or dead bacteria, or sheep red cell stroma.

Two strains of E. coli, five Salmonellae, one of Pseudomonad, one Staphylococcus and one Bacillus when injected at a level of 10^7 bacteria did not produce recognisable symptoms of disease.

S. typhimurium C5 could not be detected in the circulation twenty four hours after injection.

Phage ØX174 was still present in the blood three weeks after a primary injection. A second injection of phage was not cleared any more rapidly than the first.

CHAPTER 8.

CHEMICAL PURIFICATION OF THE BACTERICIDIN IN YABBIE SERUM FOR GRAM NEGATIVE BACTERIA.

Introduction.

Results were presented in Chapter 6 which led to the demonstration of a bactericidin in yabbie serum for some strains of gram negative bacteria. This yabbie serum bactericidal system, differed from the classical vertebrate antibody-complement bactericidal system, by having a greater heat stability, being less specific, and having no requirement for divalent cations. It was therefore considered desirable to gain some insight into the chemical nature of this factor.

This chapter deals with the various purification procedures which were employed.

Total Protein.

The total protein concentration of yabbie serum, estimated by the technique of Folin-Ciocalteu, was 30-35 mg. per ml. using a B.S.A. standard.

Protein Absorption Spectrum of Yabbie Serum.

Before estimating the protein concentration in yabbie serum on the basis of the quantity of ultra violet light absorbed at 280 mμ., a preliminary experiment was done to ascertain that there was a specific peak of light absorption at this wavelength. The quantity of light absorbed by the serum was determined at five mμ. intervals from 230-300 mμ. The absorption spectrum of the supernatant from yabbie serum which had been subjected to acid precipitation and the pH subsequently returned to 7, pig serum and B.S.A. were estimated in parallel over the same range of wavelengths. The optical densities of each protein at the different wavelengths are shown graphically in Figure 16. Maximum light absorption with each protein solution was at 230 mμ. with the characteristic second peak at 280 mu.

Effect of Incubating Yabbie Serum with Trypsin on the Bactericidal Activity Against S. typhimurium M206.

Yabbie serum was incubated with trypsin in order to determine if the bactericidin was susceptible to proteolytic digestion. An equal volume of Difco trypsin 1:250 made up to a concentration of 5 mg./ml. in 0.2 M veronal buffer pH 8.0, was added to yabbie serum. The mixture was incubated at 40^{0} for 30 minutes after which the bactericidal activity of the digested serum was estimated in the usual way.

The treated serum was no longer bactericidal. The bactericidal activity of a control serum, with buffer added but no trypsin, and incubated as above lost none of its bactericidal activity.

This result indicated that the bactericidin

Figure 16.

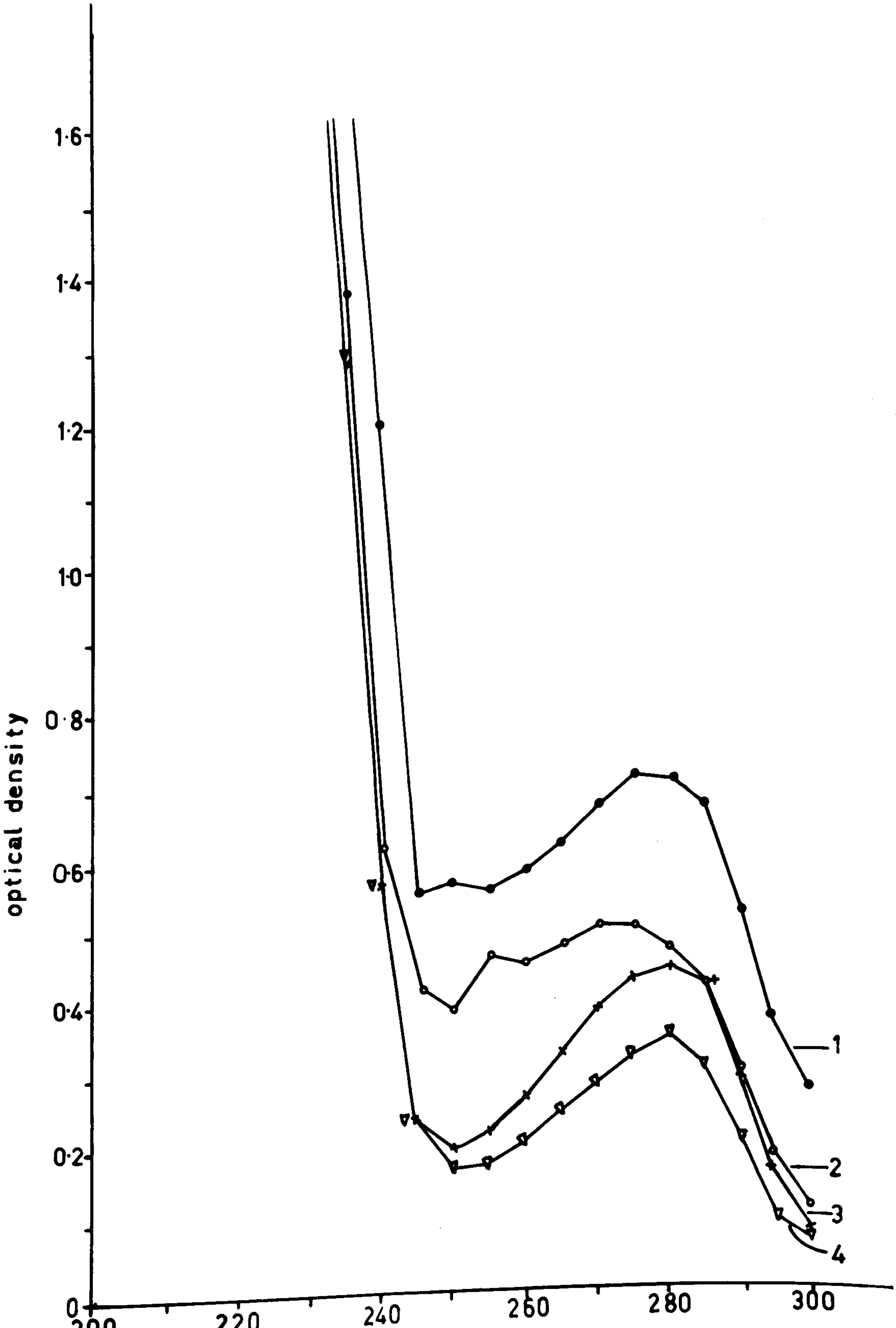
EXTINCTION CURVES FROM 230 TO 300 mμ OF YABBIE SERUM; YABBIE SERUM FROM WHICH PROTEIN HAD BEEN PRECIPITATED AT pH 3.5; PIG SERUM; AND B.S.A.

Curve 1. Yabbie serum diluted 1/100.

Curve 2. Supernatant from yabbie serum which had been subjected to acid precipitation and the pH subsequently returned to 7. Diluted 1/10.

Curve 3. Pig serum diluted 1/200.

Curve 4. B.S.A. 400 μg/ml.


optical density
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
200
220
240
260
280
300
wave length (mμ)
1
2
3
4

contained peptide linkages i.e. was a protein or polypeptide.

Starch Gel Electrophoresis.

A starch gel electrophoretic analysis of the proteins in yabbie serum was made and the position of the various protein bands was compared with those of the characterised components of pig serum (Turner and Rowley, 1963) run in parallel.

The starch gel electrophoretic patterns of yabbie and pig sera are shown in Figure 17. There was no protein present in the yabbie serum with an electrophoretic mobility corresponding to that of pig serum γ-globulin, but there was some staining material left in the well. Seven distinct bands were present, the fastest moving band corresponding in position to albumen in pig serum. The fourth band from the well was the only one giving a positive stain for copper, which suggested that this band contained the haemocyanin, (Figure 18).

Preparative Starch Gel.

Starch gel electrophoresis can also be used for the preparative separation of proteins. After electrophoresis, the various protein fractions are extracted from the gel, and tested for bactericidal activity.

The gel was sectioned as in Figure 17, each segment containing one protein band. Following extraction of the gel segments, bactericidal activity was found only in

Figure 17.

STARCH GEL ELECTROPHORETIC PATTERNS OF YABBIE AND PIG SERA.

Yabbie serum on the right.   Pig serum on the left.

Starch gel run at 4 volts/cm. for eighteen hours in the cold at pH 8.6.

The vertical numbers refer to the fractions of the preparative starch gel of yabbie serum which were extracted and assayed for bactericidal activity.

Gels stained for protein with amido black 10B.

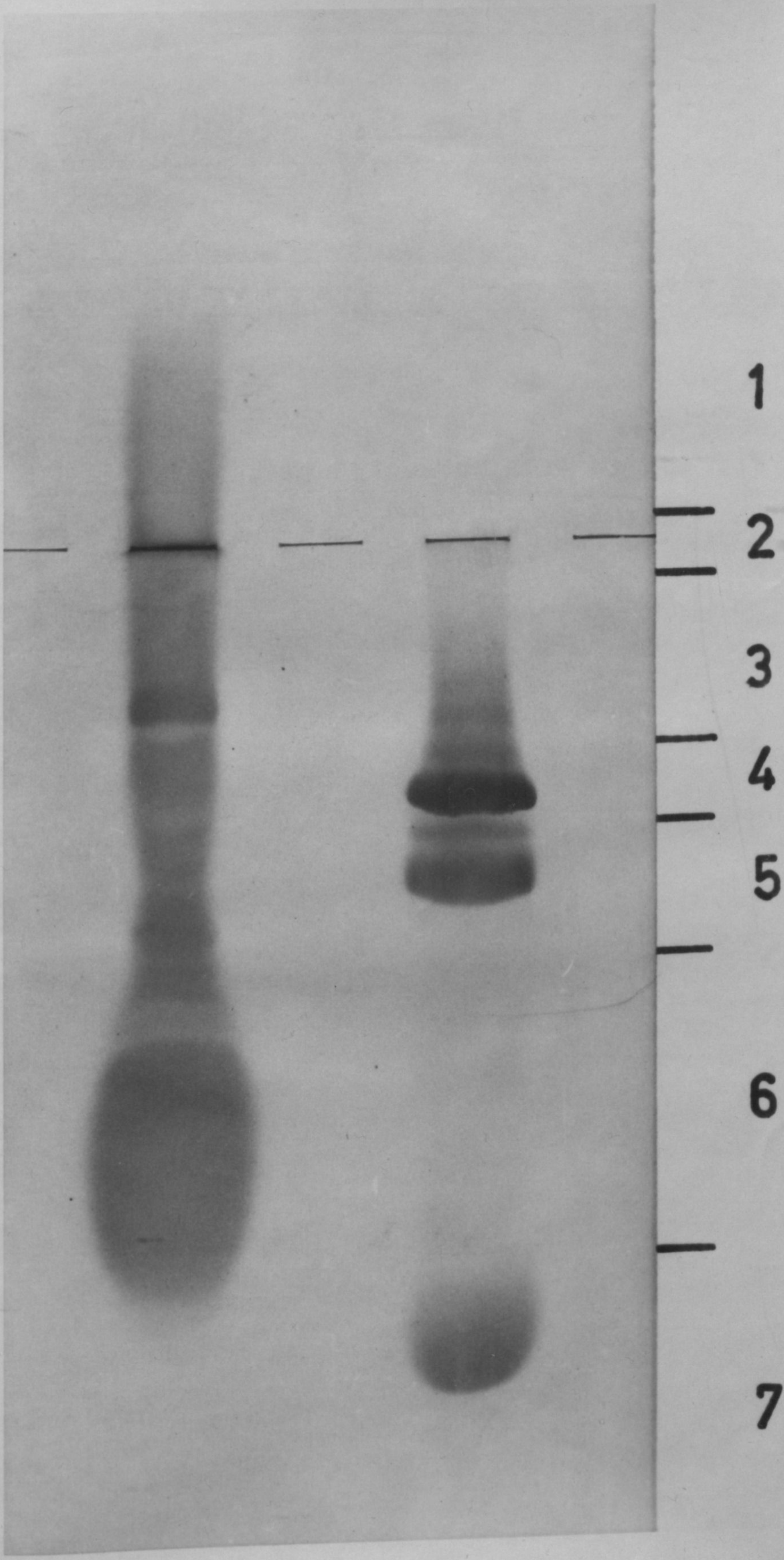

1
2
3
4
5
6
7

Figure 18.

STARCH GEL ELECTROPHORETIC PATTERN OF YABBIE SERUM.

Stained for copper with rubeanic acid.

the material remaining in the well. The recovery of activity was approximately 10%.

Three possibilities were considered which might account for the bactericidal activity remaining in the well. (1) the particle had no net charge at this pH, (2) the active molecule was too large to migrate on starch, (3) it complexed with the starch.

If the bactericidal activity did not migrate in the starch gel because the active molecules had no net charge at pH 8.6, then migration might be expected in a starch gel run at a different pH, when the molecules would be expected to carry a net charge.

Before attempting to run a starch gel at a lower pH (3.5) the effect of this pH on the stability of the bactericidin was determined. As the pH was lowered with 0.1 M hydrochloric acid, protein was precipitated. The supernatant, following the removal of the precipitate at 4,500 g. for 10 minutes, contained 50% of the bactericidal activity. No preparative starch gel was run on this material, as the other techniques to be described gave a good separation of the bactericidin, with a higher percentage recovery of activity. Experiments to be discussed subsequently suggested that the lack of migration was due to the bactericidin being adsorbed to the starch.

Purification of the Bactericidal Substance by Acid Precipitation.

Experiments with the starch gels indicated that some degree of purification of the bactericidal factor was possible by acid precipitation. However, when acid was added directly to the serum, the degree of purification obtained varied from a four-fold increase to no overall gain in purity-equal percentage fall in bactericidal activity and protein.

Retention of bactericidal activity, and ease of removal of precipitated protein, was usually better when the serum was first diluted one to five in basal medium. It was also difficult, especially when using small volumes of serum, to ensure adequate mixing of the acid and so prevent the formation of areas of very high acidity which might lead to destruction of the bactericidin. When the supernatant from the pH 3.5 precipitation was diluted directly into basal medium pH 7, there was at the most a 50% fall in bactericidal activity over that present in normal serum. If, however, the pH of the supernatant was first brought back to 7 with alkali, further protein was precipitated and the bactericidal activity in the supernatant fell. Reducing the pH by dialysis against 0.005 M citric acid-sodium citrate buffer pH 4 gave only 50% removal of protein with no fall in bactericidal activity.

The inability of this technique to give a reproducible overall gain in purity of the active factor made it appear unsuitable as a purification procedure.

Serum partially purified as above was used to determine if the bactericidal factor(s) was of very high molecular weight. The serum was diluted 1:5 in basal medium, the pH adjusted to 3.5 with 2 M hydrochloric acid, and the resultant precipitate removed by centrifugation at 12,000 g. for ten minutes. The supernatant fluid was then centrifuged at 105,000 g. for five hours in a fixed angle head in the Spinco ultra centrifuge. The supernatant fluid was taken off in four equal aliquots and each tested for bactericidal activity. The activity was evenly distributed throughout the tube, indicating that the active factor was not of very high molecular weight, which might have been inferred from its lack of migration on starch.

Paper Strip Electrophoresis.

The relative importance of the physical factors which determine how a protein will migrate in starch and paper electrophoresis differ. On paper lack of migration due to adsorption is minimised. In order to determine if the lack of migration of the bactericidin in starch gel electrophoresis was due to adsorption, and not to a zero net charge, the serum was subjected to paper strip electrophoresis.

The electrophoretic patterns of paper strips run at pH 8 and 9.5 were similar. The major portion of the staining protein migrated as a single fast moving band towards the anode, with slight trailing back to the origin, (Figure 19). The bactericidal activity isolated from the strips, sectioned as in Figure 19, and extracted by the procedure previously described, was localised mainly in the leading protein band, with some trailing of activity back to the origin.

As the bactericidal activity migrated on paper strip electrophoresis, it was thought that a preparative separation of this factor might be possible by curtain electrophoresis.

Curtain Electrophoresis.

In this technique, the protein solution is applied slowly and continuously to a tab near the upper edge of a sheet of chromatographic paper supported vertically. The individual proteins in the mixture are separated as they pass down the curtain under the influence of an electrical field applied across the curtain. A slow continuous flow of buffer down the curtain elutes the protein fractions into collecting tubes.

The protein elution pattern from the curtain is shown in Figure 20 where the optical density at 280 mμ of the contents of each tube multiplied by the fluid volume is plotted against the tube number.

As with the paper strips, there was a slight

Figure 19.

PAPER ELECTROPHORESIS PATTERN OF YABBIE SERUM.

The paper strips were subjected to electrophoresis at room temperature for nine hours at 135 volts.

Stained with bromo-phenol blue.

a. Lightly stained protein.
b. Heavily stained protein.

Vertical numbers refer to the fractions of the paper assayed for bactericidal activity.

Fraction 1 was the section of paper from fraction 2 to the cathode.

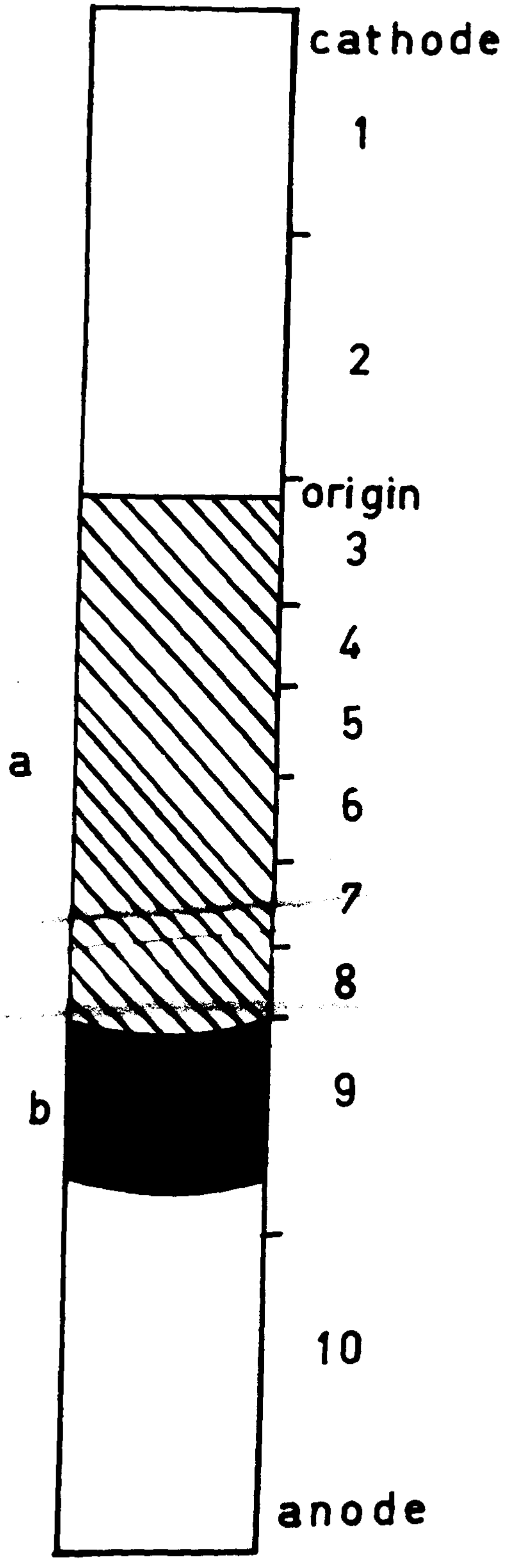

cathode
1
2
origin
3
4
5
a
6
7
8
9
b
10
anode

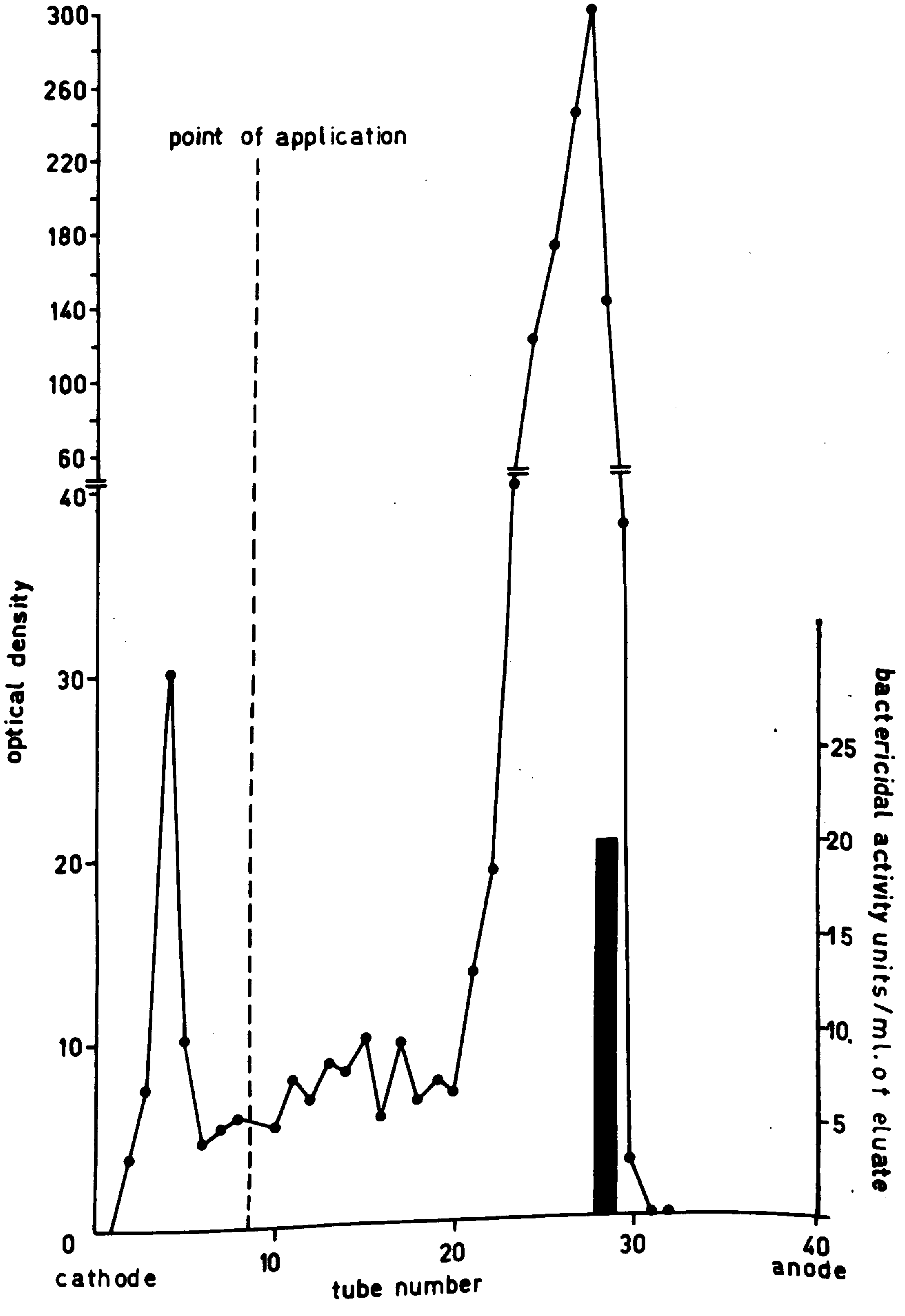
Figure 20.

YABBIE SERUM PROTEIN ELUTION PATTERN FROM CURTAIN ELECTROPHORESIS.

—●—●— optical density.

■ bactericidal activity.

One unit of bactericidal activity is contained in the highest dilution of serum which will kill 50% of the inoculated bacteria in 90 minutes.


300
260
220
180
140
100
60
40
30
20
10
0
optical density
point of application
cathode
10
20
30
40
anode
tube number
25
20
15
10
5
bactericidal activity units/ml. of eluate

trailing of protein across the paper, with a large protein peak near the point of maximum protein migration. The contents of the tubes from this peak were bluish, and during the electrophoretic run a blue band could be seen stretching across the curtain, from near the point of application to tubes 26-28 (Figure 21).

After electrophoresis had proceeded for several days a yellow pigment was eluted from tubes 13-17. This pigment may be similar to the yellow pigment described by Frentz (1958) in the serum of the crab, Carcinus maenas (Leach).

The bactericidin was present in the eluate from the leading edge of the major protein peak. Approximately 40% of the bactericidin put on the curtain was recovered. The bactericidal activity of the eluted fractions gradually declined and after four days at 4°, they were no longer active. The serum remaining in the reservoir was also inactive after this period.

Although this procedure gave an overall gain in purity of the bactericidin, the loss of bactericidal activity with time made the technique unsuitable for large scale preparations.

On paper electrophoresis the bactericidin migrated at pH 8.6 which indicated that the lack of migration in starch gel electrophoresis was not due to the particle carrying a net zero charge. There were indications that the

Figure 21.

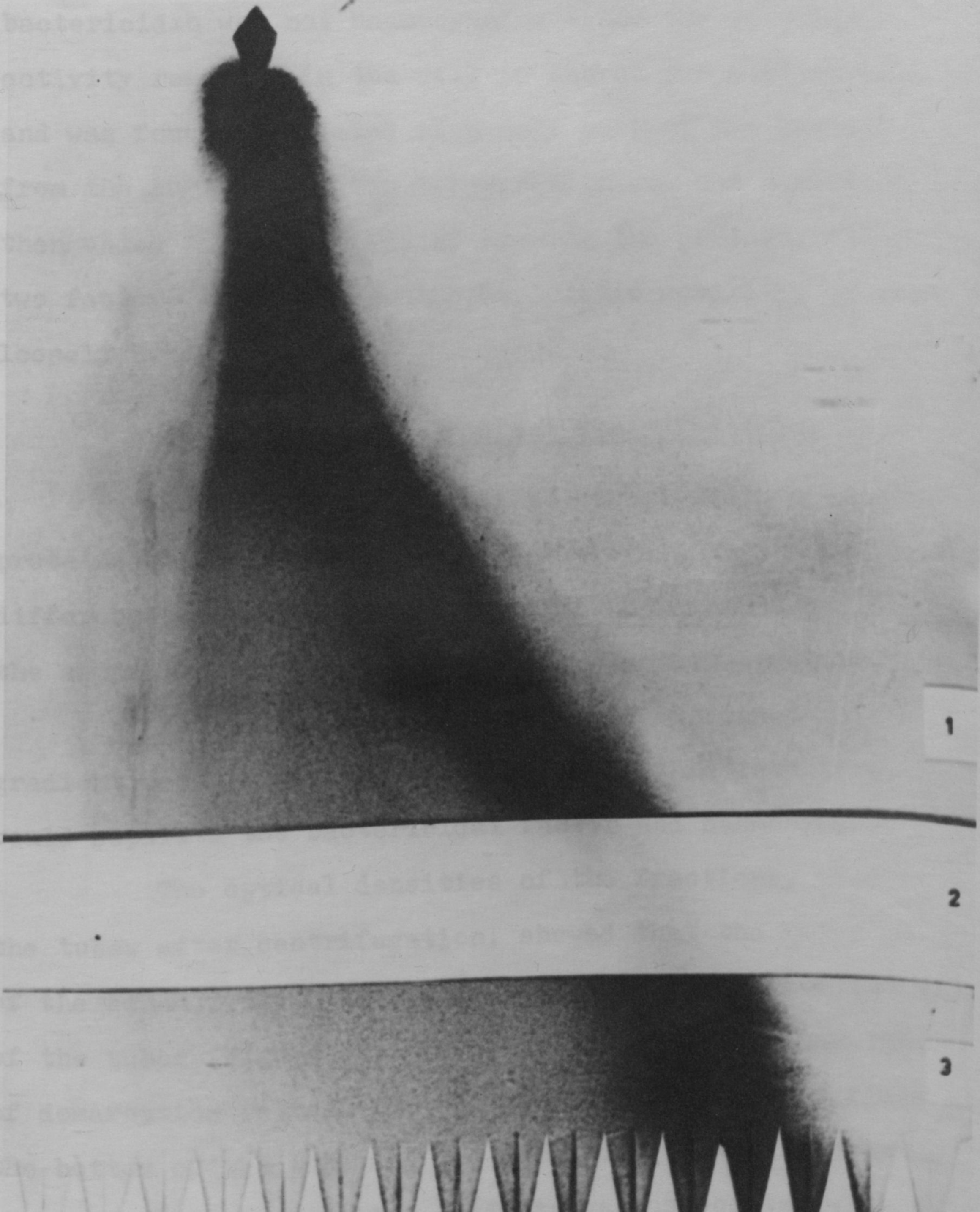
CURTAIN STAINED FOR LOCALISATION OF PROTEIN AND COPPER AT THE CONCLUSION OF THE ELECTROPHORETIC RUN.

A section of the curtain was stained for copper by the technique of Decleir (1961).

The remainder was stained for protein using bromo-phenol blue.

1 and 3 stained for protein.

2 stained for copper.


1
2
3

bactericidin was not haemocyanin, since the bactericidal activity remained in the well in starch gel electrophoresis and was found associated with only part of the haemocyanin from the curtain. If the bactericidin was not haemocyanin, then under the conditions of running the curtain, either the two factors had similar electrophoretic mobility, or were loosely bonded.

Sucrose Density Gradient Centrifugation.

Sucrose density gradient centrifugation separates proteins on the basis of molecular weight, shape, and density differences. This technique is particularly applicable to the separation of macro-globulins from other serum proteins.

Yabbie serum was subjected to sucrose density gradient centrifugation to determine if this technique would separate the bactericidal factor and haemocyanin.

The optical densities of the fractions, bled from the tubes after centrifugation, showed that the major portion of the serum protein was concentrated in the bottom 1.5 ml. of the tubes (Figure 22). Visually there was a clear line of demarcation between the one to 1.5 ml. of bluish fluid at the bottom of the tube and the remainder of the supernatant.

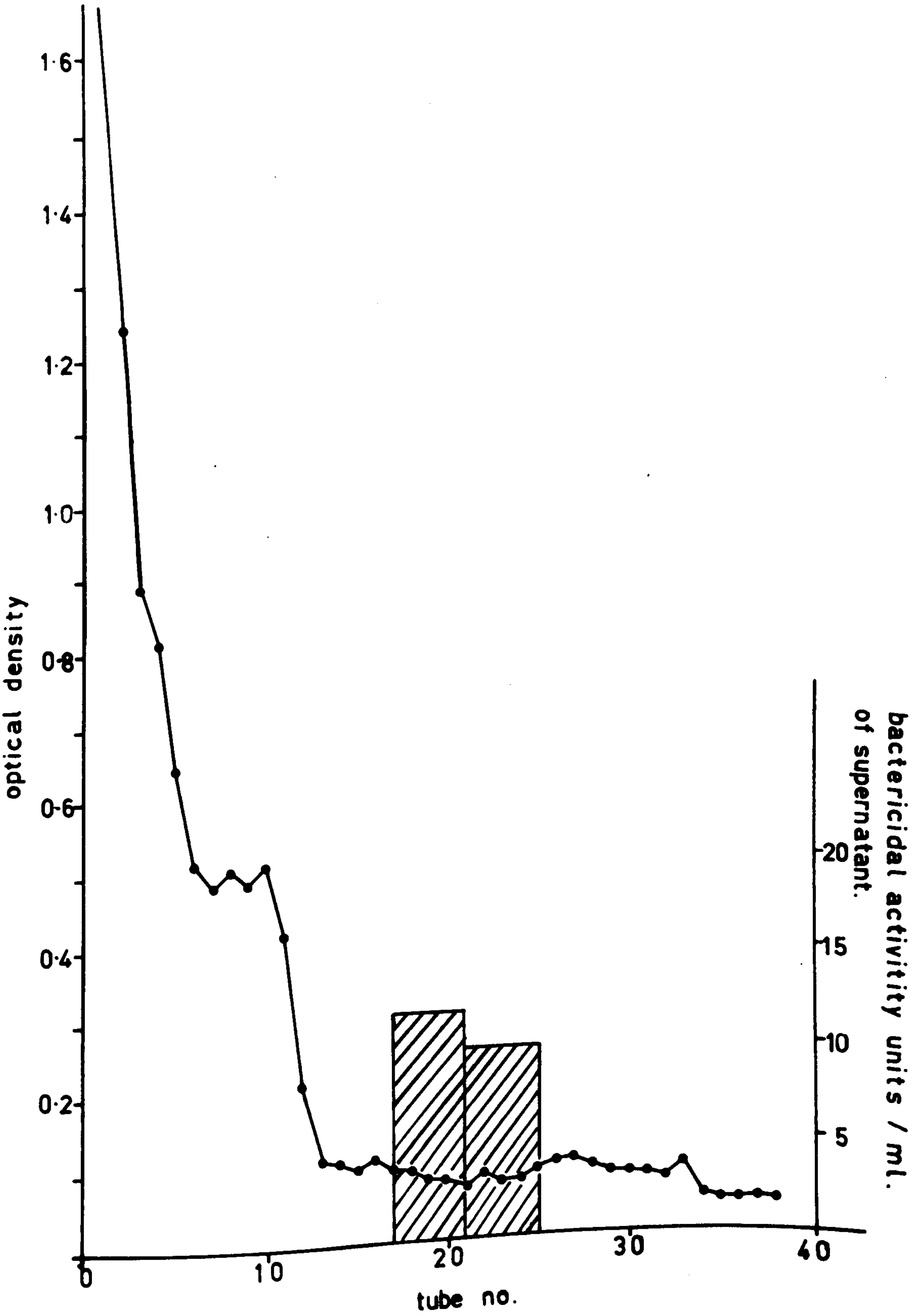
The bactericidal activity was associated with that portion of the serum proteins which remained in suspension 2.5 to 3.5 ml. from the bottom of the tube. Between 50 and 75% of the bactericidal activity was recovered. No bacter-

Figure 22.

DISTRIBUTION OF THE O.D. 280 mμ. ABSORBING FRACTIONS OF YABBIE SERUM FOLLOWING DENSITY GRADIENT CENTRIFUGATION.

—●—●— optical density.
▨ bactericidal activity.

One unit of bactericidal activity is contained in the highest dilution of serum which will kill 50% of the inoculated bacteria in 90 minutes.


optical density
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
bactericidal activitity units / ml.
of supernatant.
20
15
10
5
0
10
20
30
40
tube no.

icidal activity was associated with the haemocyanin.

This result clearly indicated that the bactericidal activity was not associated with haemocyanin, and that there was a probable molecular weight difference between the two entities, the active fraction being the smaller.

The combined results of starch and paper electrophoresis, and density gradient centrifugation, were interpreted to indicate that in yabbie serum there was a loose complexing of haemocyanin and the bactericidin. Such a complex would be more stable under the conditions of electrophoresis, i.e. alkaline pH and low ionic strength, than when subjected to density gradient centrifugation at pH 7 in molar saline, a condition which would favour the dissociation of protein-protein complexes, particularly if non-specific. The ionic strengths of the buffers used in the separations utilising Sephadex were also sufficiently high to dissociate non-specific protein-protein bonding.

G75 Sephadex Chromatography.

The technique of Sephadex chromatography employs the principle of gel filtration, separating proteins on the basis of molecular size, i.e. functioning as a molecular sieve.

Chemically, Sephadex is a long chain polymer of dextran with the individual chains cross linked. The porosity of the network depends upon the degree of cross linkage.

The diffusion through the Sephadex grains of solutes with moderately large molecular dimensions is restricted according to the porosity of the network. Large molecules are completely prevented from entering the gel grains and are eluted before smaller molecules which enter the gel grains. With increasing porosity, molecules of larger size can penetrate the grains. Therefore, by a judicious selection of pore size, molecules of different dimensions can be effectively separated. Unlike density gradient centrifugation all proteins of approximately the same molecular size are eluted together, irrespective of their density.

If the molecular weight difference between the bactericidin and haemocyanin was as great as the density gradient centrifugation suggested, then good separations of the bactericidin from haemocyanin would be expected using an appropriate grade of Sephadex. The first separations were attempted on G75 Sephadex in 0.2 M tris-HCl buffer (pH 8.0) at room temperature. Seven ml. of normal serum were layered on a 250 ml. Sephadex column and the eluates, which were collected in four ml. aliquots, were assayed individually for protein by measuring the optical density at 280 mμ.

Two protein peaks were obtained, the first and largest passed through the column in the void volume, the second smaller peak came through only after a column volume of buffer had been passed. On the basis of the protein elution pattern, 1 ml. aliquots of each series of five tubes, commencing from the beginning of the haemocyanin peak, were pooled and tested for bactericidal activity. The pools were all inactive.

A repeat column run in the cold gave a similar separation of the proteins, with bactericidal activity in one pool of the second peak (Figure 23). A summation of the optical densities of the eluates in the first and second peaks showed the second one to contain 0.7% of the total serum proteins. The total recovery of activity was low (9%) with a rapid decline and complete loss of activity after the fraction had been kept for forty eight hours in the cold.

The contents of the tubes from each peak were pooled, dialysed against three one-hourly changes of distilled water, freeze dried, and reconstituted in one ml. of saline. The electrophoretic patterns of the concentrated fractions were determined by starch and paper electrophoresis.

Starch Gel (pH 8.6).

Normal yabbie serum, and the protein from the first peak, gave similar patterns. No amido black 10B staining material could be found from the second peak notwithstanding the fact that the protein concentration estimated from the 280 mμ. optical density reading was sufficient to stain. This figure for protein may have been high

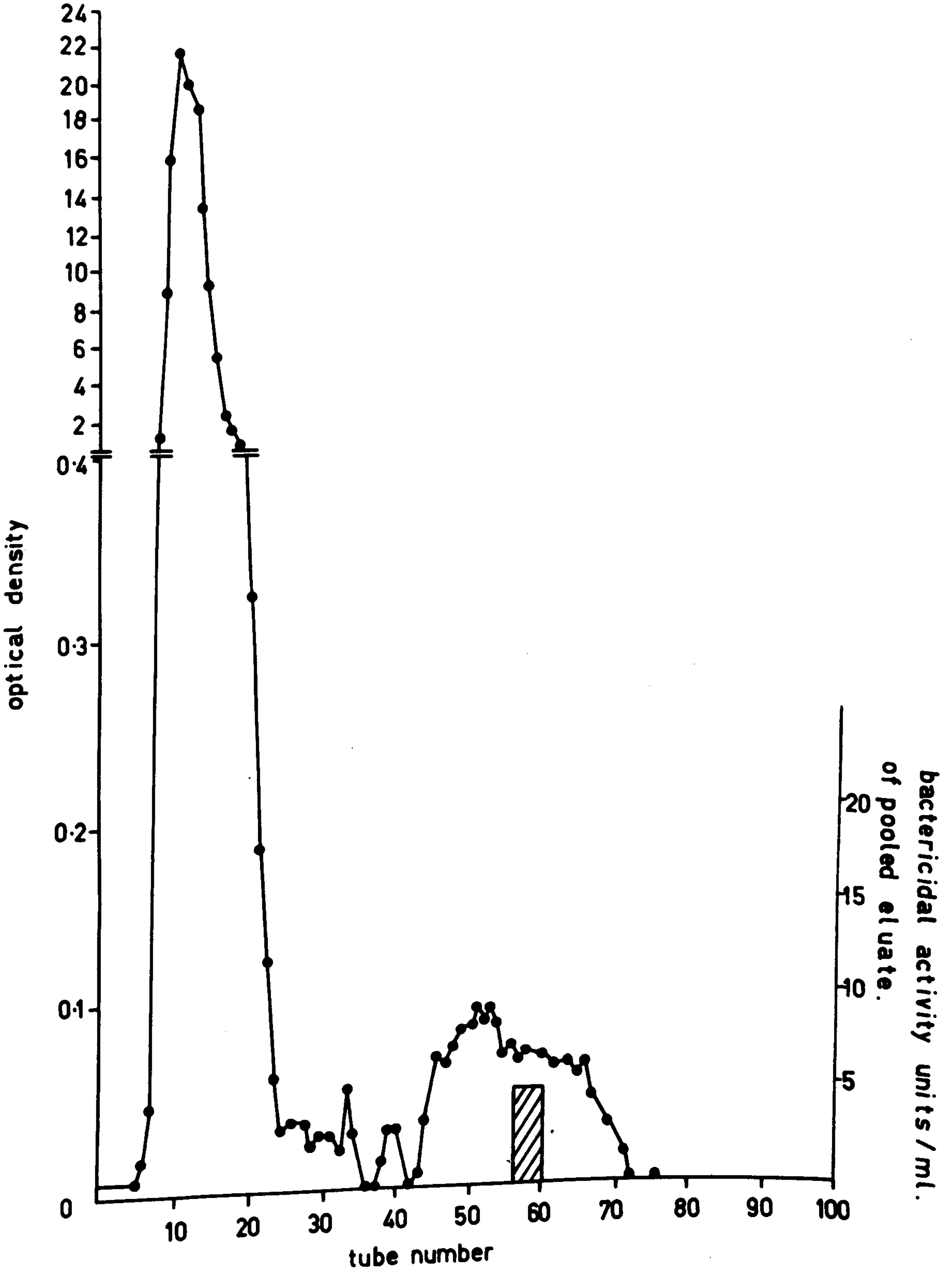
Figure 23.

PROTEIN ELUTION PATTERN OF YABBIE SERUM FROM G75 SEPHADEX.

—●——●— optical density.

▨ bactericidal activity.

One unit of bactericidal activity is contained in the highest dilution of serum which will kill 50% of the inoculated bacteria in 90 minutes.


optical density
24
22
20
18
16
14
12
10
8
6
4
2
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
90
100
tube number
bactericidal activity units/ml. of pooled eluate.
20
15
10
5

due to variations in extinction coefficients (to be discussed on page 132), or the dye binding characteristics for amido black 10B of this fraction might have been different from that of the other serum proteins.

Paper Electrophoresis (pH 8.0).

No difference was found between the electrophoretic pattern of the protein in the first fraction and normal serum when stained by bromo-phenol blue. With the second peak, the only suggestion of protein was a very faintly stained area at the origin.

More protein bands were apparent when the paper strips following electrophoresis were stained with ninhydrin. Fraction two lacked the leading densely stained band migrating towards the anode in normal serum. Both had stained material at the origin, and a band 1.5 cm. on either side of the origin. Fraction two had a green fluorescent band on the cathode side of the origin which was not apparent in normal serum (Figure 24).

Thus density gradient centrifugation, and G75 Sephadex chromatography, showed that the bactericidal activity could be separated from the haemocyanin, and further, that the active fraction constituted only a small percentage of the total serum protein.

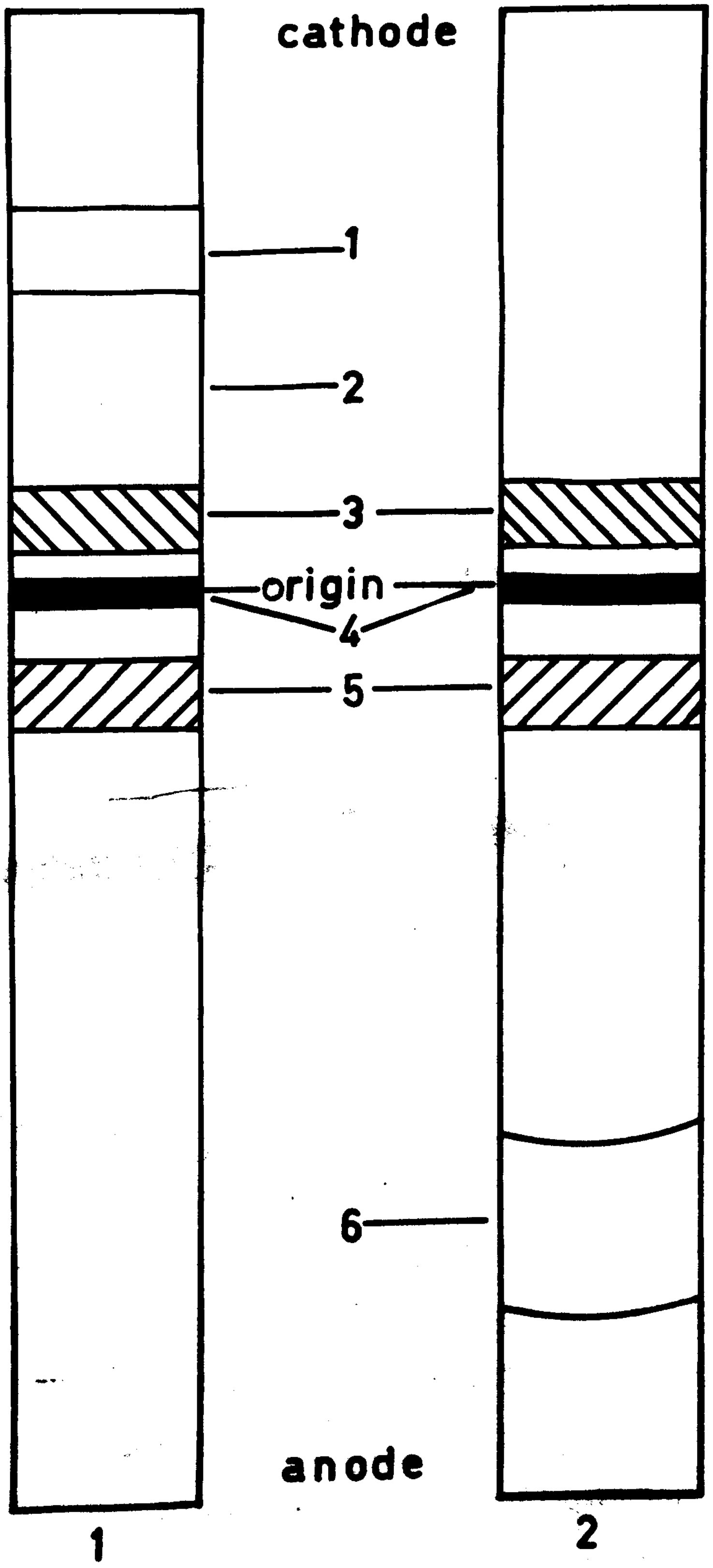
The protein loading capacity of a Sephadex column is small, and hence the technique has limited applicability

Figure 24.

PAPER ELECTROPHORESIS OF- 1. FRACTION 2. FROM G75 SEPHADEX.
2. NORMAL YABBIE SERUM.

Paper strips stained with ninhydrin.

1. Lightly stained band.
2. Green band.
3. Deeply stained band.
4. Band at origin.
5. Deeply stained band.
6. Deeply stained band.


cathode
1
2
3
origin
4
5
6
anode
1
2

as a preparative tool if the required fraction is a small percentage of the total serum protein. The effective loading capacity of a Sephadex column relative to a minor serum component can be increased by removing a major portion of the unwanted protein prior to the serum being put on the column. An alternative is to run the whole serum on very large columns, but in the present instance the instability of the active fraction at alkaline pH, and the extended period required for elution of the bactericidin precluded their use.

Precipitation of Inactive Protein at Different pH and Ionic Strengths.

As a means of increasing the effective loading capacity of the Sephadex columns relative to the minor component, it was decided to re-investigate the acid precipitation of the haemocyanin under more carefully controlled conditions of pH and ionic strength.

Serum was dialysed overnight with continuous agitation against buffers of ionic strengths 0.01, and 0.2, at pH 4, 5, 6 and 7. (The ratio of serum to buffer was 1:100). After dialysis the pH values of the retentates were measured, followed by the removal of the precipitates by centrifugation at 4,500 g. for ten minutes. The supernatants were assayed for bactericidal activity, and for total protein by the method of Folin-Ciocalteu. The actual pH reached during dialysis, total protein and bactericidal activities of the supernatants are given in Table 19. The highest protein removal of 68% occurred at pH 4 and an ionic strength of 0.2 with a loss of 50% of the biological activity. In later preparations, the precipitate following dialysis was removed by centrifugation at 39,000 g. for one hour which gave a removal of 85% of the serum protein with no further loss of bactericidal activity. These results would suggest that the difficulty experienced in getting reproducible removal of protein and retention of bactericidal activity in the earlier experiments, was possibly due to variations in the ionic strength of the supporting medium.

G50 Sephadex Chromatography.

On G75 Sephadex at pH 8 the bactericidin was retained, which indicated that its molecular weight was less than 50,000 and was possibly considerably less than this figure due to the strong retention. Because of the strong retention it was thought that a better separation might be obtained with a Sephadex of smaller pore size. A further separation of the active factor was attempted using G50 Sephadex. The effective loading capacity of the column, relative to the active fraction was increased by using the supernatant from the acid precipitation concentrated by freeze drying to a small volume.

The pre-treatment of the serum and the details of

Table 19.

THE EFFECT OF DIALYSING YABBIE SERUM OVERNIGHT AGAINST BUFFERS OF VARYING pH AND IONIC STRENGTH ON THE BACTERICIDAL TITRE AND REMOVAL OF PROTEIN.

pH of buffer.	Actual pH of retentate.		Mg. protein/ml. serum.		Reciprocal of bactericidal titre.	
	0.01 M.	0.2 M.	0.01 M.	0.2 M.	0.01 M.	0.2 M.
3	3.6	3.6	28	19	80	80
4	4.2	4.2	26	10	40	80
5	5.3	5.1	22	26	40	80
6	6.7	6.0	24	25	40	40
7	7.2	7.2	25	29	80	80
Normal serum.			31		160	

running the column are given in Table 20. The main protein peak was eluted with the void volume with a trailing off of protein until a column volume of buffer had been passed (Figure 25). Pools made with one ml. aliquots from each series of five tubes, from the commencement of the protein elution, were tested for bactericidal activity after returning the pH to 7.5 with 12.5% sodium carbonate.

Bactericidal activity was spread over 100 ml. of the eluate with the highest activity in the centre of the active band. The activity recovered approximated to 35% of that put on the column. The concentration of protein in the eluates showing bactericidal activity was estimated as a percentage of the total protein eluted. This percentage was calculated by assuming protein concentration to be equivalent to optical density. 14% of the eluted protein was present in the tubes which contained the bactericidin. As 78 mg. of protein was applied to the column and the active fraction contained 11 mg. of protein, it was calculated that the bactericidal activity was associated with less than 2% of the original serum protein. Since the active material was retained by the Sephadex, its molecular weight was probably less than 10,000.

Chemical Complexity of the Biologically Active Fraction from G50 Sephadex.

The contents of all tubes containing bactericidal

Table 20.

ACID PRECIPITATION AND G50 SEPHADEX PURIFICATION OF THE BACTERICIDIN IN YABBIE SERUM FOR GRAM NEGATIVE BACTERIA.

Serum (20 mls) = 640 mg. protein.
3,200 units of bactericidal activity.*

Dialysed overnight against 0.2 M acetate buffer
pH 4 followed by centrifugation at 39,100 g. for 1 hour.

Supernatant 102 mg. protein
3,200 units of activity.

Deposit - discarded.

20 mg. withdrawn for tests.

82 mg. freeze dried.
Reconstituted in 2 mls. distilled water.

4 mg. withdrawn for tests.

G50 Sephadex.
78 mg. protein.
1280 units of activity.
Eluate taken off in 3 ml. aliquots.

Fraction 1.
67 mg. protein.
No bactericidal activity.

Fraction 2.
11 mg. protein.
450 units of activity.

* One unit of bactericidal activity is contained in the highest dilution of serum which will kill 50% of the inoculated bacteria in 90 minutes.

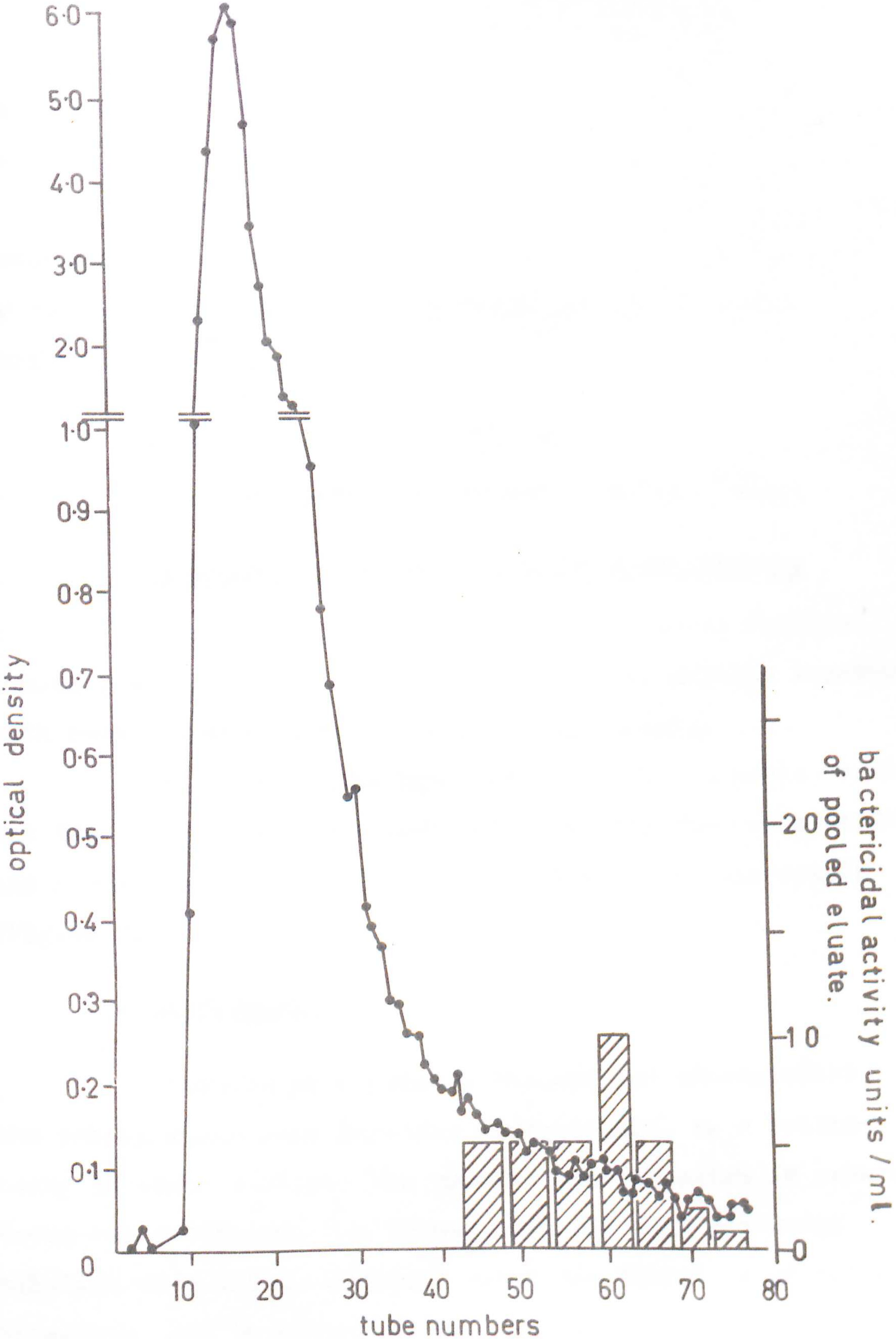
Figure 25.

PROTEIN ELUTION PATTERN FROM G50 SEPHADEX OF THE CONCENTRATED SUPERNATANT AFTER ACID PRECIPITATION OF YABBIE SERUM AT pH 4 AND IONIC STRENGTH 0.2.

—●—●— optical density.

▨ bactericidal activity.

One unit of bactericidal activity is contained in the highest dilution of serum which will kill 50% of the inoculated bacteria in 90 minutes.



optical density
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
tube numbers
bactericidal activity units / ml of pooled eluate.
20
10
0

activity were pooled, dialysed overnight against distilled water, freeze dried, and taken up in 0.5 ml. saline.

The heterogeneity of this fraction was then investigated by starch gel electrophoresis at pH 8.6 and by paper electrophoresis in pyridine-acetic acid-water buffer at pH 6.4.

Starch Gel Electrophoresis.

No amido black 10B staining band was found.

Pyridine-Acetic Acid-Water Electrophoresis.

When stained with ninhydrin the active fraction showed five bands all migrating towards the cathode together with some protein which remained at the origin.

The first three bands were present in whole serum but the fourth and fifth were not apparent. The whole serum had a much greater concentration of protein at the origin (Figure 26).

Discussion.

Attempts were made to isolate and characterise the yabbie serum gram negative bactericidin. As a preliminary to these studies, the protein concentration in yabbie serum was estimated. The values obtained were estimated relative to a B.S.A. standard using the technique of Folin-Ciocalteu, and by measuring the absorption of ultra violet light at 280 mμ. These assay techniques measure chiefly the

Figure 26.

PAPER ELECTROPHORESIS OF FRACTION 2 FROM G50 SEPHADEX AND NORMAL YABBIE SERUM RUN IN PYRIDINE-ACETIC ACID-WATER pH 6.4.

(1) Fraction 2.

(2) Normal serum.

Stained with ninhydrin.

1. Highest concentration of polypeptide - deeply stained.

2. and 3. Equal intensity of staining - about 1/5 concentration of 1.

4. Yellow band.

5. Faintly stained about 1/5 intensity of 2.

Spot at origin stained blue.

The numbered fractions (except No.4.) stained pink.

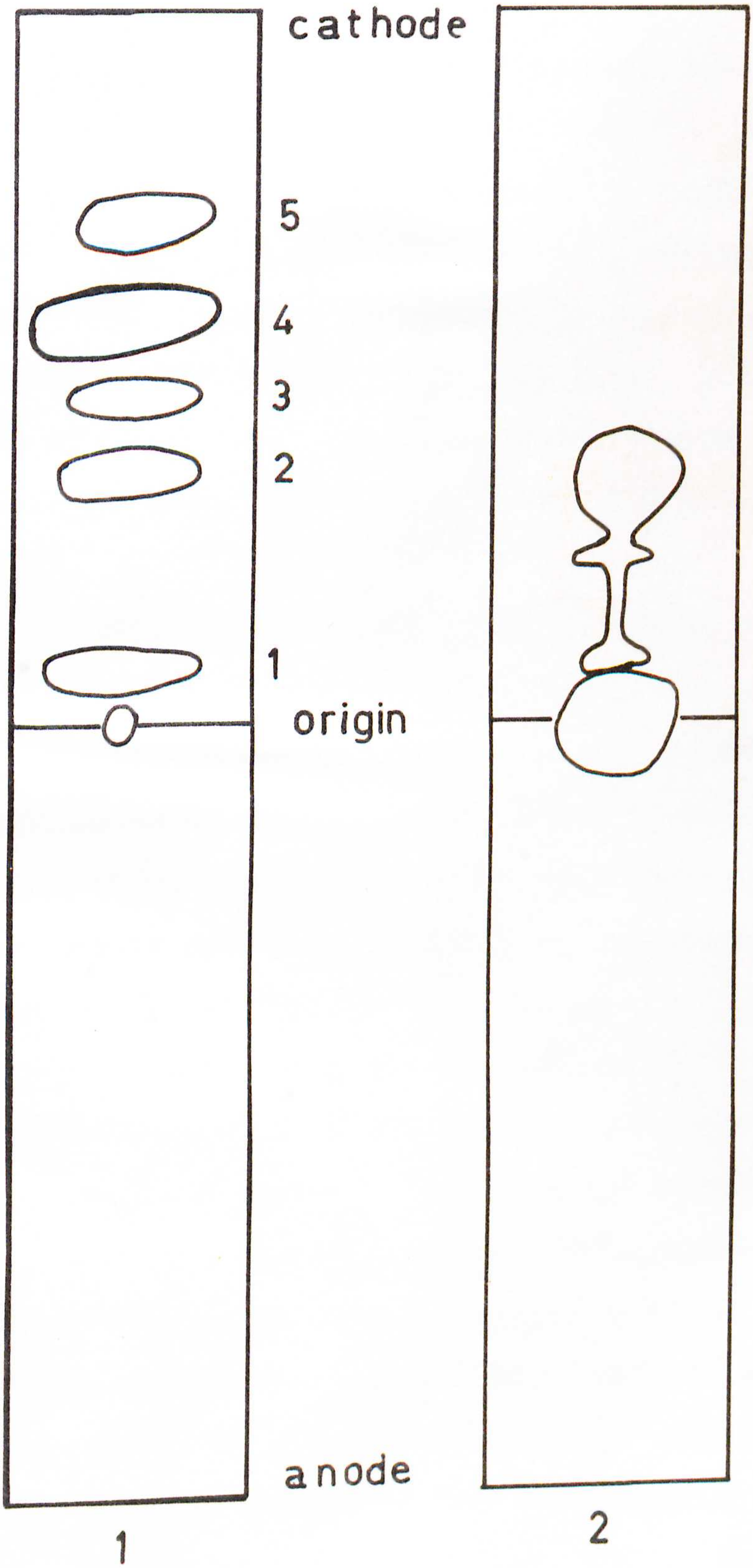

cathode
5
4
3
2
1
origin
anode
1
2

Figure 26.

PAPER ELECTROPHORESIS OF FRACTION 2 FROM G50 SEPHADEX AND NORMAL YABBIE SERUM RUN IN PYRIDINE-ACETIC ACID-WATER pH 6.4.

(1) Fraction 2.

(2) Normal serum.

Stained with ninhydrin.

1. Highest concentration of polypeptide - deeply stained.

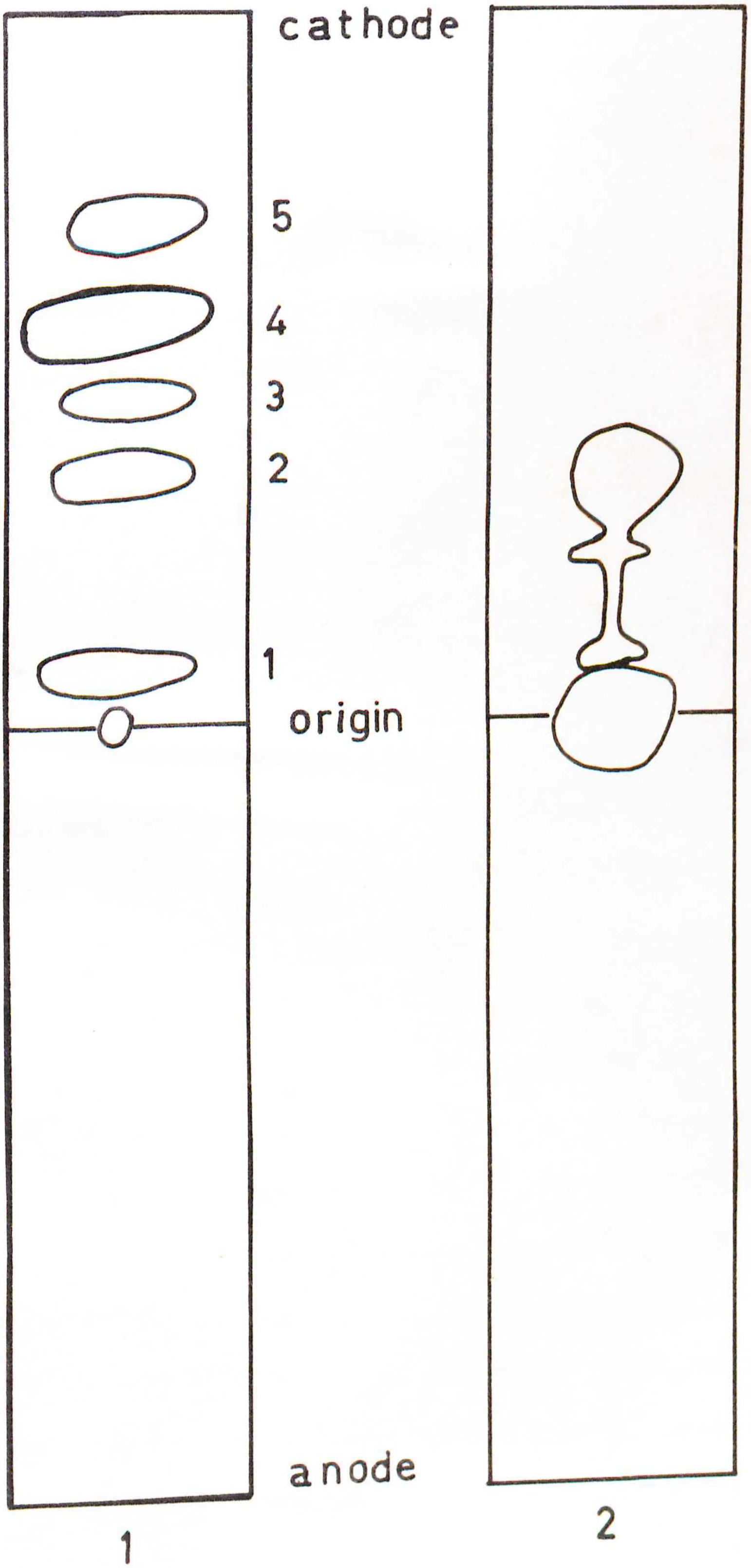
2. and 3. Equal intensity of staining - about 1/5 concentration of 1.

4. Yellow band.

5. Faintly stained about 1/5 intensity of 2.

Spot at origin stained blue.

The numbered fractions (except No.4.) stained pink.


cathode
5
4
3
2
1
origin
anode
1
2

aromatic amino acid content of proteins and thus if there is a variation in the content of these acids between the standard and unknown protein solutions, the true protein concentration in the unknown will not be estimated. Thus the absolute concentration of protein in yabbie serum may differ from the value stated. The calculated concentration of protein in yabbie serum is comparable, however, with the values quoted by Engle and Woods (1960), for crustacean sera.

The purification procedures utilising density gradient centrifugation and Sephadex chromatography clearly showed that the major protein haemocyanin was devoid of any bactericidal activity for gram negative bacteria, and that the bactericidin was associated with approximately two % of the total serum proteins. While it is not possible to state categorically if the bactericidin is a single or multicomponent system, several observations point towards it being a single entity. If the bactericidin was multicomponent, then it is unlikely that all the components would have a similar molecular weight and size range, as the separations utilising Sephadex chromatography and density gradient centrifugation suggest, particularly as the molecular weight of the bactericidin is less than 10,000.

The chemical nature of the bactericidin is still obscure. The observations that the bactericidin in whole serum was inactivated by tryptic digestion, and that the molecular weight was less than 10,000, suggest that the substance may be a polypeptide, but further investigations will be necessary before the chemical nature can be firmly established.

No protein with the electrophoretic mobility of pig serum γ-globulin could be demonstrated in yabbie serum. Woods, Paulsen, Engle and Pert (1958) also commented on the absence of these proteins from crustacean sera.

The absence from these sera of proteins with the electrophoretic mobility of vertebrate γ-globulin cannot, however, be taken as proof that these animals are unable to produce antibody, as it could well be that in these animals proteins with antibody specificity have a different electrophoretic mobility.

Summary.

Protein with electrophoretic mobility similar to that of pig serum γ-globulin was not found in yabbie serum.

The bactericidin and haemocyanin could not be separated by paper electrophoresis. Yabbie serum was sep-separated into two protein fractions by density gradient centrifugation and Sephadex chromatography. The major fraction contained the haemocyanin and was devoid of any bactericidal activity.

The minor fraction, which constituted less than 2% of the total serum proteins and had a molecular weight probably less than 10,000, was bactericidal.

The fraction containing the biological activity was heterogeneous and evidence was obtained which suggested it was a mixture of protein and peptides.

Summary of Chapters 6, 7 and 8.

The presence, specificity and chemical nature of a bactericidin in yabbie serum has been investigated.

The serum killed both gram positive and negative bacteria. Absorption tests suggested that two separate bactericidal systems were involved, one for gram positive and the other for gram negative bacteria.

Haemolytic complement activity could not be demonstrated.

The bactericidin was suggested to be a single component system with a molecular weight less than 10,000.

While all attempts to produce a demonstrable immune response in these animals were unsuccessful, the presence of some type of recognition mechanism was suggested by the ability of the animals to clear bacteria from their circulation, but not bacteriophage.

CHAPTER 8.

DISCUSSION.

This study has been concerned with a comparative examination of some aspects of the bactericidal system for gram negative bacteria in sera from various species of animals. The animals whose sera were chosen for study included representatives of six classes of vertebrates and one invertebrate. Several of the animal species whose sera were studied for bactericidal activity were native to Australia, and as far as the present author could ascertain, no information on the bactericidal activity of these sera has been published. The emphasis of this investigation has been principally to attempt to define the nature and specificity of the killing mechanism in the different sera.

Comparison of the Bactericidal Activities of Sera from Different Vertebrate Species.

The results of the screening tests for bactericidal activity described in Chapter 3 showed that all the sera were bactericidal for at least one strain of gram negative bacteria. There were variations from one preparation of pooled serum to another both with respect to the bacterial species which were killed, and to the highest serum dilution which killed 50% of the inoculated bacteria in 90 minutes. It must be emphasised that the comparative results obtained reflect differences in the bactericidal activities of particular batches of sera for certain bacteria. Had the bactericidal activities of different pools of sera from the same species of animals been compared, or had different bacteria been used, then the overall pattern of killing may quite well have been changed.

Examples of the variations found in the bactericidal activities of different preparations of pooled sera from the same animal species were presented in Table 5. Three preparations of pooled normal fowl sera were tested for bactericidal activity for the seven strains of gram negative bacteria. All seven bacterial strains were killed by one serum preparation, four bacterial strains by another and the third serum pool was bactericidal for only three of the bacterial strains. As these variations in bactericidal titres occurred with different serum preparations from one animal species, it is obviously not possible to draw any conclusions relating to the greater bactericidal activity of the serum from one animal species compared with that obtained from another.

One of the interesting findings of the present comparative study was that contrary to previous observations (Rowley, 1956; Michael and Landy, 1961) antigenically rough strains of gram negative bacteria were not uniformly more susceptible to serum killing than their smooth counterparts. This was particularly evident with one preparation of fowl serum where the bactericidal titre for the smooth strain (E. coli BV) was 1/20 compared with 1/5 for the rough strain (E. coli Lilly). Ali (1959) also found that fowl serum killed these two strains to essentially the same titre. This concept of the greater sensitivity to serum killing of smooth strains of gram negative bacteria compared with rough variants has arisen through studies with mammalian sera and has been tacitly assumed to be true of all vertebrate sera without studies being made to verify its general validity.

Michael et al., (1962) showed that in normal serum, complement and a specific sensitiser mediated in the killing of smooth strains of gram negative bacteria. This finding was corroborated in the present study, and in addition, a specific sensitiser was shown to mediate in the serum killing of rough strains of gram negative bacteria. Landy, Michael and Whitby (1962) showed that there was an appreciable difference in the level of the sensitisers for smooth strains of gram negative bacteria in different mammalian sera and it seems reasonable to assume that the level of the sensitisers for rough strains of gram negative bacteria would also differ. Such would appear to be the case from the present study.

Nature of the Bactericidal System for Gram Negative Bacteria in the Various Vertebrate Sera.

The bactericidal activity of all the vertebrate sera investigated in the present study appeared to be mediated by complement, since any treatment which led to a loss of haemolytic complement activity also resulted in a loss of bactericidal activity. Specific sensitisers were demonstrated in human serum to the smooth strains *S. typhimurium* M206 and *E. coli* BV and to the rough strain *E. coli* Lilly. Specific sensitisers were also present in lizard serum for *S. typhimurium* M206. These observations thus verify the report of Michael et al., (1962) that the serum sensitisers for smooth strains of gram negative bacteria are specific for individual bacterial serotypes. In addition, it was shown that sensitisers for rough strains of gram negative bacteria were also specific for each bacterial strain. The specificity of the natural sensitisers were determined only in human and lizard sera. These studies were not extended to the other sera since it seemed reasonable to assume that as all the sera contained complement a specific sensitiser would also mediate in the killing of both smooth and rough strains of gram negative bacteria.

A feature of these absorptions was the marked difference in the quantity of absorbing bacteria required to bring about a similar percentage decrease in the bactericidal titre of human serum for *E. coli* BV and Lilly. Less than two mg. dry weight of *E. coli* BV/ml. of human serum reduced the bactericidal titre for the homologous strain by 80% but with *E. coli* Lilly the quantity of absorbing suspension had to be increased to 50 mg. dry weight of cells/ml.

of serum in order to produce a corresponding percentage decrease in bactericidal titre for the absorbing strain. Despite this comparatively massive dose of *E. coli* Lilly the absorption was still specific, since the bactericidal titre for *E. coli* BV was largely unaffected. Wardlaw (1962), and Muschel and Jackson (1963) both used similar levels of *E. coli* Lilly to deplete the bactericidal and bacteriolytic activities of human serum for *E. coli* Lilly and presumably they also found that no marked reduction of activity was produced when smaller absorbing doses were employed.

No experimental evidence was found in the present study for the participation of lysozyme in the serum killing of rough strains of gram negative bacteria or that complement alone was bactericidal for these rough strains.

Sterzl et al., (1962) claimed that their experimental results indicated that complement alone was bactericidal for rough strains of gram negative bacteria. Their evidence for this statement was firstly that they were unable to reduce the bactericidal activity of embryo pig serum by absorption with the homologous rough strain and secondly, that the sera had no bactericidal activity for smooth strains of gram negative bacteria. Their previous studies had shown that smooth strains of gram negative bacteria were killed by a mechanism involving complement and a sensitisor and so they interpreted the lack of a bactericidal mechanism for the smooth strains tested to indicate a lack of sensitisers for all strains of gram negative bacteria. The results of the present study would indicate that in their absorption tests Sterzl and his colleagues did not employ sufficiently large quantities of bacteria to bring about any decrease in the bactericidal activity of the absorbed sera for rough strains of gram negative bacteria. Also the apparent lack of any bactericidal activity for smooth strains of gram negative bacteria would appear to be a reflection of the particular strains employed, rather than a complete lack of bactericidal activity for all smooth strains of gram negative bacteria. Thus their reasons for proposing that complement alone mediated the killing of rough strains of gram negative bacteria do not appear to be valid.

In the present study two approaches were utilised to determine if lysozyme participated in the normal serum killing of rough strains of gram negative bacteria. A comparison between the bactericidal titre of each serum for E. coli Lilly and the levels of haemolytic complement and lysozyme failed to show any evidence that the levels of serum complement and lysozyme could be correlated with the bactericidal titre. No enhancement of the bactericidal activity of human serum absorbed with E. coli Lilly or of normal lizard and toad sera was observed after adding egg white lysozyme to a final concentration of approximately 10 ug/ml. of serum.

Recently Muschel and Jackson (1963) showed that a sensitiser mediated in the killing of E. coli Lilly by normal human and rabbit sera. They claimed that the addition of egg white lysozyme to these sera augmented the bactericidal activity for E. coli Lilly. It is difficult to ascertain what effect the addition of egg white lysozyme to normal rabbit serum had on the magnitude of the bactericidal activity for E. coli Lilly as their data on this point is somewhat vague. With immune antibody and rabbit serum absorbed with E. coli Lilly as a source of complement, egg white lysozyme did significantly enhance the bactericidal activity of the system for E. coli Lilly. However, this enhancement only occurred when the concentration of egg white lysozyme was in excess of that found in normal sera.

If lysozyme is to be shown to participate in normal serum killing of rough strains of gram negative bacteria, then surely an enhancement of bactericidal activity must be demonstrated with concentrations of lysozyme within normal physiological limits. Thus from the present work it appears that in normal vertebrate sera the addition of egg white lysozyme within normal physiological concentrations does not enhance the bactericidal activity of the sera.

Discussion of the Factors Limiting the Serum Killing of Gram Negative Bacteria.

Two phenomena have to be considered in any attempt to explain the observed variations in the pattern of serum killing of gram negative bacteria. What serum factors limit the magnitude of the bactericidal activity and why it is that some bacteria are killed by one serum and not by another.

While no investigations were made in the present study to determine which serum factors limited the bactericidal activity for individual strains of bacteria, several observations did suggest that in some instances sensitiser was limiting and in others that it was the level of complement. The bactericidal activity of fowl serum for E. coli Lilly was apparently limited by the availability of sensitiser since during an absorption with E. coli Lilly the haemolytic complement level was reduced by 75%, while the bactericidal titre for the absorbing strain was unaltered. However, with lizard serum the level of complement appeared to limit the overall extent of killing since a reduction in the haemolytic complement level from 40 to 25 haemolytic units/ml. of serum led to a 50% reduction in bactericidal activity. These observations support the findings of Landy et al., (1962) that either sensitiser or complement may limit the magnitude of the bactericidal activity of normal serum.

There now seems little doubt that the natural sensitisers may arise in response to stimulation by antigens absorbed from the digestive system (Michael et al., 1962; Sterzl et al., 1962). The former workers also reported that the level of sensitisers for S. typhi, E. coli and a Shigella sp. were different in the sera of the mouse and the rat. Differences in the level of sensitiser from one preparation of pooled serum to another could thus explain the observed variations in the magnitude of killing. However, the presence in a serum of sensitisers able to fix complement in the presence of a particular bacterium does not inevitably lead to the death of that bacterium. In Chapter 3 it was shown that in the presence of S. typhimurium C5 pig serum complement was fixed but the serum was not bactericidal for this particular strain. This observation indicates that some sensitisers, although fixing complement in the presence of their homologous antigen (bacterium), are unable to initiate the reactions which lead to the ultimate death of the cell. Thus while it may well be that some bacteria are not killed by a particular serum because sensitisers are absent, complement fixing sensitisers can be present and yet killing may not occur. The possibility that S. typhimurium C5 is for some reason completely refractory to the complement sensitiser bactericidal system cannot be considered here as this bacterial strain is killed by lizard serum. A possible explanation of this phenomenon may be furnished by considering the mode of action of complement as proposed by Wardlaw (1963, 1963a). He proposed that in the first stage of the serum destruction of gram negative bacterial cells sensitiser combined with an O-antigen on the bacterial cell wall. This combination had two effects, the overall negative charge on the cell was reduced and an anchoring point was provided for complement adsorption. Complement then combined either with the lipoprotein adjacent to the antigen site or it formed a bridge with one end on the sensitiser and the other on the cell wall lipoprotein. Complement was then assumed to bring about distortion of the membrane which led to the death of the cell.

If the concentration of sensitiser for S. typhimurium C5 in normal pig serum was such that the entire bacterial cell surface was covered with sensitiser, then although complement would be fixed it is conceivable that due to steric hindrance a second attachment to the lipoprotein of the cell wall would be impossible. Thus although the bacteria would fix complement in the presence of sensitiser they would not be killed.

This situation appears unlikely to be important here since normal pig serum is bactericidal for S. typhimurium strain M206 but not C5, both of which share similar Kauffmann-White somatic antigens which Michael et al.,(1962) clearly showed were the sites on the bacterial cells towards which the specificity of the bactericidal sensitisers were directed. If the concentration of sensitisers were insufficient to inhibit the killing of S. typhimurium M206 then it seems unlikely that the concentration would be sufficiently high to inhibit the killing of the strain C5.

The inhibition of serum killing by excess immune antibody may however be due to antibody completely covering the cell surface. As the concentration of antibody in the system was decreased, then killing would be expected to occur as complement would no longer be sterically hindered from attaching to the bacterial cell wall. Such a reversal of inhibition was observed in the present study and has also been reported by other workers.

The nature of the cell surface of S. typhimurium C5 may be one of the factors determining whether or not it will be killed by a particular serum. If the participation of complement in the serum killing of gram negative bacteria is as proposed by Wardlaw (1963, 1963a), then the molecular dimensions of complement and/or sensitiser could be of importance in determining whether a particular bacterium will be killed. If the molecular dimensions of sensitiser and/or complement were such that it was impossible for complement although fixed to the sensitiser on the bacterial cell surface to then attach to the cell wall lipoprotein, the bacterium would not be killed. Thus it might well be that lizard serum kills S. typhimurium C5 because the structure of its sensitiser and complement are such that the complement after being fixed to the sensitisers on the cell surface can still unite with the cell wall lipoprotein. On the other hand, the molecular dimensions of the pig serum complement and/or sensitiser may be such that this union is sterically impossible.

There is no experimental evidence to support the validity of this hypothesis for bacterial resistance to serum killing. However, it was shown that there is a difference between the complement complex in pig and lizard sera. The optimum temperatures for both the haemolytic and bactericidal activities of the two sera differed as did the range of temperatures over which the sera were active in these reactions. As these variations in optimum temperature were observed in the haemolytic reaction where the interactions of the different complement complexes with a common antibody were measured they reflect differences in the nature of the complement complex. Other workers including Cushing (1945 a and b) and Brumfield and Pomeroy (1959) have also reported that all or part of the complement complexes derived from different species of animals are not mutually interchangeable in the lysis of sensitised erythrocytes.

It thus seems reasonable to assume that species variations in the nature of the normal serum sensitisers or of the complement complex could be a factor in determining whether serum killing of gram negative bacteria will occur.

In view of the ubiquity of the complement mediated bactericidal system among vertebrate animals, a study was made to determine if the serum of an invertebrate animal also possessed bactericidal activity.

Comparison of the Yabbie Serum Bactericidin with the Vertebrate Complement Mediated Bactericidal System.

In the latter part of this study an investigation was made of a bactericidin in normal yabbie serum which was lethal for gram negative bacteria. Evidence was obtained which suggested that this bactericidin differed from the complement mediated bactericidal system found in vertebrate sera.

In Chapter 6 results were presented which showed that yabbie serum was bactericidal for one of the nine gram positive and three of the forty four gram negative strains of bacteria which were screened for killing by this serum.

A feature of the bactericidal activities of the vertebrate sera (Chapter 3) was the constant presence of bactericidal activity for the rough strain E. coli Lilly. This strain, however, was not killed by yabbie serum.

Absorption of yabbie serum with a suspension of a gram negative bacterium depleted the bactericidal activity only for gram negative bacteria having no effect on the bactericidal activity for the gram positive strain, and vice versa. Thus yabbie serum possesses at least two bactericidal systems, one killing gram positive and the other gram negative strains of bacteria. Little work was done with the gram positive bactericidin because the emphasis of this study was on attempting to define the nature of serum killing mechanisms for gram negative bacteria.

Absorption tests indicated that the same factor killed the two gram negative strains examined even though they were apparently unrelated. The bactericidin was adsorbed to strains of gram negative bacteria for which it had no bactericidal activity and homologous or heterologous lipopolysaccharide inhibited alike the bactericidal activity of the serum for gram negative bacteria. These observations suggested that the yabbie serum bactericidin may adsorb to the gram negative bacteria through some common constituent of the lipopolysaccharide molecule. No information was obtained to suggest why it was that adsorption of the bactericidin to some strains of gram negative bacteria led to their death while adsorption to other strains produced no apparent ill effects. This enigma may be clarified if the bactericidin can be obtained chemically pure and its nature and mode of action ascertained.

The chemical purification procedures described in Chapter 8 gave some further clarification of the nature of the bactericidin. Haemocyanin, which constituted more than 95% of the total serum proteins, was devoid of bactericidal activity for the gram negative bacteria. Whether the bactericidin was a single or multi-component system was not conclusively demonstrated. However, in all the chemical manipulations bactericidal activity was found in separate aliquots with no increase in bactericidal activity by the recombination of fractions which suggested that the bactericidin was a single component system - possibly a low molecular weight peptide.

A surprising feature of the yabbie serum bactericidin was the constant level of activity in different serum preparations. The bactericidal titre was the same in sera from single animals and the pools obtained from twenty to one hundred animals. The level of activity was independent of the size and the age of the animals, the locality in which they were captured and the time of the year when they were bled. This constancy in the bactericidal titre contrasted strikingly with the observations on the vertebrate sera in which the magnitude of the bactericidal activity for any one bacterial strain often varied from one serum pool to another (Table 5).

The optimum temperature for the complement mediated bactericidal system in vertebrate sera was found to approximate to the body temperature of a homeothermic animal, and to the environment of a poiklicthermic animal. No such relationship was found with the yabbie serum bactericidin. The optimum activity temperature (35°) was far above that which the animal was ever likely to encounter in nature and still survive whilst there was little or no activity at those temperatures which the animal would normally encounter. This observation does not, however, detract from the bactericidin having in vivo function since there are many examples known where the optimum temperature for an enzyme is greatly in excess of the temperature at which the animal normally lives. Baldwin (1959) cited the example of a digestive proteinase of Tethyum (seasquirt) which over a period of two hours had an optimum temperature of 50°. This temperature was well above the thermal death point of the species which normally lives at temperatures in the neighbourhood of 15°. However, the optimum temperature for this enzyme when determined over a period of 55 hours was about 20° suggesting that the optimum activity temperature of the enzyme was, in fact, adjusted to the biological requirements of the animal.

Throughout this study an in vitro phenomenon has been investigated and no evidence has been produced to suggest that the bactericidin is active in vivo. The studies just discussed for the digestive proteinase of Tethyum suggest, however, that the observed high optimum temperature of the yabbie serum bactericidin compared with the temperature of its environment does not discount an in vivo function.

There now seems little doubt that vertebrate serum bactericidal activity arises as the result of antigenic stimulation particularly from the gut flora and from food antigens (Sterzl et al., 1962; Michael et al., 1962). If the yabbie serum bactericidin was also produced in response to antigenic stimulation then serum from individual animals would be expected to show variations in activity. Since there was no observed variation in the level of bactericidal activity in more than 20 pooled serum preparations and several individual sera, the possibility has to be considered that the bactericidin is a constitutive serum component.

In a further effort to determine whether the yabbie serum bactericidin was produced in response to an antigenic stimulation, the demonstrable immunological response of the animals to the injection of different antigenic substances was studied. When yabbies were injected with bacteria, bacteriophage or sheep red cell stroma, no demonstrable serum immune response could be observed ten days after the last injection. No comprehensive study of the possible development of an immune response at different time intervals was made since the primary purpose of the present investigation was to purify and identify the bactericidin already present in the serum.

Despite the inability to demonstrate anything resembling a vertebrate type primary or secondary antibody response, the yabbie does, however, possess some type of recognition mechanism since it appears to differentially clear particles from its circulation. Bacteria were no longer detectable in the circulation 24 hours after injection, whereas phage was still present 73 days after injection.

Two interpretations of these observations appear possible. The yabbie may remove particles from its circulation by a filtration mechanism based on particle size or it may have a vertebrate type phagocytic system involving opsonins and a reticulo-endothelial system. If yabbies do possess an active phagocytic system phage may not be cleared from the circulation because serum factors required for its opsonisation are absent. There is no experimental evidence at present to support or refute the validity of either interpretation. Clarification of this point must await further definitive experiments in the future.

Similarities Between the Yabbie Serum Bactericidin and Other Serum Bactericidins.

Serum bactericidal substances have been described which have similar properties to the yabbie serum bactericidin. Bactericidal substances have in recent years been reported in the blood of several insect species (Frings et al., 1948; Stephens and Marshall, 1962). Unfortunately these bactericidins were not fully characterised but both appeared to be of low molecular weight and that described by Stephens and Marshall (1962) was claimed to withstand heating at 100° for up to five minutes without appreciable loss of activity.

Skarnes and Watson, (1957) in reviewing the literature relating to the description of antibacterial factors in vertebrate sera and tissue fluids described several factors which were very similar to the yabbie serum bactericidin. These substances were of relatively low molecular weight and were protein or polypeptide in nature. These factors were, however, active principally against gram positive bacteria having little or no activity against gram negative species.

The work presented in this thesis has thus expanded the range of vertebrate animals in whose sera the complement mediated bactericidal system has been demonstrated. Yabbie serum was also bactericidal for some species of gram negative bacteria but the activity did not appear to be mediated by complement and a sensitiser.

This investigation has thus contributed to the knowledge of the phylogenetic distribution of serum killing mechanisms. It remains for future work to ascertain at what period of evolution the complement mediated bactericidal system was evolved.

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