THE BIOLOGICAL EFFECTS AND PROPERTIES OF K ANTIGENS OF *ESCHERICHIA COLI*

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ABSTRACT

The role of the K antigen in affecting C' sensitivity, susceptibility to phagocytosis and the virulence of Escherichia coli for man and mice was investigated. Evidence was obtained on the mechanism by which K antigens affected the pathogenicity of the bacteria.

As the K antigen content of strains of E. coli increased bacteria became more resistant to C' and less susceptible to phagocytosis. Bacteria which were resistant to C' were generally more virulent for mice than C' sensitive strains.

When sheep red cells were treated with K antigen they became resistant to lysis by C' and anti red cell antibody. This was due in part to the prevention of anti red cell antibody binding and also to the inhibitory effect of K antigen on C' at some stage subsequent to the union of antibody with antigen. The ability of K antigens to inhibit the agglutination of red cells by anti red cell antibody was due in the main to the effect of the K antigen on lattice formation although its ability to inhibit the union of antibody with antigen may have affected agglutination to a lesser extent.

From the results it was suggested that K antigens prevented anti O antibody from combining with the bacterial

cell and thus caused bacteria to be resistant to the opsonisation and to sensitisation to C' by anti O antibody.

Anti K antibody was relatively ineffective as a bactericidin compared with anti O antibody. The reason for this may be the distance from the lipopolysaccharide at which C' was activated by the K antigen-antibody complex. Anti K antibody was capable of opsonising bacteria which were resistant to opsonisation by anti O antibody.

The ability of K antigens to inhibit agglutination was related to the size of the antigen and not to the charge, although change may be a necessary contributory factor.

K antigens were also involved in the ability of E. coli to cause renal involvement in urinary tract infections in man. It is likely that K antigen acts by preventing two of the body's defence mechanisms, C' and phagocytosis, from killing bacteria in the host tissues.

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CONTENTS

ABBREVIATIONS USED IN THE TEXT

AB	- Antibody
AG	- Antigen
AS	- Antiserum
C^{9}	- Complement
D.E.A.E.	- Diethylaminoethyl
E.W.L.	- Egg white lysozyme
m.c.	- Millicurie
N.H.S.	- Normal human serum
O.D.	- Optical density
P.B.S.	- Phosphate buffered saline
R.E.S.	- Reticulo-endothelial system

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I. INTRODUCTION AND SURVEY OF PAST WORK

Introduction

The experiments reported here progressed from an initial investigation which was to determine why smooth strains of Escherichia coli of various serotypes differed in their sensitivity to the bactericidal action of human serum.

K antigens were shown to affect the sensitivity of bacteria to killing by C' and further experiments were carried out to resolve the mechanism by which they acted. The physical properties of the antigens were examined to see how they were related to their biological activity.

The relation between sensitivity to killing by C', susceptibility to phagocytosis and mouse virulence was investigated and a mechanism proposed by which they could be related. K antigens were also involved as factors contributing to the ability of E. coli to cause renal tract infections in man.

The term K antigen

After the Salmonella had been classified according to their O antigens an attempt was made to classify the coli group in the same way. Bacterial agglutination tests were used as the means of identifying different O antigens and it was found that many strains of E. coli possessed a surface antigen which prevented agglutination by anti O antibody.

These bacteria became O agglutinable after they had been heated at 100°C for one hour and antibodies directed against these surface antigens could be demonstrated in the sera of animals that had been injected with live but not heated bacteria. The antigen was therefore considered to be heat labile and was called the L antigen (Kauffmann, 1954).

Other workers found that O inagglutination of E. coli could be caused by an antigen which was not as heat labile as the L antigen. Bacteria possessing this antigen were not rendered O agglutinable unless they were heated at 120°C for 2½ hrs. This type of antigen was called an A antigen and usually existed as a visible capsule.

The term B antigen was proposed for those antigens which were responsible for O inagglutination but were intermediate between the L and A types in their heat lability. B antigens were like L antigens in that bacteria containing them were rendered O agglutinable after heating for 1 hour at 100°C. Like A antigens and unlike L antigens B antigens were still able to absorb anti B antibody after being heated at 121°C for 2 hours (Edwards and Ewing, 1962).

When the serological diagnostic scheme for typing strains of E. coli was being devised Kauffmann (1947) sought to simplify it and he proposed the term K antigen to include the L, A and B types of antigens. K antigens were defined as heat labile somatic antigens responsible for O inagglutination.

The three types of K antigens were distinguished by their differing heat labilities and their sensitivities to destruction by alcohol and hydrochloric acid. The Vi antigen of Salmonella typhi had been shown to be responsible for O inagglutination of this bacterium prior to Kauffmann's proposal of the term K antigen (Felix and Pitt, 1934b). The term K antigen has been generally accepted although it has been criticised on the basis of priority, the term Vi antigen was proposed first, and choise since K stands for Kapsel (capsule) and not all K antigens occur as capsules (Wilson and Miles, 1964). The Vi antigen is like the K antigens of the B type (Kauffmann, 1954) and can be considered to be no more than an example of these types of antigens. The term Vi antigen has been used by other authors to specifically refer to the K antigen of S. typhi and is used here in this way as suggested by Wilson and Miles (1964).

Spaun and Bentzon (1957b) demonstrated that a Seitz filtered supernatant from a heated culture of S. typhi was still capable of stimulating an anti Vi antibody response. This bacterial extract was also capable of sensitising red cells to agglutination by anti Vi antibody (Spaun, 1952). These observations confirmed those of Smith (1938) who found that the Vi antigen was not destroyed by boiling.

Ørskov and Ørskov (1968) reached the same conclusion as Spaun and Bentzon that K antigens were not heat labile when

the effect of heating K antigens on their serological reactivity tested by precipitin reactions and by passive haemagglutination was examined.

Kauffmann (1947) thought that when the chemical structure of the L, A and B antigens had been determined it would confirm the differences between the three types.

The chemical nature of some of these antigens is now known. To date most have been found to be acid polysaccharides apart from K88 which was a protein.

The acidic nature of the polysaccharide may be due to hexuronic acid, neuraminic acid, hexosaminuronic acid or phosphate. K antigens of the L, A and B types have been shown to be acid polysaccharides (Lüderitz et al., 1968).

The Vi antigen is also an acid polysaccharide composed mainly of N-acetyl-D-galactosamine uronic acid (Lüderitz et al. 1968) confirming the similarity between this antigen of S. typhi and the K antigens of E. coli.

The suggestion by Ørskov and Ørskov (1968) that some antigens designated as L may be B type antigens that are so easily eluted from the bacteria they would be designated L types seems unnecessarily contrived.

The chemical examination of K antigens of the L, A and B types have proven them to be chemically more similar than different. The suggestion by Kauffmann (1947) that chemical studies should serve to differentiate between the three groups

has in fact united them.

The term K antigen is used for the surface antigens of E. coli responsible for O inagglutination. The majority of these antigens that have been examined to date have been found to be acid polysaccharides. When K antigens are examined by bacterial agglutination tests they appear to be heat labile, they are in fact eluted from the cell when the bacteria are heated rather than destroyed.

Bactericidal action of complement

Site and mechanism

As long ago as 1893 Buchner described how the bactericidal activity of fresh serum against certain bacteria was lost after heating at 56°C. The serum of guinea pigs that had been immunised with heat killed cells was shown to be lytic for cholera vibrios by Pfeiffer in 1894, in this case the bactericidal power was specific for these organisms. In 1895 Bordet showed that the bactericidal and lytic power of an immune serum against cholera vibrio depended on two factors one being heat labile and the other being heat stable (Humphrey and White, 1964).

The heat stable fraction of Bordet was antibody and the heat labile fraction was C'. For C' to destroy cells antibody must first be bound, the C' sequence is then activated by the bound antibody. For cell destruction to

occur all 9 components the C' cascade are necessary (Muller-Eberhard et al., 1966).

Borsos et al. (1964) found that lesions were visible in red cells that had been treated with anti Forssman antibody and C'. These lesions were holes in the cell membrane 80-100 Å in diameter and were considered to be the site of cell damage by C' responsible for the destruction of the cell.

Lesions of a similar size in the surface of E. coli that had been exposed to C' and antibody were shown by Dourmashkin and Humphrey (1967). Blanden et al. (1967) isolated lipopolysaccharide from a Veillonella sp. exposed this to C' and demonstrated the characteristic C' lesions in it. The site of C' action in bacteria therefore seems to be the lipopolysaccharide component of the bacterial cell wall.

The polysaccharide component was not necessary for C' lesions to be demonstrable in extracted endotoxin. Mergenhagen et al. (1968) treated lipopolysaccharide from a wild type of Salmonella minnesota and heptoseless, O antigen polysaccharide deficient endotoxin from a mutant strain with C'. Lesions were demonstrable in both.

Humphrey and Dourmashkin (1969) found that the C' lesions made in E. coli lipopolysaccharide that had been absorbed onto carbon coated electron-microscope grids could be extracted with chloroform-methanol-acetic acid. This suggests that C' acts on the lipid component of the lipopolysaccharide of the gram negative cell wall.

Once these C° dependent lesions have been made in the lipopolysaccharide lysozyme present in normal serum is considered to be able to reach the underlying mucopeptide and to digest it thus promoting bacteriolysis (Glynn, 1969).

Cell wall structure and complement sensitivity

The sensitivity of gram negative bacteria to C° is not characteristic of any particular group of organisms but varies from strain to strain. Thus different strains of E. coli may be resistant or sensitive (Mackie and Finkelstein, 1932).

The smooth-rough mutation described by Arkwright is associated with an increased sensitivity to the bactericidal action of C° (Wilson and Miles, 1964). This mutation is now considered to be due to loss of sugars from the O antigen polysaccharide side chains (Luderitz et al., 1966).

Nelson and Roantree (1967) derived serum sensitive strains of S. typhimurium and S. enteritidis from serum resistant strains. They found that loss of sugars from the lipopolysaccharide was invariably associated with an increased sensitivity to C°. The reverse was not true, bacteria could become serum sensitive without there being any detectable differences in the sugar composition of the cell wall.

Wardlaw (1964) found that a smooth serum resistant strain

of E. coli contained nine times more lipopolysaccharide than a serum sensitive rough E. coli.

Increased endotoxin content, measured biologically, of enterobacteria of the same serotype was associated with increased resistance to C' by Michael and Landy (1961). In cases where differences in C' sensitivity were not explicable in terms of endotoxin content there were unspecified differences in either the capsular or other antigens.

The Vi antigen of S. typhi was shown to be the agent responsible for O inagglutination by Felix and Pitt (1934b). Prior to this O inagglutinable strains had been shown to be more sensitive to serum than O agglutinable strains (Felix and Olitzki, 1926).

Nagington (1956) compared strains of S. typhi for their sensitivity to the bactericidal action of C' and antibody. He found that as the Vi antigen content of strains increased both anti Vi and anti O antibodies became less effective at sensitizing bacteria to C'.

Muschel et al. (1958) compared the Vi antigen content of strains of S. typhi by making HCl extracts of the bacteria and measuring the extent to which they inhibited the agglutination by anti Vi antibody of red cells sensitized with Vi antigen. They confirmed earlier work showing that the more Vi antigen bacteria contained the less sensitive strains were to killing by antibody and C'.

The evidence relating the C' sensitivity of strains of E. coli to their K antigen content is not so well documented. Sjostedt (1946) found that 25% of strains of E. coli which possessed K antigens were more resistant to C' than the K⁻ variants derived from them. Arguing from the findings with the Vi antigen it is not the possession of K antigens that is likely to be the determinant feature but the amount the bacteria contained. Sjostedt's experiments did not take this into account.

Muschel (1960) showed that O inagglutinable strains of E. coli were less sensitive to serum than O agglutinable strains and therefore that K antigens affected serum sensitivity. He also demonstrated that bacteria could have the same O and K serotype and differ in their sensitivity to the bactericidal action of serum.

(3) Cultural conditions affecting complement sensitivity

The way in which bacteria are grown is known to affect their sensitivity to the bactericidal action of C'.

Felix et al. (1934) showed that if S. typhi was grown at 20°C instead of 37°C bacteria became more susceptible to agglutination by anti O antibody. This was considered to be due to the reduced synthesis of Vi antigen at these temperatures. Jude and Nicolle (1952) demonstrated that the Vi antigen was not produced at 18°C by showing that bacteria

lost their susceptibility to lysis by Vi specific phage after they had been grown at 18°C instead of 37°C. Muschel et al. (1958) found that S. typhi grown at 18°C or 41.5°C were more sensitive to killing by C' than bacteria grown at 37°C. Strains of E. coli grown at 14°C or 45°C instead of 37°C became more sensitive to C' (Muschel, 1960). When a strain of Paracolobactrum ballerup which produced a Vi antigen serologically identical to the Vi antigen of S. typhi was grown at 37°C it was resistant to the bactericidal action of C'. After incubation at 41°C cells became serum sensitive. Associated with this change was a reduction in the amount of Vi antigen produced. No phenotypic conversion to C' sensitivity was demonstrable in this case when bacteria were grown at 17°C (Osawa and Muschel, 1964a). Maaloe (1948b) showed that S. typhimurium became more sensitive to killing by C' if the concentration of the carbon source in the growth medium was increased and if the nitrogen in the medium was changed from an organic to an inorganic source.

The addition of certain compounds to the culture medium can affect the resulting bacteria's C' sensitivity. Phloridzin is one such compound (Maaloe, 1948a). Diphenylamine is another (Feingold, 1969). In the latter case Feingold suggested induced C' sensitivity was because synthesis of the O specific polysaccharide side chains was reduced. The situation was thus a phenotypic mimicking of

the smooth-rough mutation. He did not mention K antigens although these were known to affect C^9 sensitivity. The effect of diphenylamine was investigated further in section VIII.

Rowley and Wardlaw (1958) showed that if log and lag phase bacteria were compared the actively growing cells were the more sensitive of the two to C^9.

When Paracolobactrum ballerup was grown in inactivated guinea pig serum instead of broth it became sensitive to the bactericidal action of C^9 (Osawa and Muschel, 1964a). This may reflect differences in cell wall synthesis due to the "culture medium" used.

Bacterial virulence in experimental infections

Arkwright found that rough colonial variants of Salmonella were less virulent than the smooth strains from which they were derived (Wilson and Miles, 1964). This mutation is now known to be due to loss of the O specific polysaccharide side chains from the lipopolysaccharide in the gram negative cell wall (Luderitz et al., 1966). The term S-R mutation although originally used to describe colony morphology is now used in a general sense to describe the loss of surface antigens without necessarily meaning that the mutant colonies look rough. Besides being less virulent than the

parent strain rough mutants were also more susceptible to the bactericidal action of serum (Wilson and Miles, 1964).

Nelson and Roantree (1967) isolated serum sensitive mutants of S. typhimurium and S. enteritidis from serum resistant strains by selecting for penicillin resistance. This method of deriving serum sensitive variants had been described by Michael and Braun (1958). Although loss of sugars from the lipopolysaccharide was invariably associated with decreased virulence and increased sensitivity to C^9 the reverse was not true. Variants could be isolated that were C^9 sensitive and avirulent without there being any detectable change in the sugar composition of the lipopolysaccharide. Variants were also isolated that were avirulent and C^9 resistant, or C^9 sensitive and virulent without there being any change in the sugar composition. It would therefore appear that other factors besides O antigen structure affect the virulence and C^9 sensitive state of salmonellae independently. Nelson and Roantree suggested some of these changes in virulence and C^9 sensitivity may have been due to alterations in the chemistry or structure of the O antigen that were too small to detect by the methods they used.

The possibility was examined that the virulence of the serum sensitive mutants in normal mice was because of the lack of normal antibody in these animals. Immunisation with heat killed bacteria protected against both serum sensitive

and resistant strains (Ornellas et al., 1970). The virulence of the C^9 sensitive strain was therefore not due to host factors and was a property of the bacteria. Maaloe (1948a) found that C^9 resistant strains of S. typhimurium were more virulent for mice than C^9 sensitive strains.

Felix and Olitzki (1926) showed that O inagglutinable strains of S. typhi were more resistant to killing by serum than O agglutinable strains. Felix and Pitt (1934a) related O inagglutination to the virulence of the strain for mice. Sensitivity to serum and mouse virulence of S. typhi were therefore related.

Felix and Pitt (1934a) injected mice intraperitoneally with 10^8 bacteria, without a mucin adjuvant, and found that O agglutinable strains did not kill any of 10 mice injected whereas O inagglutinable strains killed 8 or 10 out of 10 mice. Most mice died within 48 hours. The term virulence used to describe these results was criticised by Kauffmann (1954). He thought that because of the large numbers of bacteria used and the short time to death the test should be called a toxicity test.

The Vi antigen was identified as the agent responsible for O inagglutination and hence resistance to C^9 and increased virulence in 1934 (Felix and Pitt, 1934b). The mere possession of the Vi antigen did not make strains virulent, the amount was important. The amount of Vi antigen in

different strains of S. typhi was measured by testing the ability of different bacteria to absorb anti Vi antibody from antisera. O inagglutination and mouse virulence were shown to be related to the Vi antigen content of the strain (Felix and Pitt, 1951).

The use of an intraperitoneal route for injecting bacteria into mice without mucin made it necessary for Felix and Pitt (1934a & b) to use very large challenge doses. The differences between virulent and avirulent strains was judged by the number of mice killed by a standard dose usually of about 10^8 bacteria.

Landy et al. (1957a) challenged mice intracerebrally with S. typhi. In this way they were able to set up infections using small numbers of bacteria. Strains were compared which varied in their antigenic structure. The LD_{50} of a strain which possessed the O but not the Vi antigen was 5×10^6, a rough strain, i.e. one devoid of O antigen, which contained the Vi antigen had an LD_{50} of 1×10^7. Strains considered to be virulent by Felix and Pitt (1934a) which contained both O and Vi antigens had intracerebral LD_{50}'s of <200. Landy et al. (1957a) were thus able to confirm that the Vi antigen affected the mouse virulence of S. typhi. Death was due to the multiplication of organisms in the brain and mice died when the bacterial concentration reached $10^7 - 10^8$.

Felix and Pitt (1934b) showed that anti Vi antibody raised by active immunisation or administered passively protected against challenge with O inagglutinable strains of S typhi. Anti O antibody did not give as good protection. Spaun and Bentzon (1957a) confirmed the findings that anti Vi antibody gave better protection than anti O antibody when large challenge doses of S. typhi were used. They found significant protection by anti O antibody against small challenge doses and suggested anti O antibody might therefore be important in immunity from natural infections.

Landy et al. (1957b) showed that anti Vi antibody raised actively against intraperitoneal injections or given passively intraperitoneally protected mice against intracerebral challenge with S. typhi. Anti O antibody had little protective effect. It was suggested that this might mean the blood brain barrier did not exist in mice since antibody must have been reaching the brain from the blood. Another explanation is possible. It had been suggested that in the case of mice intracerebrally infected with Bordetella pertussis when the number of bacteria in the brain reached about 10^5 the blood brain barrier broke down and antibody present in serum became available to opsonise the invading bacteria (Holt et al., 1961; Dolby and Standfast, 1961).

Bhatnager (1935) showed that O inagglutinable virulent strains of S. typhi were less susceptible than O agglutinable

avirulent strains to opsonisation by anti O antibody and subsequent phagocytosis. Anti Vi antibody was a more effective opsonin than anti O antibody for O inagglutinable S. typhi (Felix and Bhatnager, 1935).

Resistance to opsonisation by anti O antibody and subsequent phagocytosis was therefore related to the Vi antigen content and virulence of strains of S. typhi. The ability of anti Vi antibody to opsonise O inagglutinable strains parallels the protection afforded by this antibody in experimental infections with virulent S. typhi.

The evidence associating the K antigens of E. coli with the virulence of the bacteria is not as well documented as the evidence for the Vi antigen of S. typhi.

Sjostedt (1946) compared K^+ and K^- strains of E. coli by injecting graded doses of bacteria intraperitoneally without a mucin adjuvant and measuring the LD50. In general it took seven times more K^- cells than K^+ cells to kill mice.

Large doses of bacteria needed to be injected to kill the mice. The LD_{50} of one K^+ strain was 3×10^7 and that of the K^- variant was 3×10^8.

The same criticism can be levelled at these results as those of Felix and Pitt (1934a). Because such large challenge doses were used and since mice died often within 24 hours and nearly always within 48 hrs. death was probably due to endotoxic shock and not because of a genuine infection. Like Kauffmann (1954),

Sjostedt (1946) considered the test to be a "toxicity" test although it was the same test as Felix and Pitt (1934a) used to determine virulence.

Another criticism of the results of Sjostedt is that when S. typhi was examined it was not the possession of Vi antigen that was related to virulence but the amount in the cell. Comparison of K^+ and K^- strains did not take this into account.

Sjostedt (1946) also found that K^- strains were more susceptible to phagocytosis than K^+ strains. In the same way as with S. typhi anti K antibody was shown to be a more effective opsonin than anti O antibody for K^+ strains.

Experiments similar to those of Sjostedt were performed by Wolberg and De Witt (1969), except mice were challenged intraperitoneally with a mucin adjuvant. The LD_{50}'s of two strains of E. coli serotype O2:K1(L) were <10 and the LD_{50}'s of the K^- variants were 10^7 and 10^8. The K^- strains were more susceptible to phagocytosis than the K^+ strains and anti K antibody was a better opsonin for the K^+ strains than anti O antibody. Anti K antibody protected passively against challenge with the K^+ strain whereas anti O antibody had a minimal effect.

Rowley (1954) found that C^9 resistant strains of E. coli were more virulent for mice, challenged intraperitoneally with a mucin adjuvant, than C^9 sensitive strains. A characteristic

of virulent strains was their ability to multiply from a small inoculum. Medearis et al. (1968) examined the relation between the structure of the O antigen of E. coli and the virulence of the bacteria. The state of the K antigen was not considered. A mutant unable to synthesise O specific side chains containing colitose had an LD_{50} of 2×10^6. The LD_{50} of a mutant unable to synthesise total specific side chains and core polysaccharide was 2×10^7. The original strain from which the mutants were derived had an LD_{50} of 2.5×10^4. Correlated with this loss of virulence associated with different O antigen structure was an increased susceptibility to phagocytosis.

The relation between killing by C' in vitro and killing in vivo was examined by Roantree and Pappas (1960) and Roantree and Collis (1960) in an attempt to show killing by C' was important in vivo as a defence mechanism. Roantree and Pappas (1960) injected paired serum resistant and serum sensitive bacteria into rabbits and found that although viable cells of both strains rapidly disappeared from the circulation the C' resistant strains did not disappear as quickly as the sensitive strains. This experiment did not distinguish between killing in the circulation by serum factors and removal of cells from the circulation by the reticulo-endothelial system.

Roantree and Collis (1960) put strains of E. coli of differing sensitivities to C' in chambers, designed so that

serum factors but not cells could reach the bacteria, in the guinea pig peritoneum. Bacteria which were C⁹ sensitive *in vitro* were killed *in vivo*. Strains which were C⁹ resistant *in vitro* usually multiplied *in vivo* and strains of intermediate C⁹ sensitivity varied in their *in vivo* response.

It was concluded that C⁹ was effective *in vivo* as a bactericidal mechanism.

Human pathogenicity of *Salmonella typhi*

Findlay (1951) found that the mouse virulence of strains of *S. typhi* isolated from a mild outbreak of typhoid in which there were no fatalities was less than that of strains isolated from a severe outbreak in which 5 people died. It was therefore suggested that mouse virulence paralleled virulence of the bacteria for man.

Felix and Anderson (1951) came to the same conclusion. They found that strains isolated from a mild outbreak of typhoid in Oswestry were of low virulence for mice.

Because the Vi antigen had been shown to affect the mouse virulence of *S. typhi* and anti Vi antibody gave better protection for mice than anti O antibody a field trial was organised in Yugoslavia in which two vaccines were compared for their effectiveness at protecting against typhoid fever.

One of the vaccines was a heat killed phenol preserved vaccine which did not retain the Vi antigen. This gave

better protection than was given by an alcohol killed and preserved vaccine which retained the Vi antigen (Yugoslav Typhoid Commission 1962). On the basis of these results it was suggested that the Vi antigen was not as important in affecting human pathogenicity of S. typhi as it was in experimental animals.

Osawa and Muschel (1964b) found that 90% of the bactericidal activity stimulated in rabbits for S. typhi by an alcohol killed and preserved vaccine was due to anti O antibody. The anti Vi antibody response was only slight. In the sera of typhoid patients the anti Vi response is of little consequence in the bactericidal reaction (Muschel and Treffers, 1956b). It was suggested by Osawa and Muschel (1964b) that the failure of the alcoholised vaccine to give better protection in the Yugoslavia field trials was because of the poor anti Vi antibody response it stimulated. They state the results do not prove the ineffectiveness of the Vi antibody in protecting humans from infection with S. typhi.

In a later field trial in Guyana (Ashcroft et al., 1967) an acetone killed typhoid vaccine which retained the Vi antigen was compared with a heat killed phenol preserved vaccine for its effectiveness at protecting against typhoid. The vaccine which retained the Vi antigen gave 88% protection compared with the 65% protection given by the heat killed phenol preserved vaccine, but there were no doubt other

differences between the two vaccines besides possession of Vi antigen.

These findings support the experimental work showing that retention of the Vi antigen increased the protection given by a vaccine. The protection stimulated by the heat killed phenol preserved vaccine is probably due to anti O antibody which Nagington (1956) found to be bactericidal against all the strains of S. typhi he examined, although it became less effective as the Vi antigen content of the strains increased.

Human pathogenicity of Escherichia coli

Kauffmann (1954) used the term pathogenic E. coli meaning bacteria isolated from pathological material. This included peritoneal pus, appendicitic material, urine and bile. Kauffmann, Knipschildt and Vahlne all examined pathogenic E. coli and compared them with faecal strains. Pathogenic strains were more likely to be O inagglutinable than faecal strains indicating the involvement of K antigens in the pathogenicity of E. coli. Kauffmann found that 69% of pathogenic E. coli but only 26% of strains from faeces were O inagglutinable.

Pathological material was also serologically more homogeneous than faecal material. 95% of bacteria isolated

from urine samples were of the same serotype but only in 27% of cases did strains isolated from faecal samples belong to the same serotype.

Roantree and Rantz (1960) found that strains of E. coli isolated from the blood were more resistant to C' than strains isolated from faeces or urine. Resistance to C' was therefore suggested to be one of the criteria necessary for E. coli to cause a generalised systemic infection in man.

Waisbren et al. (1959) reported that bacteria isolated from three cases of generalised E. coli infections in adults were more virulent for mice than any of the enteropathogenic strains with which they were compared. They were also stated to be resistant to serum.

61 strains of E. coli from animals and man were serotyped and their LD_{50}'s determined for mice by intraperitoneal injections with mucin adjuvant. The LD_{50}'s varied from <10 to 2×10^8. Strains were divided into three groups, based on their source, enteric, non-enteric non-systemic and non-enteric systemic. Generally strains from systemic infections were more virulent than strains from enteric sources.

Mouse virulence was thus related to the ability of the bacteria to cause systemic infections in animals and man (Jacks and Glantz, 1967).

Besides the evidence relating the antigenic and chemical structure of the bacterial cell wall to its virulence for man and animals other factors have been implicated or remain to be discovered.

Roantree (1967) comments that salmonellae with the same sugars comprising the cell wall and the same antigenic structure do not necessarily cause the same sort of disease. Possibly in this case differences in the cell wall structure so far undetected are occurring or other factors may be involved.

The Vi antigen of S. typhi is also found in Paracolobactrum ballerup. It does not enable this organism to cause the same disease as S. typhi.

Biozzi et al. (1964) crossed a virulent strain of S. typhimurium with an avirulent E. coli Hfr. The recombinants examined were avirulent like the E. coli chromosome donor. The susceptibility of the strains to phagocytosis by the reticuloendothelial system of mice reflected their relative virulence. The original S. typhimurium strain was slowly cleared from the mouse circulation. The E. coli strain and the recombinants were rapidly cleared. Although avirulent the recombinants possessed the same somatic and H antigens as the virulent S. typhimurium parent. At first sight this appears to indicate some other factor besides O antigen structure is

affecting virulence in this case. The authors suggest the rapid clearance of the E. coli and the recombinants is due to opsonisation by normal antibody. It therefore seems likely that undetected changes in the cell wall structure had occurred.

There are two types of E. coli infections in calves. One is a generalised infection where colostrum deprived calves died of bacteraemia. Bacteria causing this type of infection were able to grow in precolostral calf sera. Bacteria which could not grow in precolostral calf sera did not cause the disease (Smith, 1962). The other type of disease is an intestinal infection characterised by the calves suffering from diarrhoea. Diarrhoea could be produced in colostrum fed animals experimentally. Strains which caused diarrhoea when fed orally produced an enterotoxin which could be detected by its ability to dilate ligated intestine. Bacteria which did not produce enterotoxin did not cause diarrhoea (Smith and Halls, 1967).

Smith (1969) suggested that in porcine strains of E. coli enterotoxin production was controlled by a transmissible plasmid.

The ability of the organism to cause diarrhoea was not because of its cell wall structure but because of enterotoxin production. The ability to grow in the anterior small intestine was another necessary feature of the bacteria.

E. coli enteropathogenic for human babies belong to certain O serotypes (Kauffmann, 1954). These strains are also able to dilate rabbit intestine (Taylor *et al.*, 1961) and possibly a similar mechanism based on enterotoxin production exists in the production of infantile diarrhoea and neonatal diarrhoea in animals. This may be unrelated to cell wall structure and tissue invasion.

II. K ANTIGENS AS DETERMINANTS OF SERUM SENSITIVITY

INTRODUCTION

The experiments recorded in this section were to determine whether the K antigens of *Escherichia coli* affected the resistance of bacteria to killing by serum. It had previously been shown that the Vi antigen of *Salmonella typhi* affected the resistance of strains to the bactericidal action of serum (Felix and Olitzki, 1926; Felix and Pitt, 1934b; Nagington, 1956; Muschel *et al.*, 1958). K antigens, the antigens of *E. coli* responsible for O inagglutination (Kauffmann, 1947), were associated with serum resistance by Sjostedt (1946). Muschel (1960) reported that amongst strains of E. coli O inagglutinability was related to resistance to serum.

Possession of a K antigen does not necessarily make strains resistant to serum. Muschel (1960) reported that strains of the same O and K serotype may differ markedly in their sensitivity to C°. The possibility was explored that it was the amount of K antigen produced by a strain which affected its final C° sensitivity.

Hungerer *et al.* (1967) stated that K antigens isolated from a great number of *E. coli* strains had proved to be polysaccharides and in the majority of cases acidic polysaccharides. An exception was K88 which was a protein

(Ørskov and Ørskov, 1967).

It was reasoned that if the majority of K antigens were acidic polysaccharides the more K antigen a strain contained the higher should be the surface negative charge on the cell. Strains with a higher negative charge should bind more egg white lysozyme since this is a cationic protein. The amount of lysozyme bound by different strains was compared to see if the amount bound, and hence the negative charge on the cell, was related to the resistance of the strain to the bactericidal action of human serum.

A property of acid polysaccharide antigens, independent of their serological specificity or source, is their ability to inhibit unrelated agglutination systems (Ceppellini and Landy, 1963). If the K antigen content of bacteria affected the sensitivity of the strain to C^9 then extracts of serum resistant strains should be more effective at inhibiting an unrelated agglutination system than extracts of serum sensitive strains.

These two properties of K antigens, negative charge and agglutination inhibiting activity, made it possible to estimate K antigen content non specifically, i.e. independently of the K serotype, in the various strains of E. coli studied.

When serum resistant strains of E. coli were grown at 18°or 45°C they became serum sensitive (Muschel, 1960).

Strains were examined to see if this phenotypic change to a serum sensitive state was associated with a reduction in the K antigen content of the cell.

Antigen concentrations can be measured by radial immunodiffusion (Mancini et al., 1965). Four strains of E. coli with the same O and K serotype but different sensitivities to serum were suspended in saline to give the same concentration in mg. dry weight per ml. The bacteria were extracted using a homogeniser and the amounts of K antigen in the extracts were measured by radial immunodiffusion. The amount of K antigen per mg. dry weight of cells was calculated to see if the K antigen content of the strains was related to differences in serum sensitivity.

MATERIALS AND METHODS

Antisera

Haemolysin. Rabbit anti sheep red cell serum was either obtained from Staynes Laboratories or raised in rabbits as described by Kabat and Mayer (1961).

Antibacterial antisera

All antibacterial antisera were raised in rabbits according to the methods of Edwards and Ewing (1962).

Anti O sera. Bacteria were grown overnight at 37°C on nutrient agar. The cells were suspended in 0.15M saline and

heated at 100°C for 2½ hours. Heated cells were harvested by centrifugation, washed and resuspended in 0.15 M saline and injected intravenously into rabbits.

Anti OK sera. Bacteria were grown overnight at 37°C on nutrient agar, suspended in 0.15 M saline and injected intravenously into rabbits. Antisera raised against suspensions of live cells contained both anti O and anti K antibodies.

Measurement of the complement sensitivity of strains of E. coli

Bacteria. The strains used, their source and serotype are listed in Table 1. Bacteria were grown into the logarithmic growth phase at 37°C in nutrient broth in shaking L tubes (Gorrill and Needs, 1958). 0.5 ml of a 37°C overnight nutrient broth standing culture was inoculated into 5.0 ml of fresh nutrient broth and incubated at 37°C for 1 hour. The bacteria were harvested by centrifugation, washed and resuspended in water. The O.D. of the suspension was adjusted to give 1 x 10^7 organisms per ml.

Complement source. Normal human sera (N.H.S.) was used as a source of C' and antibody. Blood from one donor (C.J.H.) was taken by venepuncture, defibrinated quickly by shaking with glass beads, centrifuged and the serum (N.H.S.) stored at -60°C. The haemolytic titre of this serum as measured by Mayer's method (Kabat and Mayer, 1961) was 36 $C'H_{50}$ per ml.

Sensitivity of bacteria to C'. The method of Muschel and Treffers (1956a) was used to determine the sensitivity of a bacterial strain to the bactericidal action of C'. The C' concentration was varied and the antibody concentration kept constant by diluting N.H.S. in N.H.S. that had been heated at 56°C for 30 min. This degree of heat inactivates the C' but is not enough to affect normal IgM antibody (Michael, Whitby and Landy, 1962).

0.1 ml of the bacterial suspension was added at 1 minute intervals to a series of L tubes which contained 0.9 ml of various concentrations of C' and antibody (N.H.S. in heated N.H.S.). Bacteria were also added to a control tube with no C' (0.9 ml of heated N.H.S.). The bacterial concentration in the L tubes was 1 x 10^6 cells per ml.

The mixture of C', antibody and bacteria was incubated at 37°C for 15 minutes after which the bactericidal action of the C' was stopped by the addition of 5.0 ml of nutrient broth (Muschel and Treffers, 1956a).

Incubation of the rocking L tubes at 37°C was continued until the optical density of the heated serum control reached 0.4 to 0.5 on an absorbtiometer (Spekker) using a neutral grey filter.

The O.D. of the other tubes was then read and the C' sensitivity of a strain expressed as the number of $C'H_{50}$ units required to kill 50% of the standard inoculum (10^6 cells) in 15 minutes.

Measurement of the agglutination inhibiting activity of strains of E. coli

Bacteria were grown on nutrient agar plates overnight at 37°C and acetone dried. Standard extracts of the bacteria were made by suspending the dried cells at a concentration of 10 mg/ml in 0.15M phosphate buffered saline pH 7.2. The suspension was homogenised on ice for 3 minutes using an "Ultra Turrax" homogeniser. Bacteria and debris were removed by centrifugation at 1200 g for 30 minutes and the clear supernatant tested for inhibiting activity. A series of doubling dilutions of 0.5 ml of each extract was made in phosphate buffered saline. 0.5 ml of 5% v/v sheep red cells in buffered saline was added to the dilutions of the bacterial extract and to a control tube which contained only buffer. Extracts and red cells were incubated together at 37°C for 30 minutes. 0.04 ml of red cells that had been incubated with the bacterial extract and control cells were added to 0.2 ml amounts of doubling dilutions of rabbit anti sheep red cell serum in haemagglutination trays (W.H.O. pattern). The arrangement used was that antibody dilutions were read horizontally and extract concentrations vertically. The trays were incubated for 1 hour at 37°C and overnight at 4°C.

The agglutination inhibiting activity of the extract was taken as being the reciprocal of the dilution required to double

the minimal agglutinating dose of the anti red cell serum.

Chemical examination of standard extracts of various strains of E. coli

Extracts of acetone dried bacteria were made in 0.15M NaCl using a homogeniser as described previously. Carbohydrate estimations (Dische, 1955). Total sugar given as glucose equivalents was estimated by the Molisch reaction which detects hexoses, pentoses and methyl pentoses but not hexuronic acids or aminosugars. Total hexose, also given as glucose equivalents was measured by the Anthrone reaction. Hexuronic acids were measured by the carbazole reaction and hexosamines were measured by the method of Reissig, Strominger and LeLoir (1955).

Measurement of ^{125}I-lysozyme bound by bacteria

Crystalline egg white lysozyme (E.W.L.) (Armour) was labelled with ^{125}I by the method of McFarlane (1958). The concentration was measured by optical density ($O.D._{280}$ E.W.L. 100 mg/ml = 0.25) and the radioactivity measured in a scintillation counter.

Effect of egg white lysozyme concentration on amount bound

Two strains of E. coli one C° sensitive, WF96, and one C° resistant, WF82, were grown into the log phase harvested

by centrifugation, washed with water and adjusted by O.D. to give 5 x 10^8 cells per ml. 2.0 ml amounts of various concentrations of ^{125}I-lysozyme were added to 2.0 ml samples of E. coli, mixed and kept at 0°C for 5 min. The bacteria were then centrifuged and washed by resuspension in water 3 times. The number of bacteria present in the final suspension was measured by optical density and the radioactivity measured in a scintillation counter.

From these results and the specific activity of the lysozyme the quantity of E.W.L. bound per bacterium was calculated and expressed as fg/cell.

E.W.L. bound by different bacteria

The estimation of lysozyme bound by different strains was done in the same way. 2.0 ml of bacterial suspension was mixed with 2.0 ml of lysozyme, to give a final concentration of egg white lysozyme in the mixture of 150 µg per ml. The number of fg of lysozyme bound per bacterium was calculated as above.

Egg white lysozyme was used as a molecule with a positive charge and not because of its enzymatic activity.

Estimation of the K antigen content of four strains of the same serotype by radial immunodiffusion

Bacteria. The 4 strains of E. coli WF98, WF8, WF86, WF26, were grown on nutrient agar overnight at 37°C and acetone dried. Standard extracts of the bacteria were made in 0.15M saline using a homogeniser in the same way as for estimating agglutination inhibition activity, except that suspensions of 20 mg. dry weight bacteria per ml. were extracted.

Standard. The acid polysaccharide from E. coli WF26 was prepared from a saline extract of the bacteria by fractional precipitation with cetavlon according to the method of Hungerer et al. (1967).

Antibody. An antiserum raised in a rabbit against live E. coli WF26 was used as the source of anti K antibody. Anti O antibody was removed by absorption with E. coli WF98 that had been steamed for 2½ hrs and washed with 0.15M saline (Edwards and Ewing, 1962).

Estimation of K antigen concentrations in bacterial extracts

The method used was that of Mancini et al. (1965) Antiserum was mixed with Oxoid ion agar No. 2 in 0.15M saline to give a final concentration of 1/10 antiserum in 1% w/v ion agar. 10 ml of the agar-antiserum was pipetted onto an 8 cm square glass plate and wells 3 mm diameter, into which samples and standards were pipetted were cut in the agar.

After incubation overnight at room temperature the precipitin rings were photographed and the diameters measured. A standard curve of the square of the ring diameter against the log of the antigen concentration was drawn and the K antigen concentrations in the bacterial extracts were calculated from this.

Effect of growing strains at 45°C and 18°C on their C' sensitivity and agglutination inhibition activity

Bacteria used for estimating C' sensitivity were grown into the log phase at 18°, 37° and 45°C in L tubes from an overnight nutrient broth standing culture grown at the same temperature. Bacteria used to estimate agglutination inhibiting activity were grown on nutrient agar plates at the appropriate temperatures and acetone dried. Agglutination inhibiting activity estimations were carried out on the dried cells as previously described. Sensitivity to C' was estimated in this case by measuring the increase or decrease in viable count after 30 minutes incubation at 37°C with a 1/5 dilution of N.H.S.

RESULTS

Complement sensitivity of strains of E. coli

Different strains of E. coli varied markedly in their

sensitivities to the bactericidal action of human serum. This was true whether strains of the same or different serotypes were compared (Table 1).

For example, 0.8 $C'H_{50}$ units was sufficient to kill 50% of the standard inoculum of WF96 (07:K1). For strain WF98 (06:K13) 8 $C'H_{50}$ units were necessary to kill the bacteria to the same extent. Strain WF26 (06:K13) was unaffected by a concentration of 32.4 $C'H_{50}$ units per ml.

Carbohydrate analysis of crude extracts of strains of E. coli related to C' sensitivity

In Table 2 the strains have been arranged in order of increasing resistance to C', WF41 being the most sensitive and WF26 the most resistant. It is apparent that increased resistance to C' was associated with an increase in the amount of carbohydrate extracted per unit weight bacteria.

Hexuronic acids were only detectable in two cases. Other bacterial extracts may have contained hexuronic acids but the brown colour produced by hexoses present in the extract and concentrated sulphuric acid masked any uronic acid reaction that might have been occurring.

Egg white lysozyme bound by strains of E. coli

The effect of varying the egg white lysozyme

concentration on the amount bound by bacteria was examined (Fig. 1). Two strains of E. coli were used one of which was resistant to C⁹, the other was sensitive. For both strains the amount of lysozyme bound increased as the concentration was raised to 100 µg per ml. Above this concentration the properties of the bacteria and not the concentration used governed the amount which was bound to the bacteria.

The amount of ^{125}I labelled lysozyme bound by various strains of E. coli in the presence of an excess of lysozyme is recorded in Table 3. There were up to eight fold differences in the amounts found by different strains.

There was a significant increase in the lysozyme bound as the strains became more resistant to C⁹ indicating that the more resistant strains had a more negative surface charge ($P = < 0.05$).

The line drawn through the points in Fig. 2 is the regression line calculated from these values. The correlation coefficient for the values in Fig. 2 was 0.59. From this value P was calculated to be <0.05 (Moroney, 1967).

Agglutination inhibiting activity of standard extracts of strains of E. coli

The activities of the extracts is given in Table 1 as the reciprocal of the highest doubling dilution of the bacterial extract which would reduce the agglutination titre

of the anti sheep red cell serum used by 50%. Marked differences were found when strains of the same or different serotypes were examined.

Increased resistance to C' was associated with an increased inhibitory activity of the bacterial extract with one obvious exception which was WF70 (Fig. 3).

Measurement of K antigens by radial immunodiffusion

For four strains of E. coli serotype 06:K13 as the bacteria became more resistant to C' more K antigen was extractable from the cells (Table 4). A direct relation between C' resistance and the K antigen content of bacteria had therefore been demonstrated.

Effect of growing strains at 18° or 45°C on C' sensitivity and K antigen synthesis

When bacteria which were resistant to the bactericidal action of human serum when grown at 37°C were grown at 18° or 45°C they may be induced to become more sensitive to serum (Table 5). Associated with this phenotypic conversion to C' sensitivity was a reduction in the agglutination inhibiting activity of bacterial extracts.

Induced serum sensitivity due to growth at these temperatures is therefore related to the reduced amounts of K antigens synthesized.

Table 1. C° sensitivity, serotype and agglutination inhibiting activity of strains of E. coli

Strain No.	Serotype	C° sensitivity $C°H_{50}$ killing 50% of the inoculum	Agglutination Inhibition Activity
K235L⁻O(m)	O1:K⁻ :M	< 0.7	0
K235L⁺T	O1:K1	14	32
K235L⁺O(m)	O1:K1 :M	-	16
WF20	O2:K? :H4	32.4	64
WF98	O6:K13:H16	8	16
WF6	O6:K13:H⁻	12	64
WF86	O6:K13:H1	27	32
WF8	O6:K13:H⁻	> 32.4	64
WF26	O6:K13:H31	> 32.4	64
WF60	O6:K? :H1	9.5	32
WF90	O6:K? :H7	15	64
WF96	O7:K1 :H6	0.8	2
WF77	O8:K88:H10	0.8	0
WF70	O22:K? :H21	32.4	2
WF58	O28:B? :H⁻	1.4	0
WF16	O32:K? :H4	>32.4	64
WF88	O62:K? :H1	0.5	1
WF53	O83:K? :H27	25	32
WF95	O117:K? :H32	1.2	-
WF82	O117:K? :H27	29	64
WF61	O126:K? :H2	0.38	0
5396/38	Vi	12.2	32
# 136	Vi	-	4-8
WF38	?	11	64
MRE162	?	1.2	0
MRE600	?	< 0.7	0

Where the serotype has been recorded as ? it was unknown.

Strains K235 were provided by Dr. Goebel (Goebel, 1963).

Strain # 136 was provided by Dr. Baker (Baker et al., 1959).

Strain 5396/38 was provided by Dr. Landy (Landy, 1952).

Strains MRE were provided by Mr. Elsworth of M.R.E. Porton.

Table 2. Carbohydrate analysis of crude extracts of strains of *E. coli* related to their complement sensitivities

Strain	Serotype	C^9 sensitivity C^9H_{50} killing 50% of the inoculum	Total carbohydrate µg/ml as glucose		Hexuronic acid µg/ml
			Molisch	Anthrone	
WF41	017:K16:H18	<0.7	287	376	-
WF96	07:K1 :H6	0.7	312	480	-
WF98	06:K13:H16	7.9	450	400	-
WF86	06:K13:H1	27	425	525	-
WF82	0117:K? :H27	29	588	N.D.	65
WF20	02:K? :H4	32.4	400	325	-
WF8	06:K13:H$^-$	>32.4	625	663	-
WF26	06:K13:H31	>32.4	1000	1210	60

N.D. = not done

\- = no detectable hexuronic acid

Table 3. ^{125}I-egg white lysozyme bound by strains of Escherichia coli

Strain	Serotype	C' sensitivity $C'H_{50}$ killing 50% of the inoculum	Lysozyme bound fg per bacterium
WF61	0126:B16	0.4	31
WF41	017:K16	<0.7	108
WF77	08:K88	0.8	43
WF96	07:K1	0.8	94
WF58	028:B?	1.4	120
WF98	06:K13	8.0	134
WF60	06:K?	9.5	89
WF6	06:K13	12	140
WF90	06:K?	15	167
WF76	0128:B12	28	120
WF82	0117:K?	29	160
WF70	022:K?	32.4	56
WF20	02:K?	32.4	250
WF26	06:K13	>32.4	220

Table 4. Complement sensitivity of 4 strains of E. coli serotype 06:K13 as a function of their K antigen content

Strain	C' sensitivity $C'H_{50}$ killing 50% of the inoculum	µg K antigen extracted per mg. bacteria
WF98	8.0	8.0
WF86	27.0	14.7
WF8	>32.4*	27.5
WF26	>32.4*	51.0

* 32.4 $C'H_{50}$ units killed 20% of the inoculum of E. coli WF8. There was no demonstrable killing of WF26.

Table 5. Relation of complement sensitivity and agglutination inhibiting activity of extracts of strains grown at different temperatures

Strain	Growth temperature	Agglutination inhibiting activity	Per cent growth (+) or killing (-) after 30 minutes in 7C'H_{50} units of complement
WF60	37°	32	+74
	18°	0	-41
	45°	4	-40
WF26	37°	64	+170
	18°	0	+20
	45°	0	-50
WF38	37°	64	+120
	18°	1	-12.5
WF20	37°	64	+5
	18°	0	-30
WF82	37°	64	+20
	18°	8	-10

Fig. 1. The effect of egg white lysozyme concentration on the amount bound by bacteria.

+ - fg lysozyme bound to *E. coli* WF82.

0 - fg lysozyme bound to *E. coli* WF96.

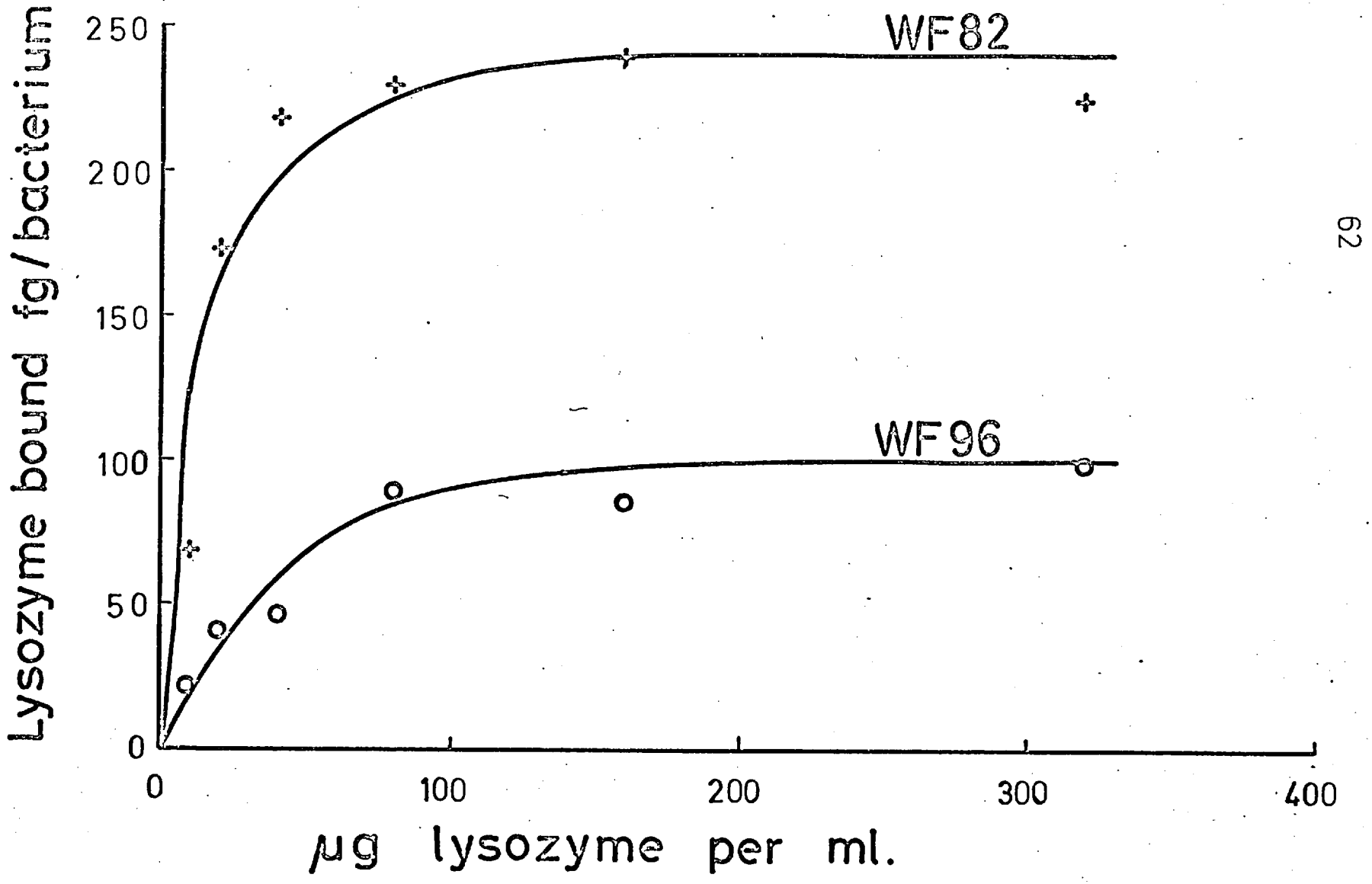
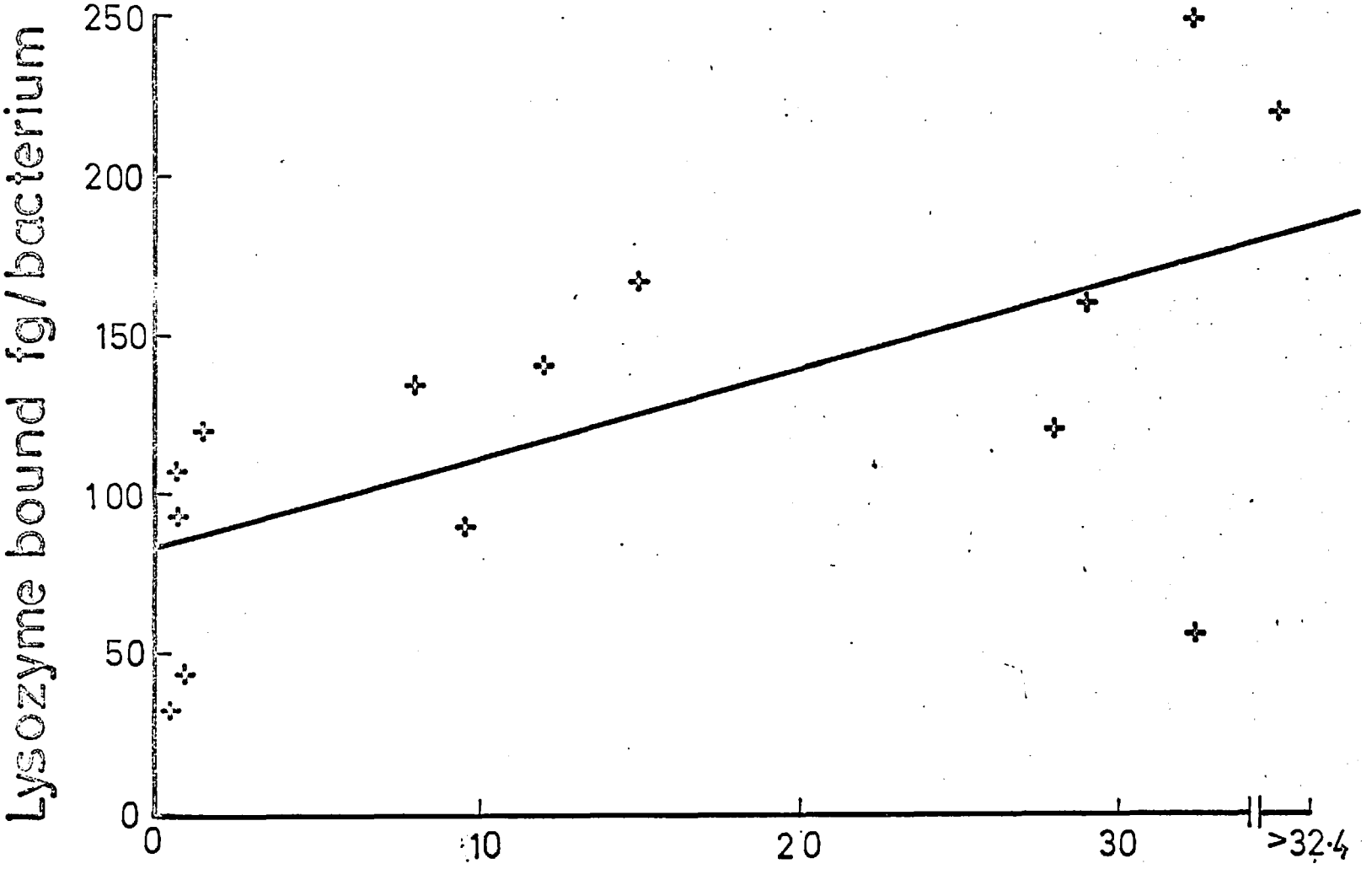
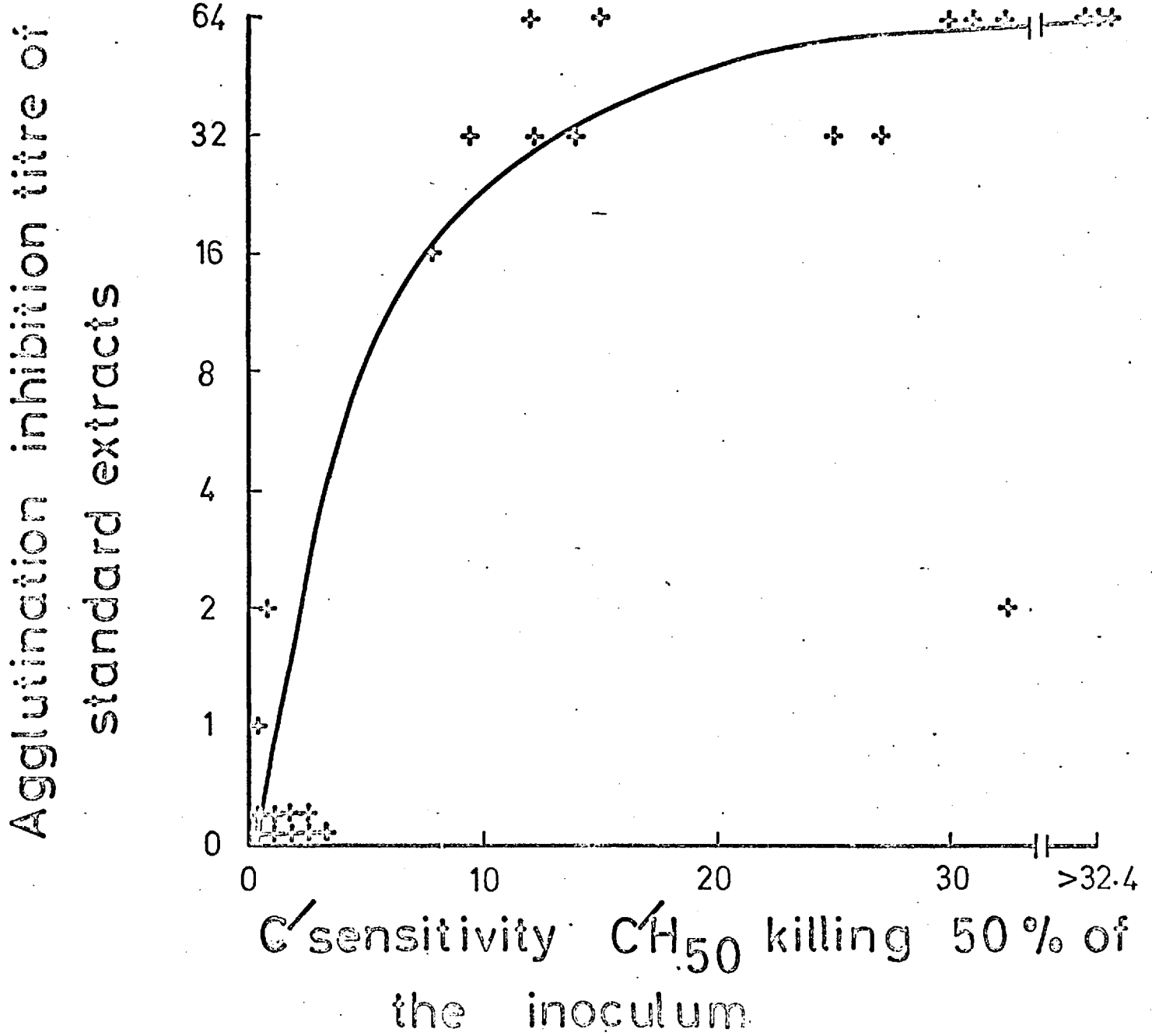

Lysozyme bound fg/bacterium
250
200
150
100
50
0
WF82
WF96
0
100
200
300
400
µg lysozyme per ml.

Fig. 2. Egg white lysozyme bound to *E. coli* as a function of C^{0} sensitivity.



C′ sensitivity. $C'H_{50}$ killing 50% of the inoculum.

Fig. 3. Complement sensitivity of strains of E. coli related to the agglutination inhibiting activity of standard bacterial extracts.


Agglutination inhibition titre of standard extracts
64
32
16
8
4
2
1
0
0
10
20
30
>32.4
C' sensitivity C'H50 killing 50 % of the inoculum

DISCUSSION

Strains of E. coli differed markedly in their sensitivity to the bactericidal action of serum. This was evident whether strains of the same or different serotypes were compared. The same conclusions were reached by Muschel (1960). The possession of a K antigen, therefore, does not necessarily make bacteria resistant to C°.

The possibility was explored that it was the amount of K antigen present in the cell which caused it to be resistant. Since the majority of K antigens have been found to be acid polysaccharides (Luderitz et al., 1968) it was reasoned that strains with more K antigen should have a more negative surface charge.

From the results obtained measuring the amount of ^{125}I-labelled egg white lysozyme that was bound to bacteria it was shown that as strains became more resistant to C° they bound more of this cationic protein. This indicates the more resistant strains have a more negative surface charge and thus supported the hypothesis that amongst smooth strains of E. coli resistance to C° was related to the K antigen content of the bacteria.

The acriflavine effect reported by Glynn and Priest (1970) to be related to the C° sensitivity of E. coli was also suggested to be due to the negative charge on the bacterial cell imparted by K antigens

The K antigen content of different serotypes of E. coli was compared by measuring the extent to which bacterial extracts inhibited the agglutination of sheep red cells by anti sheep red cell antibody. The results fitted the theory that increased resistance to C' was related to the amount of K antigen present.

The amounts of K antigen extracted from strains of E. coli of the same serotype was measured by radial immunodiffusion. The more resistant the bacteria were to killing by serum the more K antigen they were found to contain.

The carbohydrate estimations were performed on crude extracts of E. coli containing both O and K antigens. It was therefore difficult to make precise deductions from these results. However it is of interest that White (1929) observed that smooth strains of salmonella reacted more vigorously than rough strains with Molisch's reagent in a crude qualitative test. The quantitative results reported here were more likely to be detecting extra K antigens than extra lipopolysaccharide since the comparison was between smooth strains only.

When bacteria which were resistant to C' when grown at 37°C were grown at 18°C or 45°C they became C' sensitive. Associated with this induced C' sensitivity was a fall in the agglutination inhibiting activity of the extracts. Presumably

elevation or depression of the incubation temperature resulted in a decreased production of K antigen so the bacteria became less resistant to C'.

Other reports have appeared relating K antigens to the serum sensitivity of E. coli. Sjostedt (1946) compared strains of E. coli containing both O and K antigens with K^- variants derived from them. He found that 25% of the strains with a K antigen were more resistant to serum than the K^- variants. Although this indicated K antigens affected C' sensitivity it did not take into account the amount of K antigen present and this has been found to be the controlling factor. If no difference is found in the C' sensitivity of K^+ and K^- strains this cannot be taken to indicate that K antigens are not important as determinants of C' sensitivity since there might not have been sufficient K antigen present to make the bacteria resistant.

Muschel (1960) examined various serotypes of E. coli for their C' sensitivity and related resistance to C' with O inagglutination. He argued that since O inagglutination was due to K antigens these antigens were responsible for resistance to C'.

O inagglutinable strains of S. typhi were more resistant to the bactericidal action of serum than O agglutinable strains (Felix and Olitzki, 1926). Subsequently the Vi antigen was shown to be the agent responsible for O inagglutination (Felix

and Pitt, 1934b).

Nagington (1956) found that as the Vi antigen content of strains of S. typhi increased anti O antibody became less effective at sensitising bacteria to C' confirming earlier observations associating the Vi antigen with resistance to C'.

Muschel et al. (1958) compared the amount of Vi antigen in strains of S. typhi by making HCl extracts of the bacteria and measuring the extent to which bacterial extracts inhibited the agglutination by anti Vi antibody of red cells sensitised with Vi antigen. The resistance of S. typhi to the bactericidal action of C' was shown to be related to the amount of Vi antigen they contained.

Other reports have appeared in the literature showing that the amount of Vi and K antigen produced by bacteria was affected by the temperature at which the organisms were grown.

Felix et al. (1934) found that an O inagglutinable strain of S. typhi became O agglutinable when grown at 20°C. Jude and Nicolle (1952) used a bacteriophage specific for Vi antigen and showed that this antigen was not produced when the bacteria were grown at 14°C or 45°C.

Muschel et al. (1958) compared the C' sensitivity of S. typhi grown at 18°, 37° and 45°C with the amount of Vi antigen produced at these temperatures. Increased sensitivity to C' due to growth at these abnormal temperatures was

associated with a reduction in Vi antigen synthesis.

A strain of Paracolobactrum ballerup was found to be resistant to C^9 when it had been grown at 37°C. If it was grown instead at 45°C it became C^9 sensitive. Associated with this change was a reduction in the amount of Vi antigen synthesised (Osawa and Muschel, 1964a). Muschel (1960) found that strains of E. coli became O agglutinable when grown at 18°C or 45°C, they also became more sensitive to C^9.

Much evidence therefore has been accumulated to show that the temperature at which bacteria were grown affected the amount of Vi or K antigen synthesised. Associated with the reduction in K antigen content of bacteria due to the growth temperature was an increased sensitivity to the bactericidal action of C^9. These experiments therefore support the theory that K antigens affect the sensitivity of bacteria to killing by C^9.

K antigens have not been the only factor shown to be related to C^9 sensitivity. The fact that rough enterobacteria are more sensitive to C^9 than smooth strains has been generally accepted (Wilson and Miles, 1964). Rowley (1956) compared smooth strains of S. paratyphi B and E. coli with rough variants derived from them. The smooth strains were resistant to killing by 1/5 guinea pig serum and the rough ones were killed.

Selection of penicillin resistant mutants of enteric bacilli revealed a minority which were more sensitive to serum that the parent strain (Michael and Braun, 1958).

Nelson and Roantree (1967) used this method to isolate serum sensitive mutants from serum resistant strains of S. typhimurium and S. enteritidis. Loss of sugars from the lipopolysaccharide, S — R mutation, was associated with an increased sensitivity to C^9 although strains could become C^9 sensitive without there being any associated detectable change in the sugars of the O antigen.

Michael and Landy (1961) compared the endotoxin content, measured biologically, in paired strains of enterobacteria of the same serotype but different C^9 sensitivities. In some cases increased resistance to C^9 was associated with an increased endotoxin content. In other cases no difference in endotoxin content was demonstrable but there were unspecified differences in either the capsular or other antigens.

A smooth resistant strain of E. coli was shown to contain nine times the amount of lipopolysaccharide contained by a rough C^9 sensitive strain of E. coli by Wardlaw (1964). He suggested the amount of lipopolysaccharide present affected the C^9 sensitivity of E. coli.

Although the state of the O antigen can affect the C^9 sensitivity of E. coli the results reported here show that the

amount of K antigen present is another contributory factor and may well be more important amongst smooth strains. The K antigen content of bacteria was related to C^9 sensitivity whether strains of the same or different serotypes were compared or whether strains induced to be C^9 sensitive by growth at abnormal temperatures, which affected the production of K antigens were examined. The amount of K antigen was the determining feature and not the fact that it was present.

III. INHIBITION OF HAEMAGGLUTINATION AND HAEMOLYSIS BY K ANTIGEN

INTRODUCTION

In section II evidence was presented associating resistance to C' amongst strains of *E. coli* with the amount of K antigen present in the bacterial cell. One of the methods used to estimate the K antigen content of different strains was based on the ability of acid polysaccharides to inhibit antigenically unrelated agglutination systems.

K antigens are known to prevent the agglutination of live bacteria by anti O antibody (Kauffmann, 1947) and Muschel (1960) reported that O inagglutination of *E. coli* was related to resistance to serum.

The mechanism by which K antigens inhibited the agglutination of sheep red cells by anti sheep red cell serum was investigated in the hope that the results would provide a model for the way in which K antigens *in situ* on the bacterial cell affected the C' sensitivity of *E. coli*.

K antigens could inhibit the agglutination of sheep red cells either by preventing the union of antibody with antigen or by affecting subsequent lattice formation. In order to distinguish between these two possibilities the effect of K antigens on haemolysis was investigated.

If K antigen prevented antibody binding then haemolysis

should be inhibited as well as agglutination. However, just as agglutination might be affected at either or both of two stages so could haemolysis. Haemolysis would be reduced either if less antibody could combine with the red cell or if K antigen prevented the antibody-antigen complex fixing C'. These processes would not be mutually exclusive and haemolysis could be affected by both.

The effect of K antigens on the binding of ^{125}I-labelled anti sheep red cell antibody was investigated and the possibility that C' was affected directly was examined.

MATERIALS AND METHODS

Preparation of an extract of *E. coli* WF82

Bacterial extracts were made in 0.15M saline using a homogeniser as described previously (Section II). The extract was concentrated by precipitation with 3 volumes of ethanol, the precipitate dissolved in 0.15M saline and dialysed against saline to remove residual alcohol.

In order to compare the concentrations of bacterial extracts made on different occasions the carbohydrate content was measured by the method of Dubois *et al.* (1956) using glucose as standard.

Purification of K antigen

The K antigen in saline extracts of bacteria prepared

as above was purified according to Hungerer et al. (1967). Lipopolysaccharide was removed by centrifugation at 100,000 g for 2 hours and the K antigen was isolated by fractional precipitation with cetavlon (cetyltrimethyl-ammonium bromide).

The separation of K antigen from O antigen present in the extracts could be demonstrated by immunoelectrophoresis using anti O and anti OK sera.

Oxoid ion agar No. 2 was dissolved in 0.05M veronal buffer pH 8.2 (Grabar and Burtin, 1964) to give a final concentration of 1% w/v agar. After electrophoresis antisera were added, slides incubated overnight at room temperature and the precipitin lines photographed.

Antibacterial antisera

Antisera containing both anti O and anti K antibodies were raised in rabbits against live bacteria. Anti O antisera were raised against bacteria that had been heated at 100°C for 2½ hours. Anti K antisera were prepared by absorbing anti OK sera with bacteria that had been heated at 100°C and washed in saline (Edwards and Ewing, 1962).

Anti sheep red cell antiserum (haemolysin)

Haemolysin was raised in rabbits according to Kabat and Mayer (1961) and used as the source of γ globulin, IgG and

IgM antibodies.

γ globulin

The γ globulin fraction of haemolysin was prepared by precipitation with 12% sodium sulphate (Kekwick, 1940).

IgG and IgM antibodies

Haemolysin was fractionated on D.E.A.E. cellulose in order to separate the IgG and IgM antibody fractions (Adinolfi et al., 1966).

Haemolysin was dialysed against 0.017M phosphate buffer pH 7.5 and put on a D.E.A.E. cellulose (Whatman DE52) column that had been equilibrated with the same buffer. The column was washed through with 0.017M buffer followed by 0.1M phosphate buffer pH 6.5 and then with 0.3M phosphate buffer pH 6.5.

The 0.017M fraction and the 0.3M fraction were concentrated by negative pressure dialysis against 0.15M saline. The 0.017M fraction was stored at 4^{o}C and used as the source of IgG.

The 0.3M fraction was passed through Sephadex G200 (Flodin and Killander, 1962) to further purify the IgM which is excluded from the Sephadex gel. The IgM was concentrated by negative pressure dialysis against 0.15M saline, stored at 4^{o}C and used.

γ globulin, IgG and IgM were labelled with ^{125}I by the method of McFarlane (1958).

Inhibition of Haemagglutination

This was performed as described in Section II except that instead of bacterial extracts various concentrations of K antigen in phosphate buffered saline were incubated with the red cells. The titre of the haemolysin against untreated and K antigen treated red cells was compared and the extent of inhibition was recorded as being the number of doubling dilutions by which the titre of the anti red cell serum was reduced.

Inhibition of haemolysis by bacterial extracts

Complement. Normal human serum was prepared as described in Section II. Prior to use it was absorbed on ice with packed washed sheep red cells to remove "normal" anti sheep red cell antibody.

Inhibition of haemolysis

5% v/v sheep red cells in phosphate buffered saline (P.B.S.) were incubated with various concentrations of the bacterial extract at 37°C for 30 min. The red cells were then washed with P.B.S. and resuspended in the veronal, calcium, magnesium buffer (Oxoid) used for complement estimations.

Antigen coated and uncoated red cells were sensitised with haemolysin by incubation at 37°C for 15 min in the standard way for C' titrations (Kabat and Mayer, 1961).

0.5 ml of coated sensitised cells together with controls (coated unsensitised, uncoated unsensitised, uncoated sensitised cells) were added to 7.0 ml amounts of a suitable dilution of human C' and incubated at 37°C for 90 min. The cells were then spun down, the haemoglobin in the supernatant measured by reading the O.D.541 nm and the % haemolysis calculated. The C' and haemolysin concentrations used were that found by a preliminary titration to give approximately 80% lysis of cells not coated with extract.

In similar experiments the IgG or IgM fractions of haemolysin were used to see if K antigen affected haemolysis mediated by both of these types of antibody.

In other experiments the red cells were sensitised with antibody before being coated with bacterial antigen. Batches of red cells sensitised to the same extent could be treated with different amounts of bacterial extract and the effect of the extract on C' subsequent to the binding of antibody could be examined.

Purified K antigen was incubated with red cells in the same way as for the crude bacterial extract to examine its effect on haemolysis

Haemolysis as a function of the amount of antibody bound to the red cells in the presence and absence of bacterial extract

5% v/v sheep red cells in phosphate buffered saline were divided into two portions, one of which was incubated at 37°C for 30 minutes with an equal volume of 0.15M saline, and the other with an equal volume of bacterial extract in 0.15M saline. The cells were washed and resuspended in phosphate buffer saline to give a 5% v/v suspension. Extract treated or untreated red cells were incubated with an equal volume of a range of concentrations of ^{125}I-labelled γ globulin for 15 minutes at 37°C. The cells were washed with veronal, calcium, magnesium buffer, and the radioactivity measured. The cells were then incubated with C' as above and the % haemolysis measured.

The count (= antibody bound) was plotted against % haemolysis to see if K antigen affected C' per se rather than via its effect on antibody.

Inhibition of ^{125}I-labelled antibody binding by bacterial extract

5% v/v red cells in phosphate buffered saline, pH 7.2, 0.15M were incubated with various concentrations of bacterial extract for 30 min at 37°C and washed in the same buffer. 0.5 ml of 5% v/v red cells that had been treated with bacterial extract and untreated control cells were incubated

with 0.5 ml of ^{125}I-labelled γ globulin, IgG or IgM antibody for 15 min at 37°C. After washing three times with phosphate buffered saline the radioactivity was measured (= antibody bound to the red cells).

RESULTS

Inhibition of agglutination by K antigen

The effect of the K antigen from E. coli WF82 on haemagglutination is shown in Fig. 4. As the antigen concentration increased the extent to which agglutination was inhibited also increased, showing inhibition of agglutination was directly related to the antigen concentration used.

Red cells that had been incubated with antigen were added without washing to the haemolysin. The extent to which agglutination was inhibited was compared for these cells and for red cells which had been incubated with antigen and then washed once and three times with phosphate buffered saline. Washing had no effect on the extent to which the K antigen inhibited agglutination. It was therefore concluded that the K antigen was absorbed onto the red cell surface and then inhibited agglutination. It was not active in solution by "neutralising" anti red cell antibody.

Inhibition of haemolysis

Fig. 5 shows that a saline extract of E. coli WF82 was

capable of inhibiting C' dependent lysis of sheep red cells.

The acid polysaccharide bearing the K antigen determinant site was isolated from the saline extract as described previously. Removal of the O antigen during the purification procedure was demonstrated by immuno-electrophoresis (Fig. 6). The purified K antigen was capable of inhibiting haemolysis (Fig. 7), the extent of inhibition increased as the antigen concentration was increased. Although endotoxin has been demonstrated to be "anticomplementary" (Pillemer et al., 1955) removal of O antigen from the saline extract did not remove the haemolysis inhibiting activity so endotoxin was not responsible for the inhibition of haemolysis by the saline extract in this case.

Fig. 8 shows that the bacterial extract was capable of inhibiting haemolysis mediated by IgG or IgM. There was no apparent difference in the extent to which the extract inhibited haemolysis due to either type of antibody.

When red cells were sensitised with antibody before they were incubated with bacterial antigen the bacterial extract still reduced haemolysis. The effect was not as great as when the red cells were incubated with bacterial antigen prior to sensitisation with haemolysin (compare Figs. 5 and 9). As the red cells were incubated with antibody before exposure to antigen it was assumed that in this case the bacterial antigen was not inhibiting antibody binding. The reduction

in haemolysis was presumably because K antigen inhibited the C^9 sequence at some stage subsequent to the union of antibody with the red cell.

The inhibitory effect of the bacterial extract on C^9 itself rather than on the union of antibody with antigen was demonstrated in another way (Fig. 10). A suspension of red cells was divided into two parts, one of which was incubated with bacterial extract and the other with saline. The extract treated cells were washed and 0.5 ml amounts of these and untreated red cells were incubated with 0.5 ml of various concentrations of ^{125}I-labelled antibody. The red cells were washed and the amount of antibody bound was estimated by measuring the radioactivity. Cells were then lysed by the addition of C^9 and the % haemolysis calculated.

% haemolysis was plotted against the amount of antibody bound (Fig. 10). When untreated and extract treated cells were compared for the same amount of antibody present on the red cell, indicated by the radioactivity, extract treated cells were lysed less than untreated cells. Since the reduction in lysis was not due to prevention of antibody binding it was concluded that K antigen affected C^9 directly after antibody had been bound to the red cell.

Inhibition of antibody binding

Bacterial extracts were capable of preventing ^{125}I-

labelled antibody from combining with the red cell (Figs. 11 and 12). The extent to which the extract prevented the union of antibody with antigen was directly related to the extract concentration. The γ globulin fraction of haemolysin or IgG and IgM prepared from it were all affected.

Fig. 4. Inhibition of agglutination by the K antigen from *E. coli* WF82.

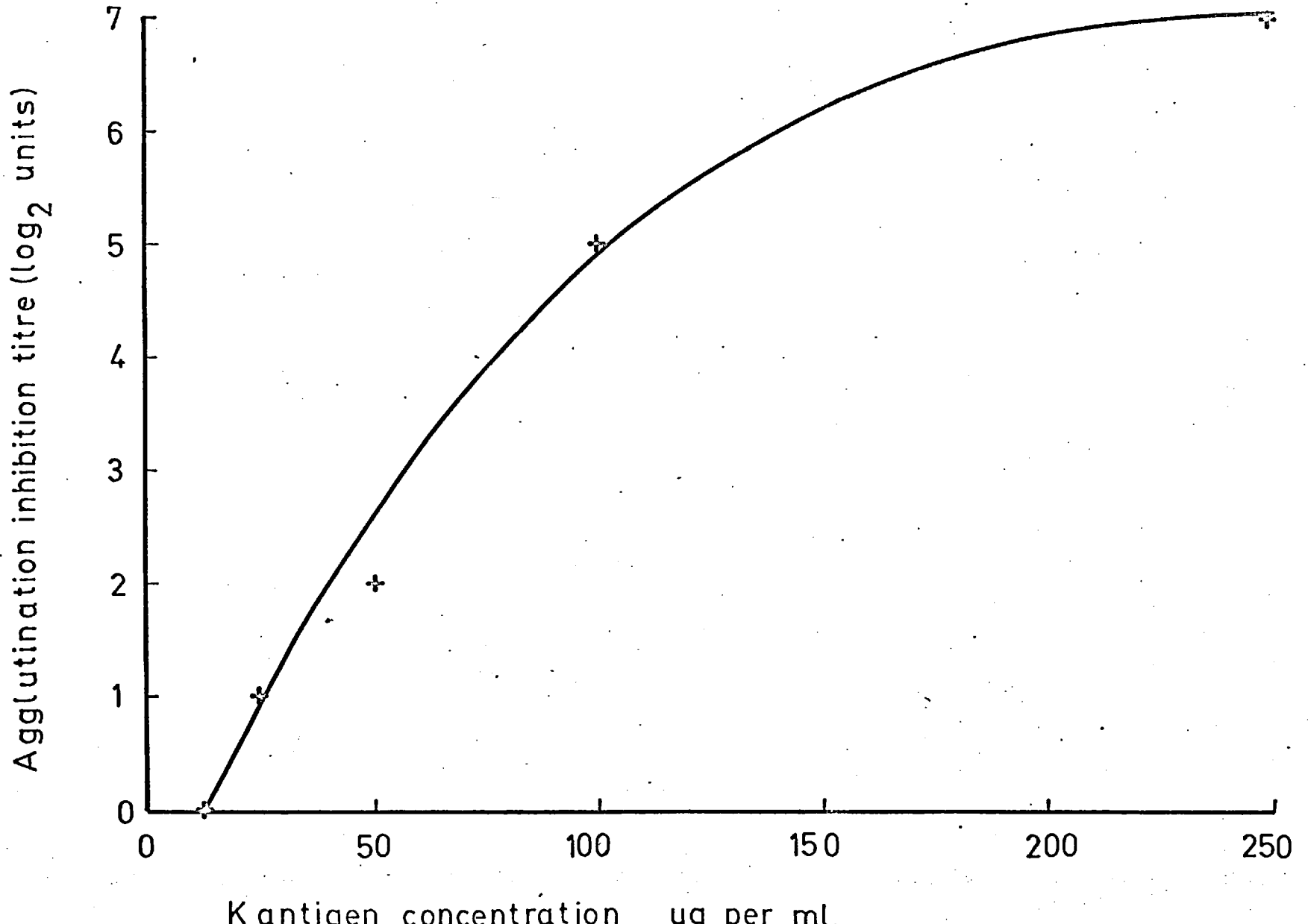

Agglutination inhibition titre (log_2 units)
7
6
5
4
3
2
1
0
0
50
100
150
200
250
K antigen concentration ug per ml.

Fig. 5. Effect of a saline extract of *E. coli* WF82 on the immune haemolysis of sheep red blood cells.

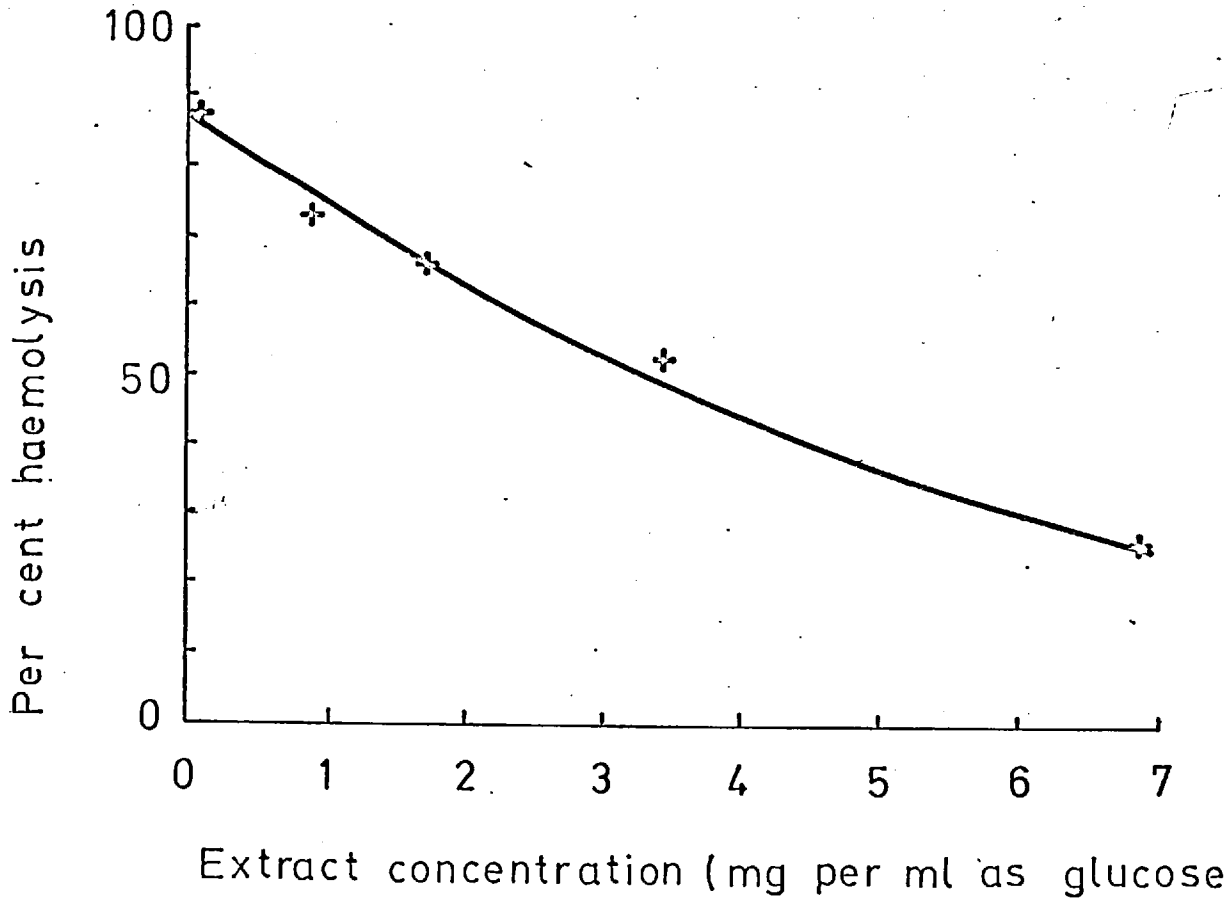
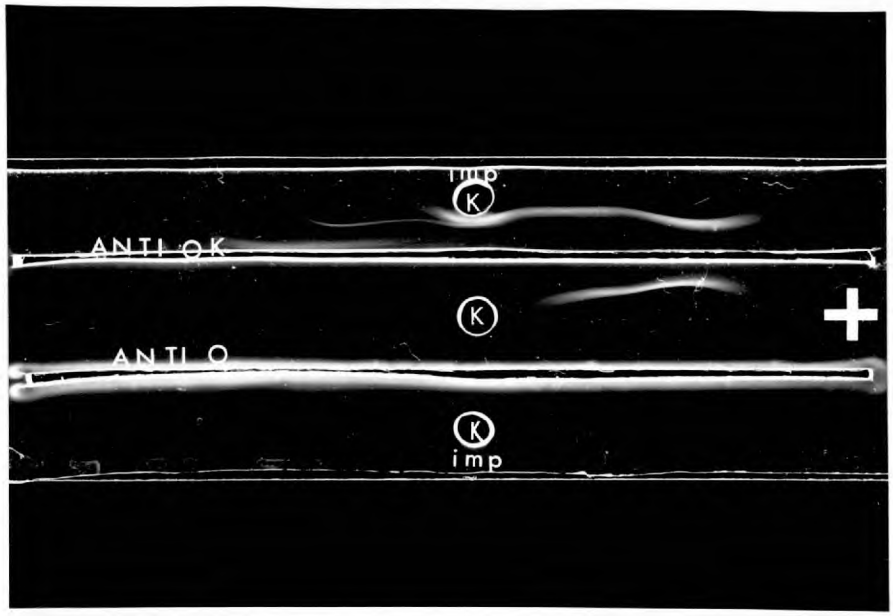

100
50
0
Per cent haemolysis
0
1
2
3
4
5
6
7
Extract concentration (mg per ml as glucose)

Fig. 6. Immunoelectrophoresis of a saline extract of *E. coli* WF82 and of the purified acid polysaccharide isolated from the bacteria.
In the troughs: anti O, anti OK and anti K sera.
In the wells: Imp K is a saline extract of *E. coli* WF82. K is the purified K antigen.


imp
K
ANTI O K
K
+
ANTI O
K
imp

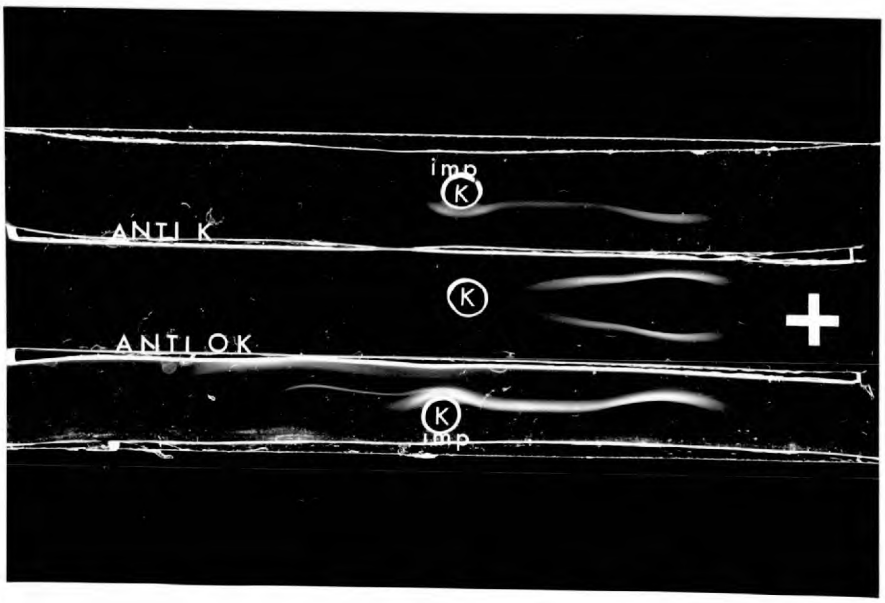

imp
K
ANTI K
K
+
ANTI O K
K
imp

Fig. 7. Effect of purified K antigen on immune haemolysis.

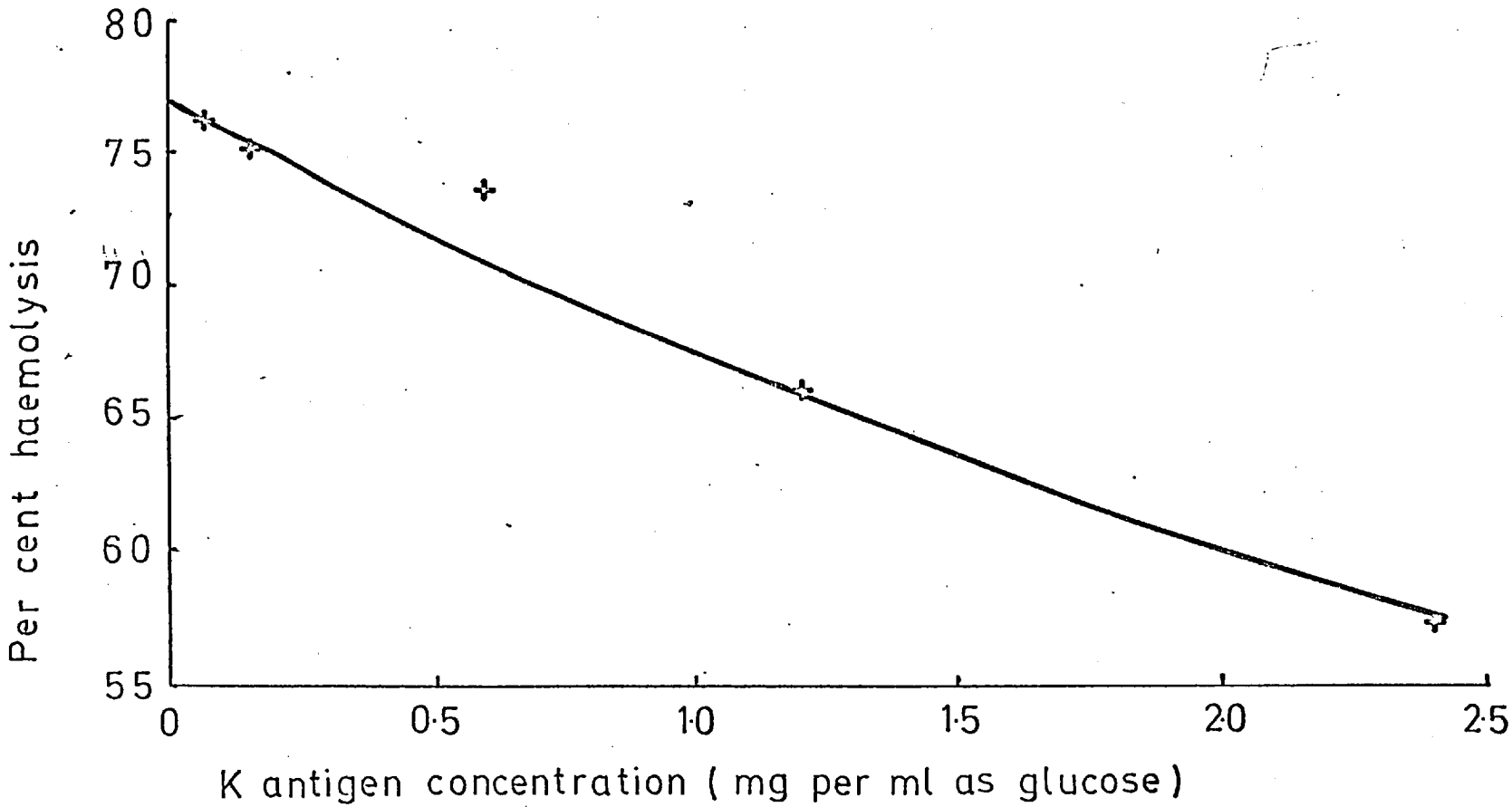

80
75
70
65
60
55
Per cent haemolysis
0
0·5
1·0
1·5
2·0
2·5
K antigen concentration (mg per ml as glucose)

Fig. 8. Inhibition by an extract of *E. coli* WF82 of complement lysis of sheep red cells sensitised with IgG and IgM antibody.

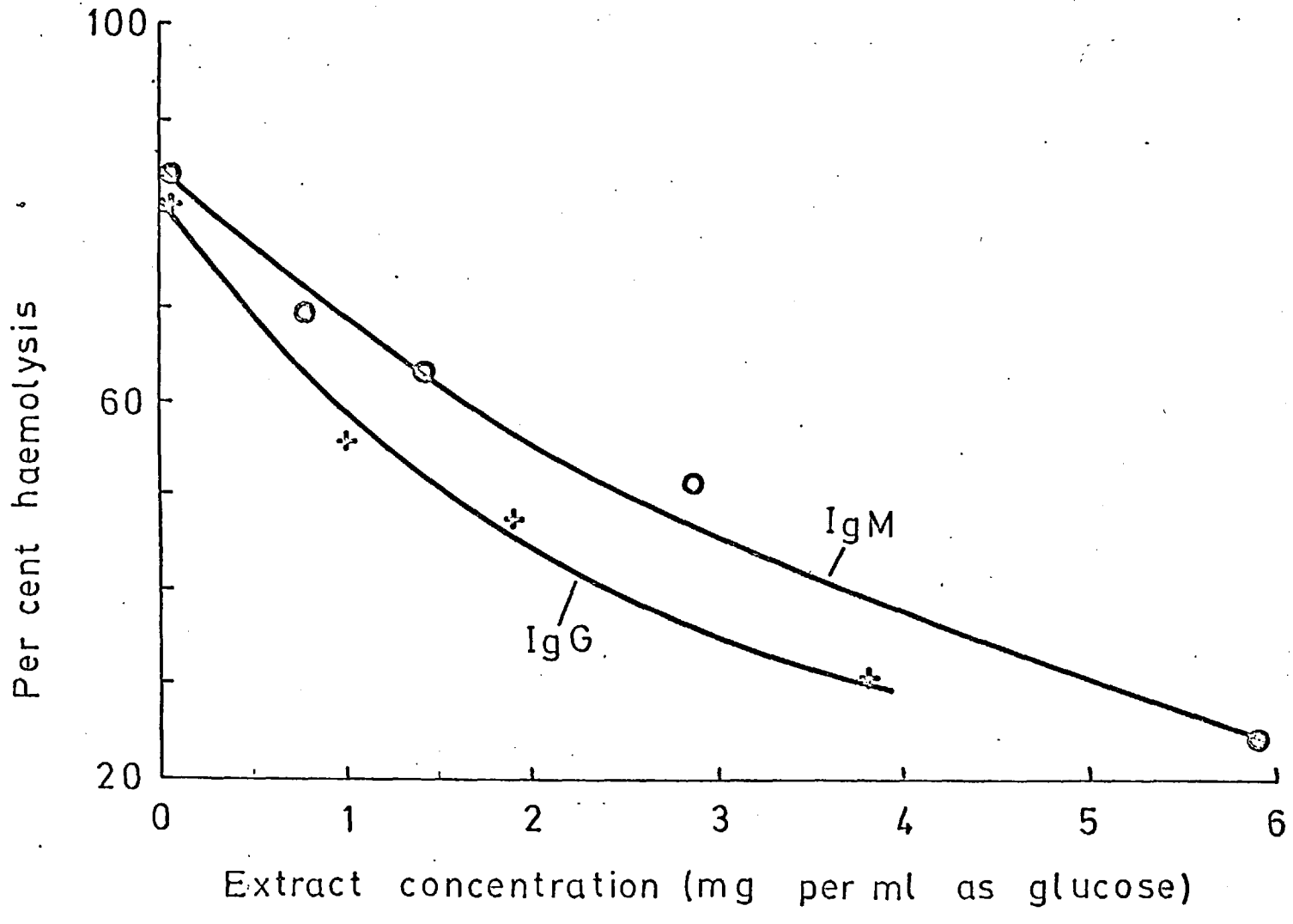

100
60
20
Per cent haemolysis
IgM
IgG
0
1
2
3
4
5
6
Extract concentration (mg per ml as glucose)

Fig. 9. Inhibition of complement dependent lysis of sensitised sheep red cells by an *E. coli* extract.

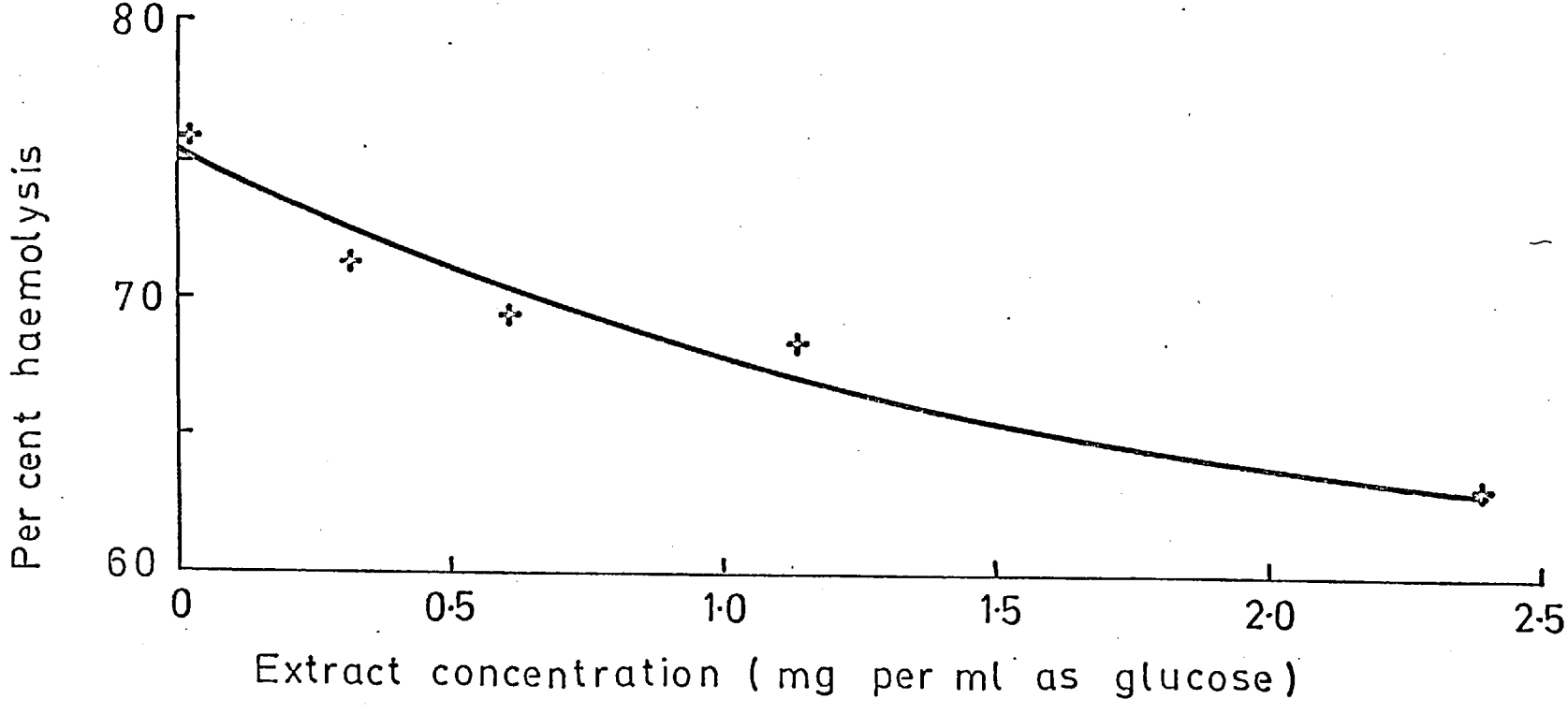

80
70
60
Per cent haemolysis
0
0·5
1·0
1·5
2·0
2·5
Extract concentration (mg per ml as glucose)

Fig. 10. Degree of haemolysis of sheep red cells as a function of haemolysin bound in the presence and absence of E. coli extract.

+ - red cells treated with extract,

o - red cells treated with saline.

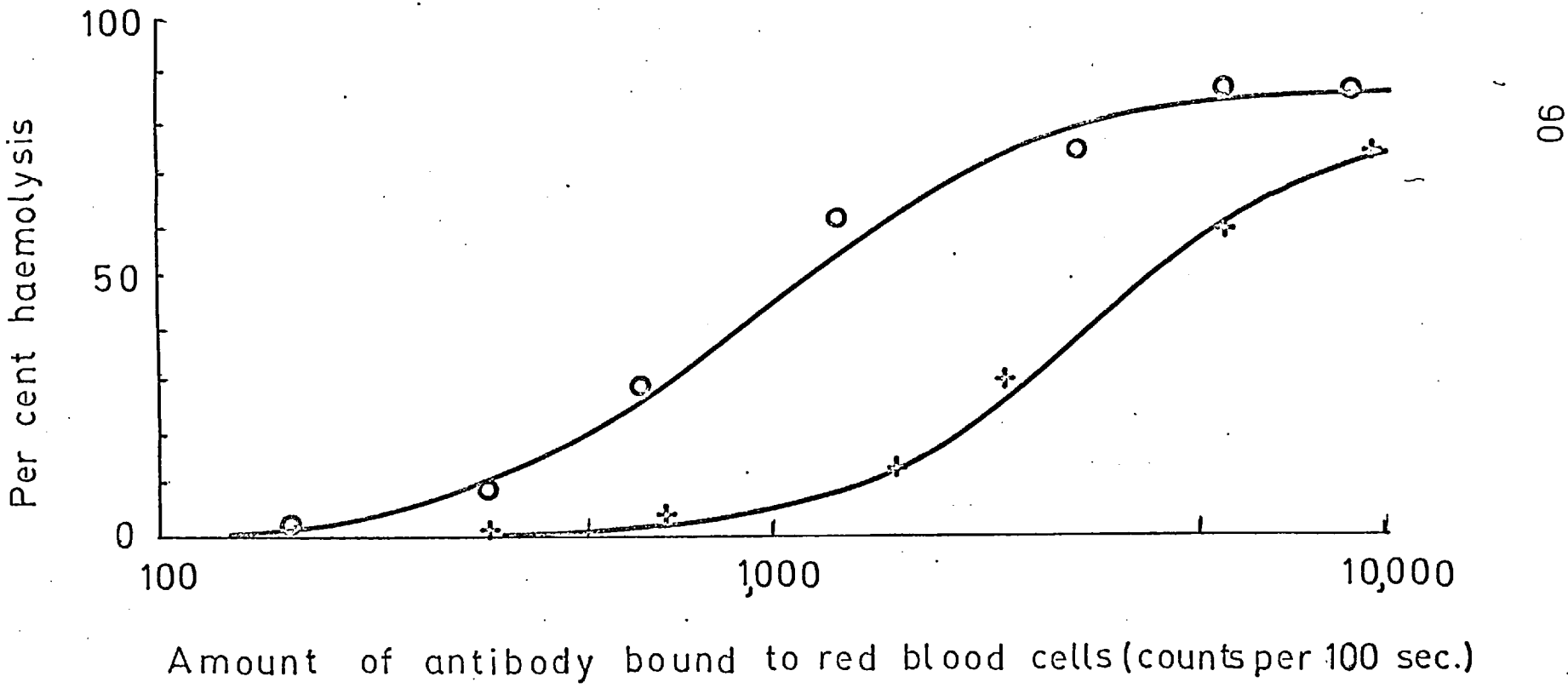

100
50
0
Per cent haemolysis
100
1,000
10,000
Amount of antibody bound to red blood cells (counts per 100 sec.)

Fig. 11. Effect of a saline extract of E. coli on antibody binding.

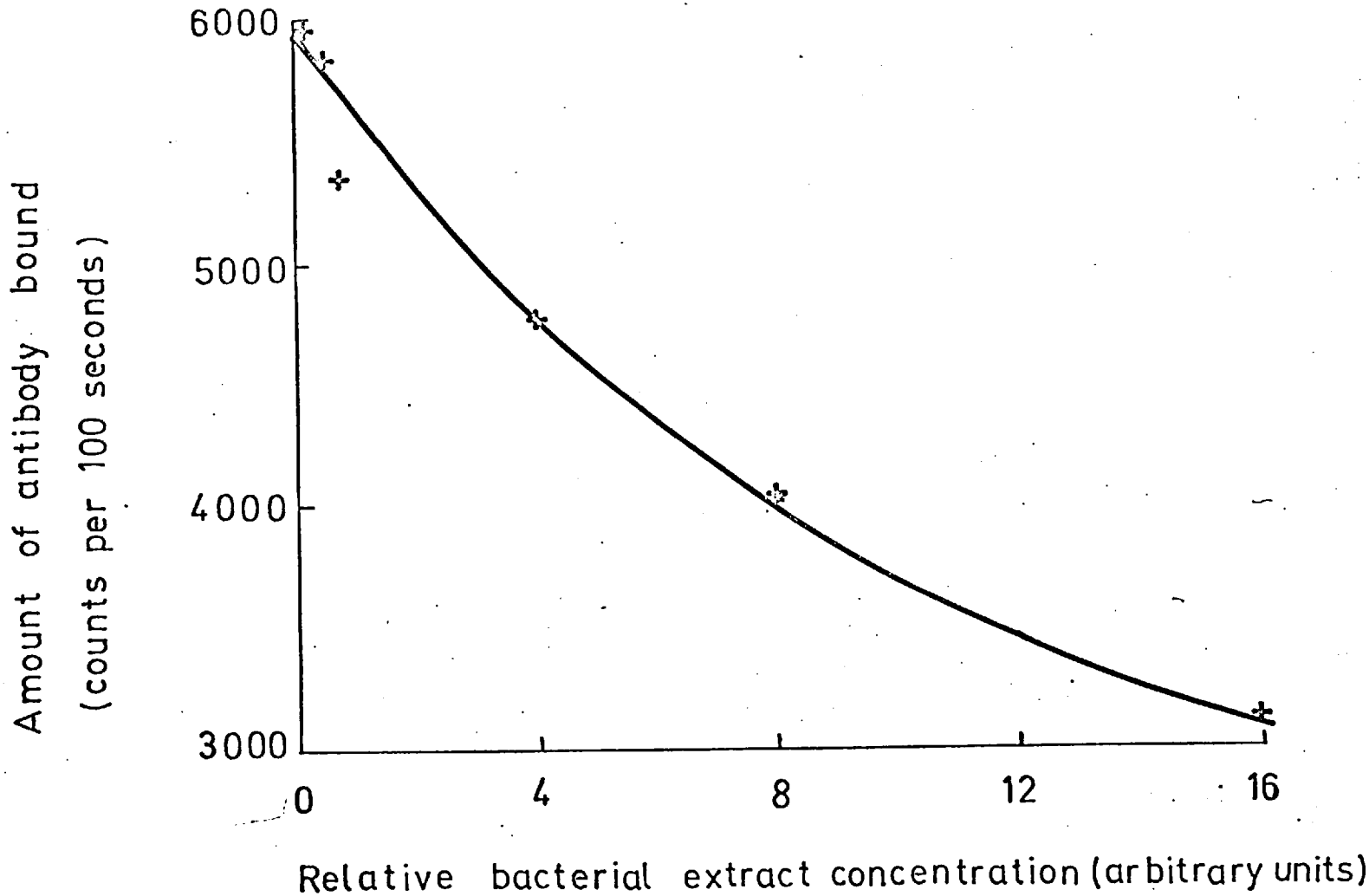
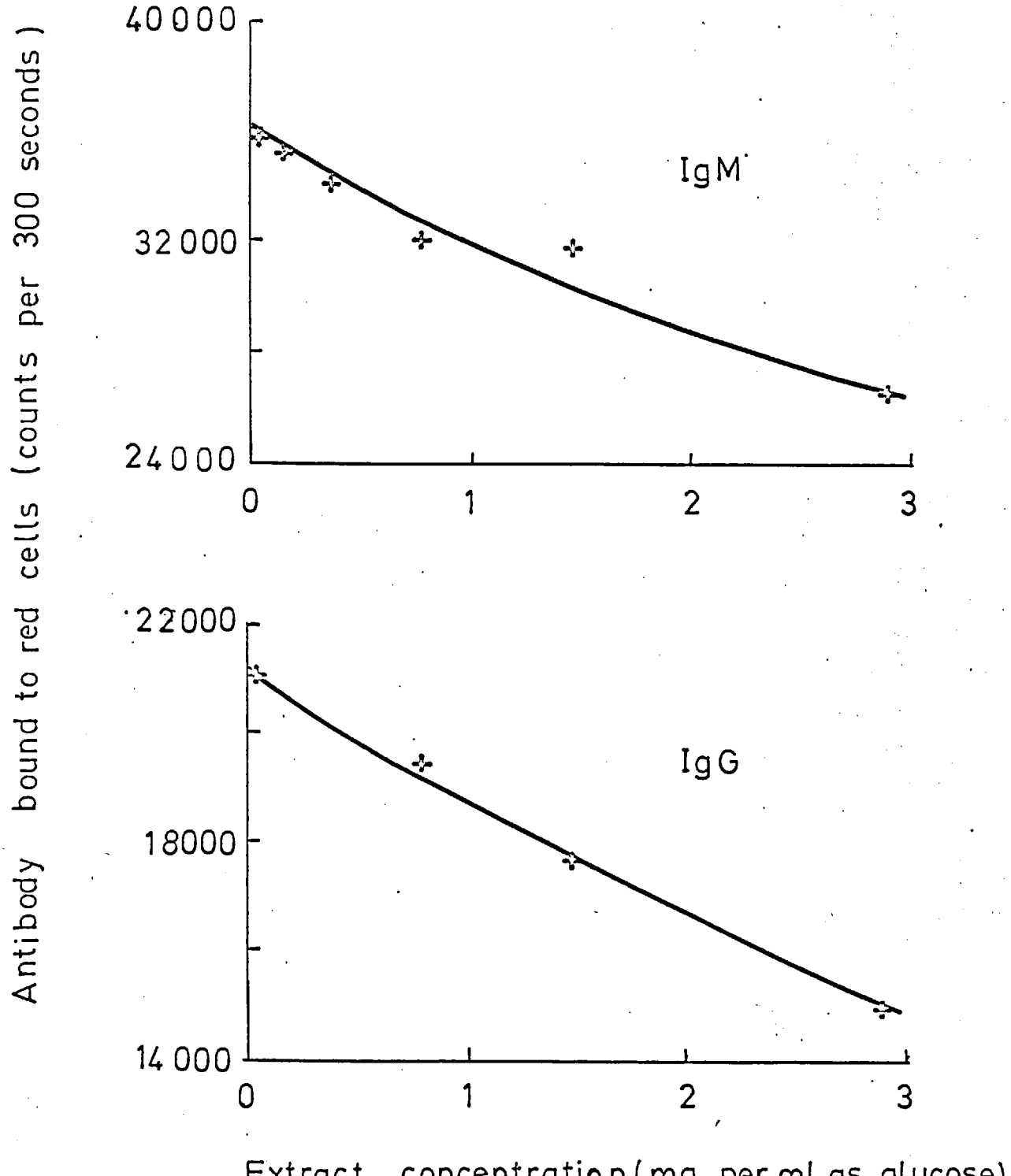

Amount of antibody bound
(counts per 100 seconds)
6000
5000
4000
3000
0
4
8
12
16
Relative bacterial extract concentration (arbitrary units)

Fig. 12. Inhibition of IgG and IgM antibody binding by a saline extract of *E. coli* WF82.


40000
32000
24000
IgM
0
1
2
3
22000
18000
14000
IgG
0
1
2
3
Antibody bound to red cells (counts per 300 seconds)
Extract concentration (mg per ml as glucose)

DISCUSSION

The K antigen of E. coli WF82 inhibited both the agglutination of sheep red cells by anti sheep red cell sera and the lysis of sheep red cells by haemolysin and C'. The extent to which each system was inhibited was directly related to the K antigen concentration.

Haemolysis could have been inhibited either because the K antigen prevented anti sheep red cell antibody combining with its substrate, consequently the red cells would not be sensitised to C', or the K antigen could affect C' directly at some stage after the union of antibody with antigen.

Using ^{125}I-labelled γ globulin, IgG or IgM antibody a reduction in antibody binding could be demonstrated the extent of which was dependent on the bacterial extract concentration.

If red cells were sensitised with antibody before incubation with bacterial antigen there was still a reduction in haemolysis suggesting C' was being affected directly. This was confirmed by another experiment in which the extent of haemolysis for a constant amount of ^{125}I-labelled bound antibody was compared for normal red cells (i.e. untreated) and cells that had been incubated with the bacterial extract. Haemolysis was less in the presence of bacterial antigen when the same amount of antibody had been bound to the red cells.

These results suggest that inhibition of haemolysis is due partly to the prevention of anti red cell antibody binding and partly to some direct inhibitory effect of the K antigen on C'. Possibly the negatively charged polysaccharide has some general repellent effect on proteins.

The inhibitory action of K antigens on agglutination cannot be explained in terms of the reduction in the amount of antibody which combines with the red cell. If Figs. 4, 7 and 12 are compared it can be seen that although a K antigen concentration of 25 µg per ml reduced the agglutinating activity of haemolysin by half a K antigen concentration in excess of 2.5 mg per ml was required to reduce the lytic activity of the same antiserum to the same extent or to produce the same effect on antibody binding. It is therefore concluded that inhibition of agglutination is due in the main to the inhibitory effect of the K antigen on the second stage of agglutination, i.e. it prevents lattice formation, although the reduction in the amount of antibody bound may affect agglutination to a lesser extent.

The inhibitory effect of the K antigen on O agglutination can be reproduced <u>in vitro</u>. Spaun (1952) made 100°C saline extracts of two strains of <u>S. typhi</u> one of which contained O antigen but not Vi antigen and the other produced Vi antigen but little or no O antigen. When either extract was used alone red cells could be sensitised so they became

agglutinable with a homologous anti serum, i.e. an anti O serum or an anti Vi serum. When extracts containing O and Vi antigen were incubated together with red cells the red cells could be agglutinated with anti Vi antibody but not by anti O antibody. If diluted Vi antigen extract and the O antigen extract were incubated with red cells the sensitised cells could be agglutinated by both anti O and anti Vi antibodies.

Similar experiments were performed by Landy and Ceppellini (1955). They showed that the order in which O and Vi antigen were incubated with red cells did not affect sensitisation. Inhibition of agglutination by Vi antigen was not therefore because Vi antigen blocked O receptor sites on the red cell. They suggested agglutination was inhibited either by steric hinderance or because of a non specific stabilisation of the cell due to the peculiar physicochemical properties of the Vi antigen.

Neter (1962) using red cells sensitised with O antigen reasoned that as Vi antigen did not inhibit haemolysis by anti O antibody and C^{9} to the same extent as agglutination by anti O antibody lattice formation was affected rather than antibody binding.

Ceppellini and Landy (1963) came to the same conclusion. They found that although Vi antigen inhibited the agglutination of red cells by anti red cell sera they could still detect bound antibody by building up globulin-

antiglobulin layers. In some cases they found a reduction in antibody binding but this was not a general effect and varied depending on the blood group used. Besides this they found that red cells coated with Vi antigen were less effective than uncoated red cells at absorbing antibodies from haemolysin. Although some evidence was presented showing that the Vi antigen could affect the ability of antibody to combine with antigen they also concluded that in the main agglutination was inhibited by the prevention of lattice formation. They suggested that when Vi antigen coated the red cell it extended the ionic halo around the cell so there was a wider gap to be bridged by the globulin and the cells could not come close enough to agglutinate.

O inagglutinability of bacteria has been related to resistance to the bactericidal action of serum (Felix and Olitzki, 1926; Muschel, 1960). If K antigens affected bacterial agglutination only by their effect on lattice formation and had no effect on antibody binding there would be no reason why they should affect C^9 sensitivity. With the red cell experiments, although agglutination was inhibited in the main because of inhibition of lattice formation, K antigens were demonstrated to reduce the amount of antibody which bound to the cell and to affect haemolysis.

According to Rowley and Turner (1968) only 30 molecules of IgM antibody per bacterial cell were sufficient for

complement killing of a gram negative bacterium. In the haemolytic reaction only one or two molecules of IgM or about 800 molecules of IgG (Humphrey and Dourmashkin, 1969) were required per red cell.

The effect of K antigen on antibody binding described clearly does not diminish the number of antibody molecules per red cell to anything like this extent yet in some strains of E. coli K antigen may completely abolish killing. It is likely that the K antigens normally in situ on the surface of the bacteria are present in greater quantity and are more efficiently arranged sterically than the K antigen artificially coated onto the red cell.

From these results a theory on the mechanism of resistance to serum involving the K antigen can be proposed. It has been known since the work of Burgii in 1907 and Gibson in 1930 that normal sera from man and animals contains antibodies capable of agglutinating many different groups of bacteria (Wilson and Miles, 1964). Brumfitt and Percival (1965) tested sera from twenty individuals for their agglutination titre against eleven different O serotypes of E. coli. All the sera tested contained bacterial agglutinins usually with a titre of about 1/160.

It is suggested that the ability of K antigens to prevent the binding of non homologous antibody prevents normal anti O antibody present in human serum from combining with the

bacterial cell and sensitising bacteria to C^{7}.

Further experiments designed to test this theory are reported in Section IV.

IV. THE INVOLVEMENT OF K ANTIGENS IN DETERMINING THE SUSCEPTIBILITY TO PHAGOCYTOSIS AND VIRULENCE OF E. COLI AND THE BACTERICIDAL AND OPSONIC ACTIVITIES OF ANTI O AND ANTI K ANTIBODIES.

INTRODUCTION

In Section III it was suggested that if K antigens were present in the bacterial cell in sufficient quantity they could prevent normal anti O antibody binding to the cell. In this way K antigens made the organism resistant to the bactericidal action of normal human serum.

If this were true then there should be a relation between sensitivity to C°, K antigen content, and susceptibility to phagocytosis because K antigen would be expected to prevent opsonisation of the bacterial cell by normal anti O antibody.

Halpern and Biozzi's quantitative clearance technique (Biozzi et al., 1960) was chosen to compare the susceptibility of strains of E. coli to phagocytosis by the reticulo-endothelial system. The disappearance of ^{51}Cr labelled bacteria from the blood stream of mice was examined to see if it was related to C° sensitivity and K antigen content.

In order to investigate further the mechanism by which K antigens affected C° sensitivity the opsonising activities of

anti O and anti K antibodies were compared. If K antigens prevented anti O antibody from combining with the O antigen then anti O antibody would not be expected to opsonise C' resistant bacteria. Anti K antibody would be expected to bind to the bacterium and to be opsonic. The bactericidal activities of anti O and anti K antibodies and the possibility that anti K antibody might augment the killing of bacteria by normal human serum was examined.

It was feasible that anti K antibody might neutralise the K antigen and so enable more anti O antibody to combine with the O antigen. This possibility was examined by using ^{125}I-labelled anti O antibody.

Finally the LD_{50} of strains of E. coli for mice challenged intracerebrally was calculated to examine the relation between mouse virulence and sensitivity to C'.

MATERIALS AND METHODS

Antisera

Anti O and anti OK antisera were raised in rabbits according to the methods of Edwards and Ewing (1962) as described in Section II. Anti OK sera were used as the source of K antibody in the bactericidal and phagocytosis experiments unless otherwise stated.

Anti WF96 K antibody was prepared by absorbing an anti OK antiserum with E. coli WF96 that had been heated at 100°C for

one hour and washed with saline. Anti WF96 OK antiserum was also absorbed with live E. coli WF96. This removed both anti O and anti K antibody.

Anti WF82 K antibody was prepared by incubating an antiserum raised against live E. coli WF82, anti OK antiserum, with an alkali treated phenol-water extract of WF82 (see Section VII).

The agglutination titres of the antisera used are recorded in Table 6.

Bacterial agglutination tests

Live bacteria or bacteria that had been heated at 100°C for 1 hour and washed with saline were suspended in 0.15M saline to give an optical density of 0.6 on an absorbtiometer (Spekker) using a neutral grey filter. 0.2 ml of bacteria were added to 0.2 ml of doubling dilutions of antisera in 0.15 M saline in a haemagglutination tray. The trays were incubated at 37°C for 2 hours and overnight at 4°C after which the agglutination titres were read.

Phagocytosis

Bacteria: Logarithmic phase bacteria were grown in nutrient broth at 37°C, harvested by centrifugation and washed by resuspension in 0.15 M saline containing 1/10,000 merthiolate.

The bacteria were finally resuspended to give a concentration of 2×10^{10} cells per ml by optical density.

Radioactive labelling: Bacteria were labelled by adding 0.15 m.c. of ^{51}Cr per 1 ml of suspension and incubating the mixture for 2 days at 37°C (Howard et al., 1959). The cells were washed three times with saline and the optical density adjusted to give 2.5×10^{9} cells per ml.

Experiment: The disappearance from the mouse circulation of various strains of E. coli was measured after injecting 5×10^{8} bacteria per 20 g. mouse into the lateral tail vein. Albino mice weighing 20-25 g. were used. Standard blood samples were taken at timed intervals from the retro-orbital plexus using a capillary tube (Schieffelin & Co. 0.8 m.m. internal diameter) that had been coated with heparin.

The blood samples were lysed in 2.0 ml of 0.1% w/v sodium carbonate and the radioactivity measured in a scintillation counter.

The phagocytic index, K, was calculated from the equation

$$K = \frac{\log C_o - \log C_t}{t}$$

The phagocytic index is related to the rate at which bacteria

are cleared from the mouse circulating by phagocytosis due to the reticulo endothelial system (Biozzi et al., 1960).

The effect of anti O and anti K antibody on phagocytosis was examined by injecting 0.2 ml of the appropriate antiserum into the tail vein 15 minutes before injecting the bacteria. The antisera used are listed in Table 6.

The effect of anti K antibody on the killing of E. coli by normal human serum

Log phase bacteria suspended in water were adjusted to the required concentration by optical density. Antisera were diluted in 0.15 M saline so that the final concentrations would be 1/500, 1/1000 and 1/2000.

For strains WF98 and WF26 0.1 ml of bacteria were incubated at 37°C in an L tube with a mixture of 1.8 ml of N.H.S. and 0.1 ml of antibody or 0.1 ml of 0.15 M saline. 0.1 ml samples were taken from the L tube at timed intervals, diluted in nutrient broth and the number of viable cells determined by plate counts. In the control tubes heated serum was substituted for N.H.S.

For strain WF96, which is very sensitive to C^9, the C^9 concentration in the reaction mixture was reduced by using a mixture of 0.2 ml of N.H.S. and 1.6 ml of heated serum instead of 1.8 ml of N.H.S.

The antisera used were raised against live WF96 and WF98. They contained both anti O and anti K antibodies (Table 6).

Bactericidal and bacteriolytic action of anti O and anti K antibody and piglet C' against E. coli WF96

Complement: Precolostral piglet serum, provided by Miss J. Poland of the Royal Veterinary College, was used as a source of C' which was devoid of normal antibacterial antibody. The serum was stored at -60°C and samples thawed as required.

Antibodies: Anti O antibody, anti OK antibodies, anti K antibody and anti OK serum that had been absorbed with live E. coli WF96 to remove both O and K antibody were examined for bactericidal and bacteriolytic activity.

Experiment: Logarithmic phase E. coli WF96 in 0.04 M tris buffer pH 8.4, piglet serum, Ca^{++}, Mg^{++}, egg white lysozyme (Armour), antibody, saline and water were mixed in a cuvette so that the final volume was 3.0 ml. The final concentrations of the constituents were:

	Final Concentration
Bacteria in tris buffer	1×10^8 per ml
Piglet serum	1/5
Lysozyme in H_2O	20 µg per ml
Ca^{++}	0.00015 M
Mg^{++}	0.0005 M
Antibody in 0.15 M NaCl	1/100

The volumes of water and saline added were calculated to give a final ionic strength of about 0.06 (Section VIII and Glynn and Milne, 1967).

In another experiment bacteria were sensitised with 1/20 antibody before being added to the cuvette. The volumes of water and saline added were adjusted to keep the final ionic strength the same.

To study bacteriolysis the % transmission at 542 nm was measured in an absorptiometer (Vitatron) and recorded automatically. % lysis at any given time could be calculated from the % transmission values.

The bactericidal reaction was followed by counting the number of viable organisms present in the cuvettes at timed intervals.

Lysis of red cells sensitised with K antigen by anti K antibody and C'

Red blood cells: Sheep red cells were obtained from Tissue Culture Services Ltd. They were washed three times in C' diluent (Oxoid C' fixation test diluent tablets) and suspended in C' diluent to give a final concentration of 1% v/v.

K antigen: The K antigen from E. coli WF82 was isolated from a phenol-water extract of the bacteria by fractional precipitation with cetavlon (see Section VII).

Anti K antibody: An anti OK serum was used as the source of anti K antibody (Table 6). 1.0 ml of the antiserum that had been heated at 56°C for 30 minutes was absorbed on ice with 0.1 ml of packed washed red cells prior to use to remove any normal anti sheep red cell antibody.

Anti red cell antibody: Haemolysin was obtained from Staynes Laboratories Ltd.

Complement: Normal human serum which contained 36 C'H_{50} per ml was used as the source of C'. Prior to use it was absorbed on ice with packed red cells to absorb natural antibody

directed against sheep red cells. The absorbed N.H.S. was diluted 1/5 in C^{9} diluent before use.

Experiment: Red cells were incubated with an equal volume of K antigen solution containing 1 mg, 500 µg, 100 µg and 50 µg of K per ml. Control red cells were incubated with an equal volume of C^{9} diluent. After incubation at 37°C for 30 minutes the red cells were washed and resuspended in C^{9} diluent to give a 1% v/v suspension.

0.5 ml of sensitised red cells and control cells were incubated at 37°C for 30 minutes with 0.5 ml of 1/100, 1/400 and 1/1600 dilutions of anti WF82 OK serum. 1.0 ml of a 1/5 dilution of absorbed N.H.S. and sufficient C^{9} diluent to bring the final volume to 7.5 ml were added to the red cells. Control tubes contained red cells that had been treated with K antigen, anti red cell antibody and C^{9}, K antigen treated red cells with and without anti K antibody or haemolysin and untreated red cells to which anti K antibody or haemolysin had been added.

The tubes were incubated for 90 minutes at 37°C, centrifuged, the haemoglobin in the supernatant measured by reading the optical density at 541 nm and the % haemolysis calculated.

The effect of anti K antibody on the binding of anti O antibody

Bacteria: An overnight culture of E. coli WF82 in nutrient broth at 37°C was harvested by centrifugation, washed and resuspended in 0.15 M saline to give 3 x 10^8 cells per ml.

Antibodies: Anti O117 antiserum raised in a rabbit against heated E. coli WF82 was used as the source of anti O antibody. The γ globulin was prepared from this by precipitation with sodium sulphate (Kekwick, 1940) and labelled with ^{125}I by the method of McFarlane (1958). The labelled antibody had an agglutination titre of 320. Anti K antibody was prepared as described on p. 101.

1.0 ml amounts of various dilutions of anti O antibody in 0.15 M saline were mixed with 1.0 ml volumes of various dilutions of anti K antibody in saline. In the control 1.0 ml of saline was mixed with 1.0 ml of anti O antibody.

1.0 ml of bacterial suspension was added to the mixture of anti O and anti K antibodies. The bacteria were incubated with the antibodies on ice for 1 hour and then washed three times by centrifugation and resuspension in 0.15 M saline.

The radioactivity and optical density of a 2.0 ml sample of the bacterial suspension was measured and the counts per 100 sec (equals bound antibody) per 2 x 10^8 bacteria calculated.

LD_{50} of strains of E. coli for mice

The virulence of strains of E. coli for female albino mice weighing 15-20 g was assessed by intracerebral challenge. Log. phase bacteria suspended in 0.15 M saline were adjusted to the required concentrations by optical density. The number of viable cells were counted by diluting and plating samples. 0.025 ml of bacteria suspended in saline were injected intracerebrally and the times of death recorded. The LD_{50} was calculated by the method of Reed and Muench (1938).

RESULTS

Susceptibility to phagocytosis and the opsonic activity of anti O and anti K antibody

The susceptibility to phagocytosis of strains of E. coli was studied by following their disappearance from the mouse circulation following intravenous injection. The phagocytic index varied for strains within a serotype as well as for strains of different serotypes.

When four strains of E. coli serotype 06:K13 were compared (Fig. 13) as the K antigen content of the strain increased there was an associated decrease in susceptibility to phagocytosis by the reticulo endothelial system.

The phagocytotic index of strains with different serotypes

was compared with their sensitivity to C' (Table 7 and Fig. 14). Decreased susceptibility to phagocytosis was associated with increased resistance to the bactericidal action of human serum.

The effect of anti O and anti K antibody on the phagocytic index is shown in Table 8. The C' sensitive strain WF96 was presumably being cleared so rapidly that the addition of anti O or anti K antibody had no stimulatory effect on the rate of disappearance of bacteria from the blood stream.

The other three strains WF26, WF8 and WF82 were all resistant to C' and the same result was observed with each of these strains. Although homologous anti O antibody had no marked effect on the phagocytic index anti serum containing homologous anti K antibody markedly increased the rate of disappearance of C' resistant bacteria from the circulation.

The effect of anti K antibody on the bactericidal action of normal human serum

E. coli WF96 was very sensitive to C' and was rapidly killed by a 1/10 dilution of N.H.S. Addition of an antiserum containing anti O and anti K antibody did not augment the killing achieved by N.H.S. alone (Fig. 16). This lack of any detectable enhancement of killing by the addition

of anti K antibody could have been because killing was so rapid anyway. Anti K antibody might still have been expected to improve the killing of strains that were less sensitive to C' such as E. coli WF98 and E. coli WF26. No such effect was detectable for E. coli WF26 (Fig. 17). For WF98 a very slight enhancement of bactericidal activity was achieved when an anti OK serum concentration of 1/500 was used (Fig. 18). The addition of antiserum containing anti K antibody did not cause WF98 to be killed by C' and antibody to anything like the same extent as WF96.

Bactericidal and bacteriolytic action of anti O and anti K antibody and piglet C'

When precolostral piglet serum was incubated with E. coli WF96 it had only a slight bacteriolytic effect (Figs. 19 and 20). It was not demonstrated to be bactericidal (Figs. 21 and 22). If antisera containing either anti O or both anti O and anti K antibodies were added to the piglet serum both killing and lysis of the bacteria could be demonstrated (Figs. 19-22). Heated piglet serum to which anti O and anti K antibodies had been added produced negligible lysis (Fig. 20) and no killing (Fig. 22).

When the anti OK antiserum was absorbed with live bacteria it did not cause any more lysis or killing of bacteria than was achieved by piglet serum alone. This was so whether

antibody was added to the cuvette containing the bacteria or if bacteria were sensitised with antiserum prior to mixing with piglet serum (Figs. 19-22).

If anti OK antiserum was absorbed with heated bacteria to remove anti O but not anti K antibody the bactericidal and bacteriolytic activity of the antiserum was markedly reduced (Figs. 19-22).

No lysis was demonstrated if the anti K antiserum was added to the bacteria in the cuvette (Fig. 19). If bacteria were sensitised with anti K antibody before they were incubated with C' the extent of lysis achieved was 18% after 45 minutes, compared with the 11% lysis produced by piglet serum alone (Fig. 20).

Absorption of anti WF96 OK serum with heated bacteria reduced the anti O antibody titre from 1280 to <20. The anti K antibody titre remained at 5120. Whether the anti OK or anti K serum were added to the cuvette to give a final concentration of 1/100 or if bacteria were sensitised with 1/20 antisera prior to mixing with C' a reduction in killing from 99.9% to 40-50% was associated with the removal of the anti O antibody (Figs. 21 and 22).

Lysis by anti K antibody and C' of sheep red cells sensitised with K antigen

When the ability of anti K antibody to fix C' was

examined no haemolysis could be demonstrated using an antiserum concentration of 1/400 although the agglutination titre of the antiserum used was 1000 against red cells sensitised with K antigen. Haemolysis due to anti K antibody could be demonstrated using an antiserum concentration of 1/100. Anti K antibody was therefore capable of fixing C' (Fig. 23).

These results contrast with the usual observation that the haemolytic reaction is a more sensitive method of detecting antibody than agglutination reactions (Neter et al., 1952).

The effect of anti K antibody on the amount of anti O antibody bound to bacteria

From Fig. 24 it can be concluded that the addition of anti K antibody to a mixture of anti O antibody and bacteria neither increased or reduced the amount of anti O antibody bound to the bacteria.

The opsonic effect of anti K antibody (Table 8) is therefore due to the anti K antibody per se and is not because the anti K antibody "neutralises" the K antigen making it possible for more anti O antibody to bind to the cell and opsonise it.

Virulence of E. coli related to C' sensitivity

When mice were infected intracerebrally with E. coli

there was up to 7 $\log_{10}$ units difference in the LD_{50} of the strains (Fig. 15 and Table 7). Increased resistance to the bactericidal action of N.H.S. was associated with increased mouse virulence. An exception was E. coli WF95 which was more virulent than would be expected from its C^9 sensitivity.

Table 6. Agglutination titres of antisera

Bacteria against which antisera were raised	Antisera absorbed with:	Antibodies present	Bacterial agglutination titres	
			Against live cells	Against heated cells
Live WF82 O117:K?	-	anti OK	640	2560
Heated WF82 O117:K?	-	anti O	<10	2560
Live WF82 O117:K?	alkali treated phenol-water extract of WF82	anti K	640	<20
Live WF98 O6:K13	-	anti OK	2560	2560
Heated WF96 O7:K1	-	anti O	<10	2000
Live WF96 O7:K1	-	anti OK	5120	1280
Live WF96 O7:K1	heated WF96	anti K	5120	<20
Live WF96 O7:K1	live WF96	anti (K)	40	<20

- indicates the antisera were not absorbed

(K) anti K antibody concentration low.

Table 7. Phagocytic index and virulence of strains of E. coli related to their complement sensitivity

Strain	Serotype	C° sensitivity $C°H_{50}$ killing 50% of the inoculum	Phagocytic Index	LD_{50} (i.c.)
WF41	017:K16	<0.7	0.180	5×10^6
WF96	07:K1	0.8	0.150	5×10^5
WF95	0117:K?	1.2	0.130	6×10^2
WF98	06:K13	8	0.144	1×10^3
WF86	06:K13	27	0.110	1×10^2
WF82	0117:K?	29	0.044	5×10^3
WF8	06:K13	>32.4	0.063	1×10^2
WF26	06:K13	>32.4	0.051	1×10^1

Table 8. Anti O and anti K antibodies as opsonins

Strain	Serotype	Phagocytic Index		
		Normal mice	+ Anti O antibody	+ Anti K antibody
WF26	06:K13	0.051	0.063	0.166
WF8	06:K13	0.063	0.069	0.103
WF82	0117:K?	0.044	0.058	0.140
WF96	07:K1	0.150	0.130	0.159

Fig. 13. Phagocytic index of four strains of *E. coli* serotype 06:K13 as a function of their K antigen content.

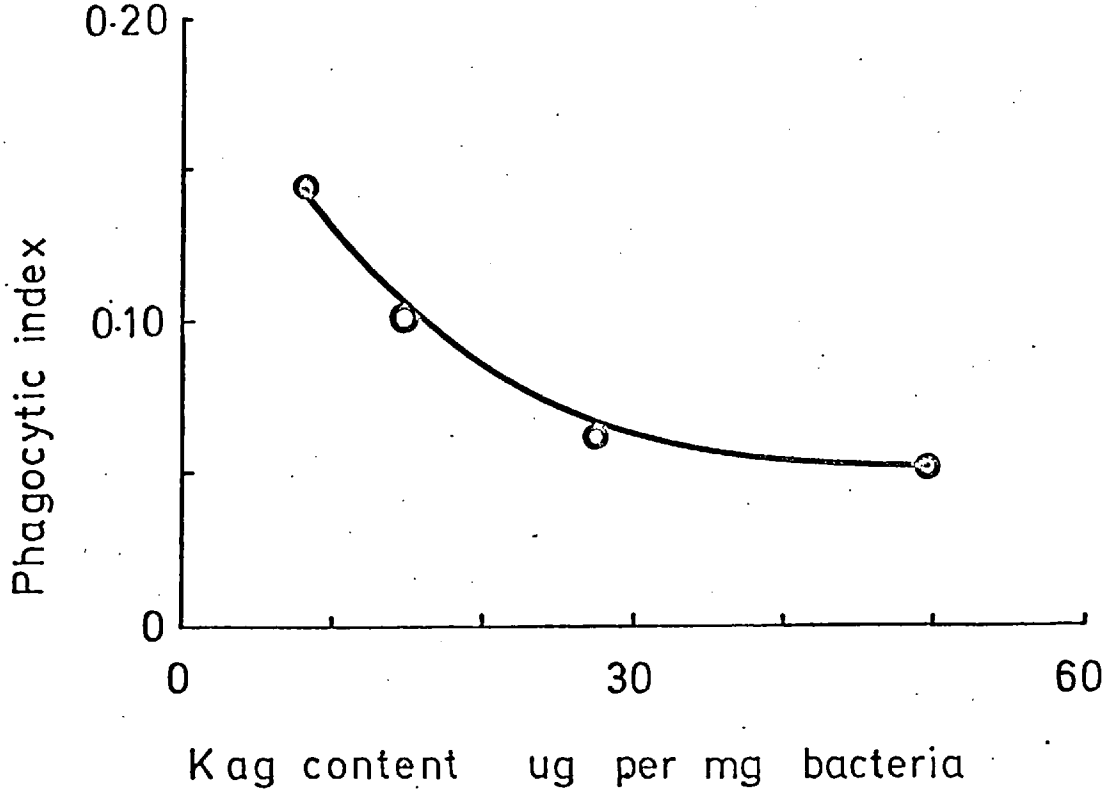
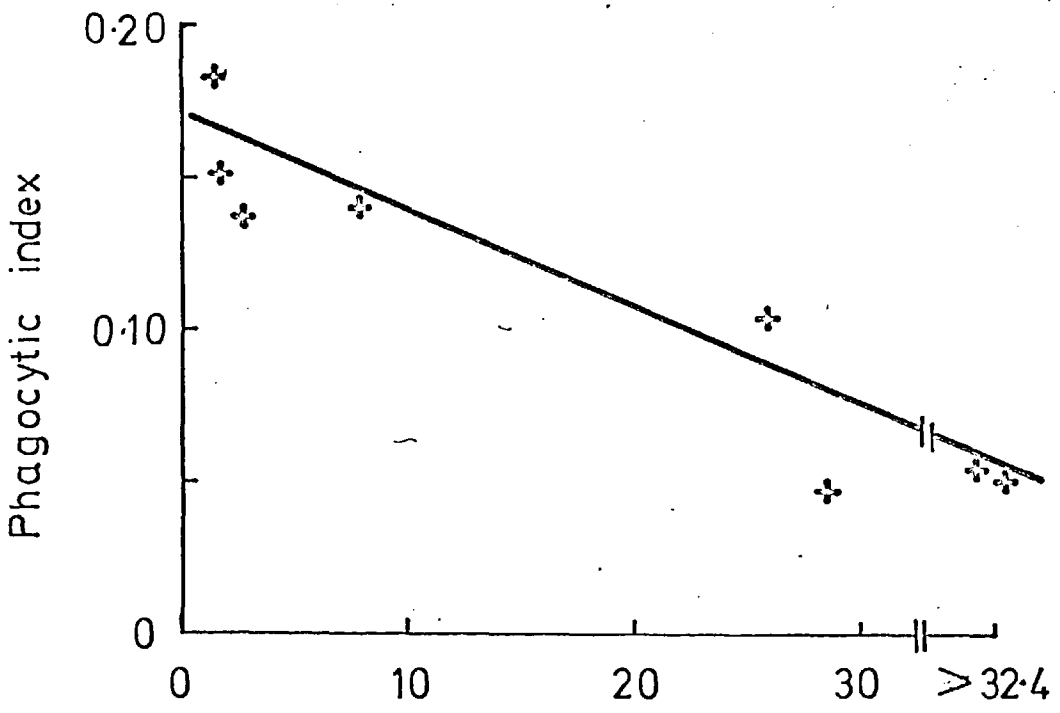

0·20
0·10
0
Phagocytic index
0
30
60
K ag content ug per mg bacteria

Fig. 14. Phagocytic index of strains of E. coli related to their C^9 sensitivity.



C′sensitivity. $C\bar{H}_{50}$ killing 50% of the inoculum

Fig. 15. Virulence for mice of 8 strains of *E. coli*, assessed by intracerebral challenge, related to C^0 sensitivity.

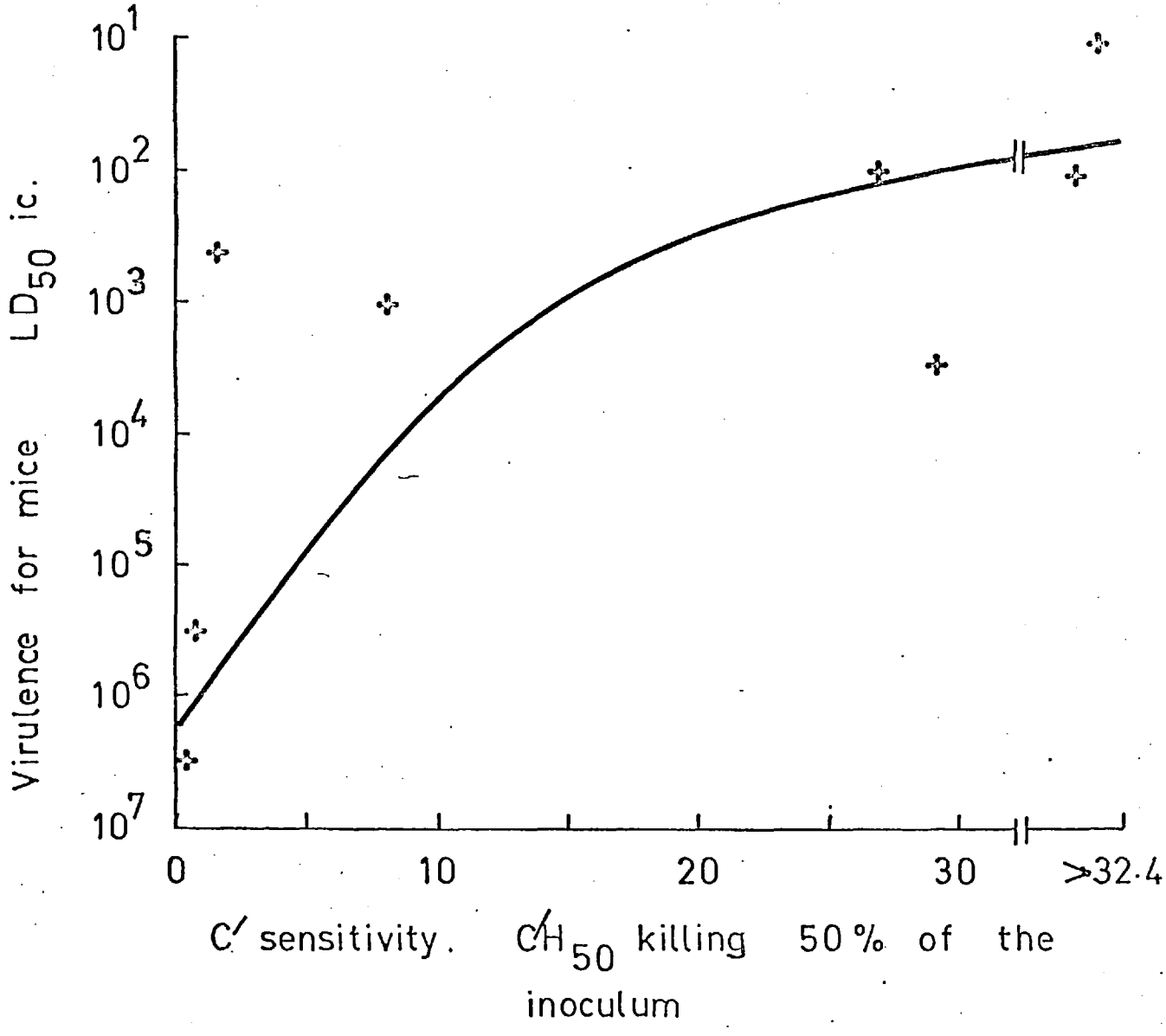

Virulence for mice LD_{50} ic.
10^1
10^2
10^3
10^4
10^5
10^6
10^7
0
10
20
30
>32·4
C′ sensitivity. CH_{50} killing 50 % of the inoculum

Fig. 16. Effect of an anti OK serum on the killing of *E. coli* WF96 by normal human serum.

H.S. - bacteria incubated with heated serum

C^{9} - bacteria incubated with normal human serum

1/500 ab - antibody concentration added.

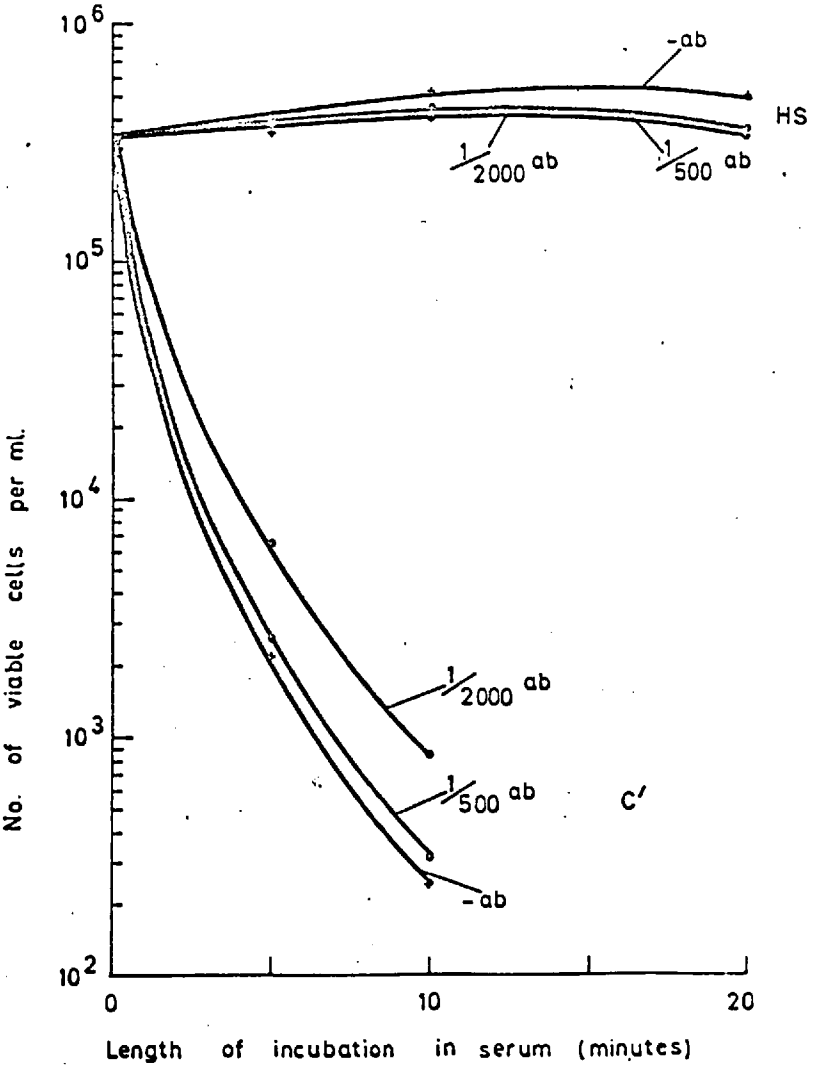

10^6
10^5
10^4
10^3
10^2
-ab
HS
1/2000 ab
1/500 ab
1/2000 ab
1/500 ab
C′
-ab
0
10
20
No. of viable cells per ml.
Length of incubation in serum (minutes)

Fig. 17. Effect of an anti OK serum on the bactericidal activity of normal human serum for E. coli WF26.

H.S. - bacteria incubated with heated serum

C° - bacteria incubated with normal human serum

1/500 ab - antibody concentration added.

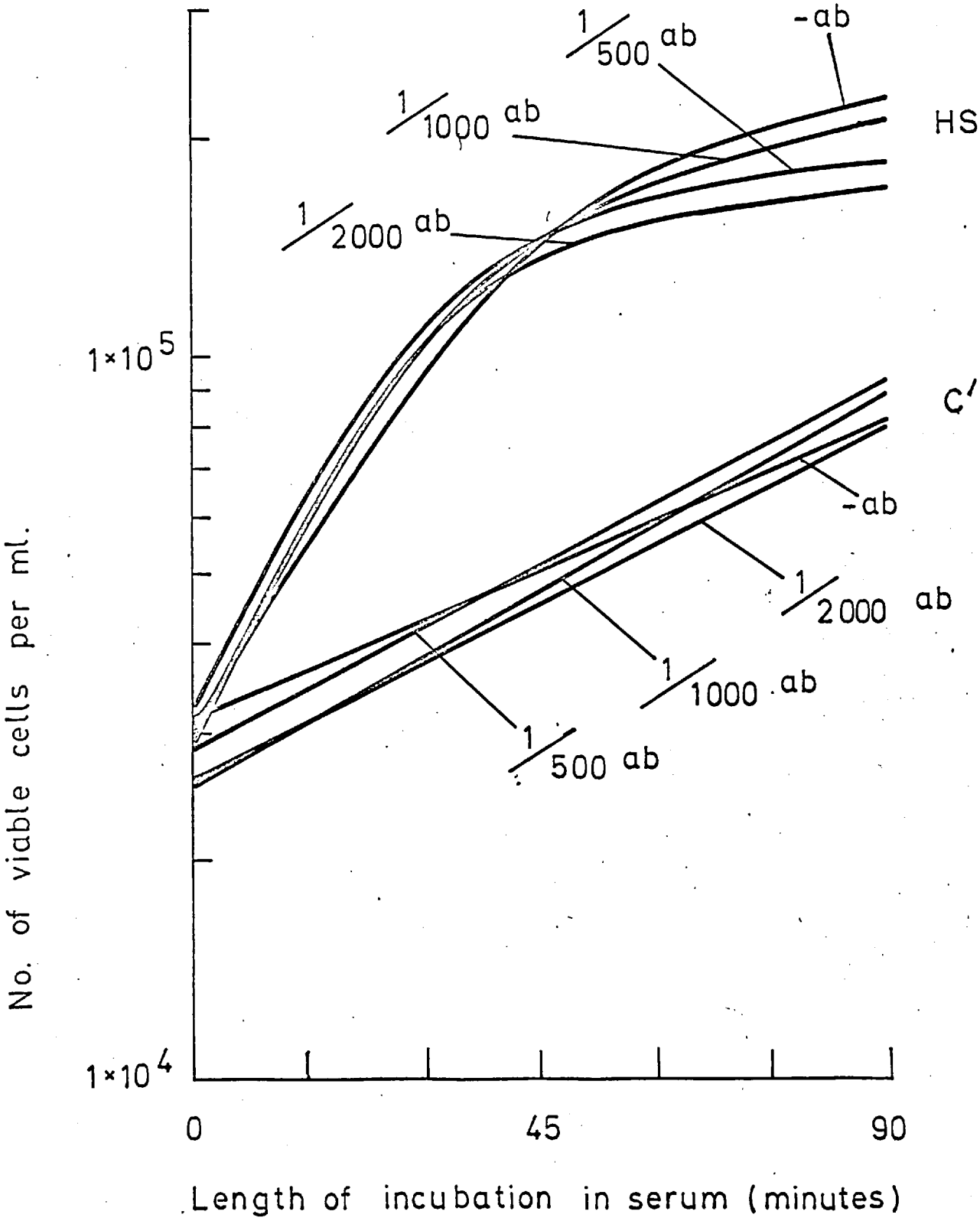

1/500 ab
-ab
1/1000 ab
HS
1/2000 ab
1×10^5
C'
-ab
1/2000 ab
1/1000 ab
1/500 ab
No. of viable cells per ml.
1×10^4
0
45
90
Length of incubation in serum (minutes)

Fig. 18. Effect of adding anti OK serum on the bactericidal activity of normal human serum for *E. coli* WF98.

H.S. - bacteria incubated with heated serum

C° - bacteria incubated with normal human serum

1/500 ab - antibody concentration added.

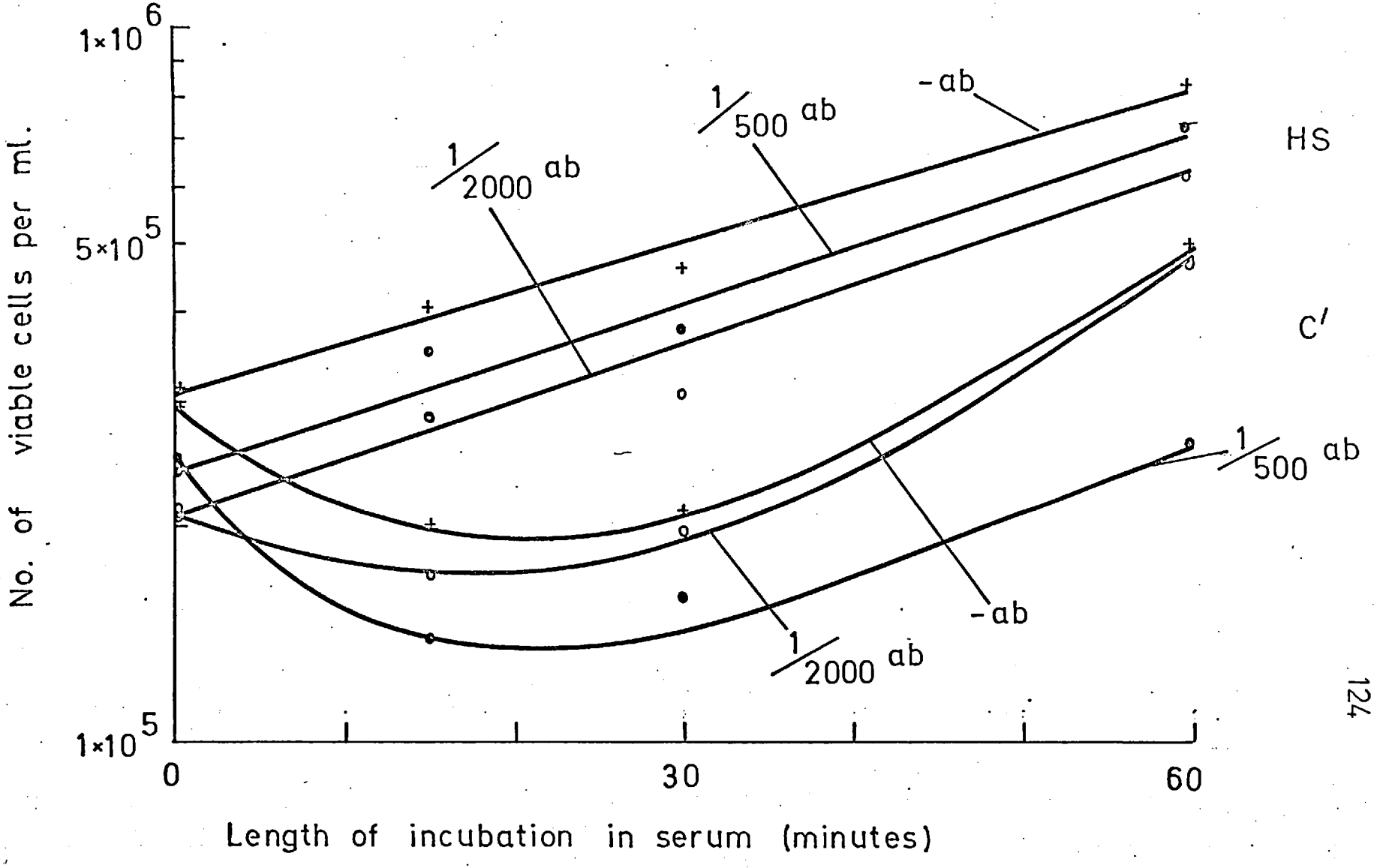

1×10^6
5×10^5
1×10^5
No. of viable cells per ml.
-ab
1/500 ab
1/2000 ab
HS
C′
1/500 ab
-ab
1/2000 ab
0
30
60
Length of incubation in serum (minutes)

Fig. 19. Bacteriolysis of *E. coli* WF96 by anti O and anti K antibody and piglet complement.

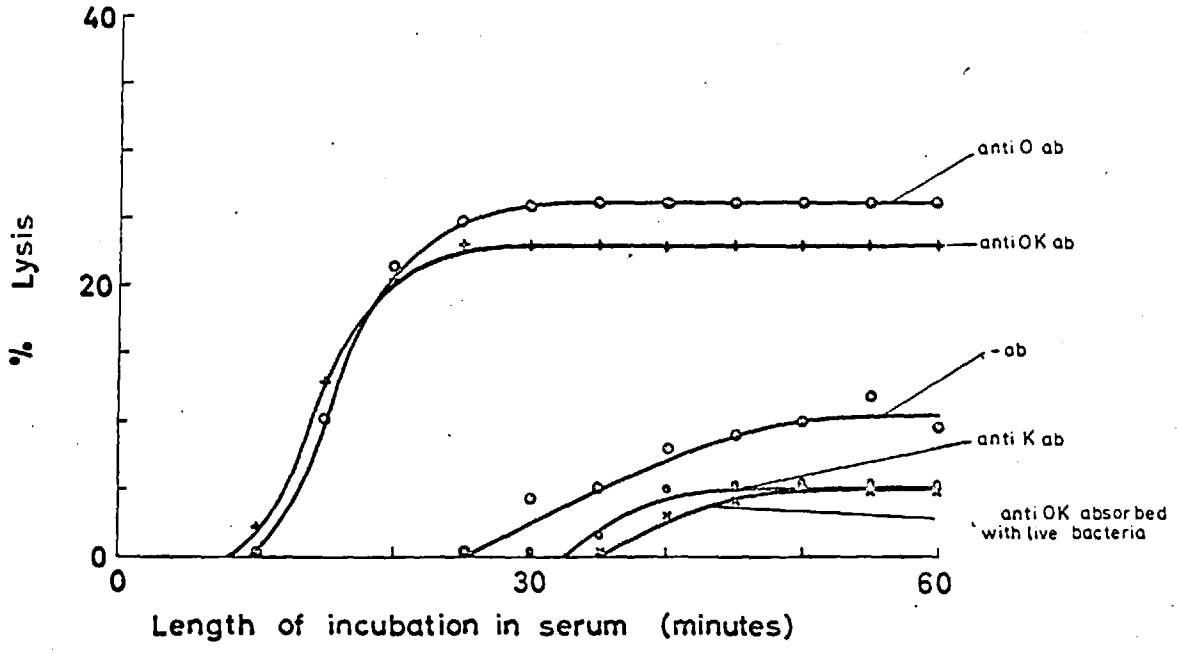

40
20
0
% Lysis
0
30
60
Length of incubation in serum (minutes)
anti O ab
anti OK ab
- ab
anti K ab
anti OK absorbed with live bacteria

Fig. 20. Complement dependent lysis of *E. coli* WF96 sensitised with anti O and anti K antibody.

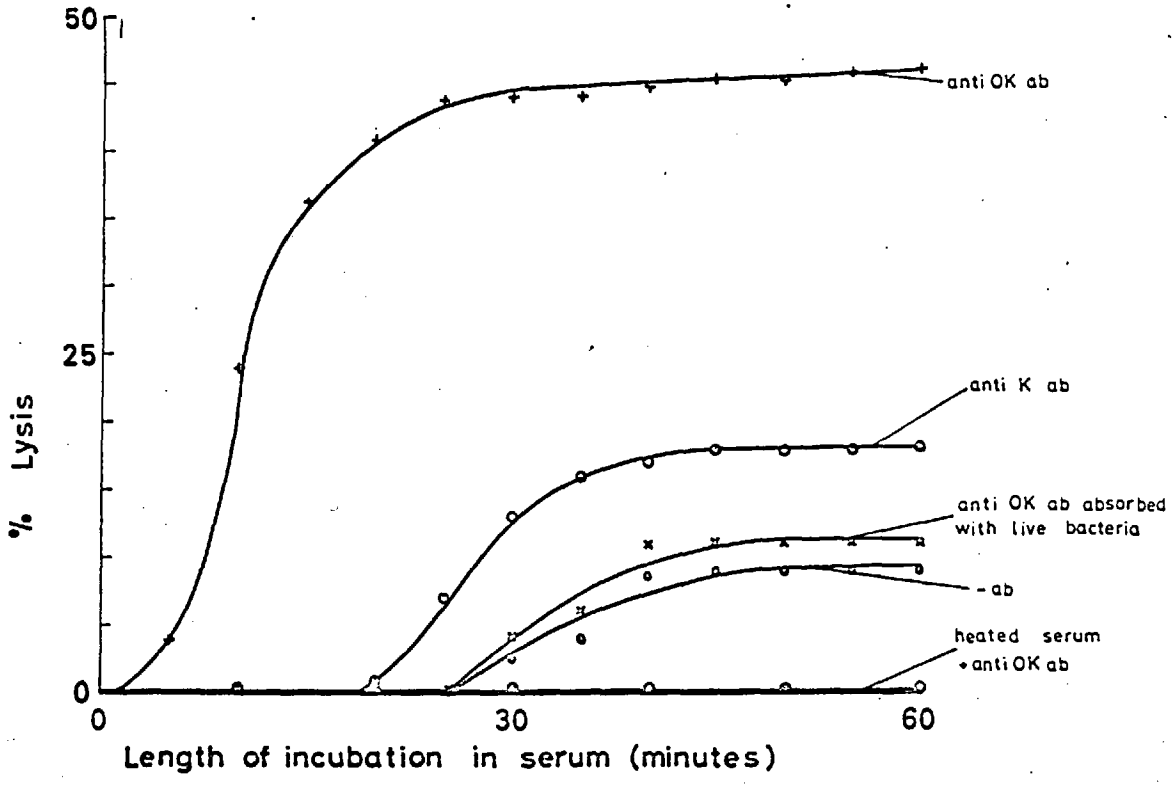

50
25
0
% Lysis
0
30
60
Length of incubation in serum (minutes)
anti OK ab
anti K ab
anti OK ab absorbed with live bacteria
- ab
heated serum + anti OK ab

Fig. 21. Bactericidal action of anti O and anti K antibody and piglet complement for *E. coli* WF96.

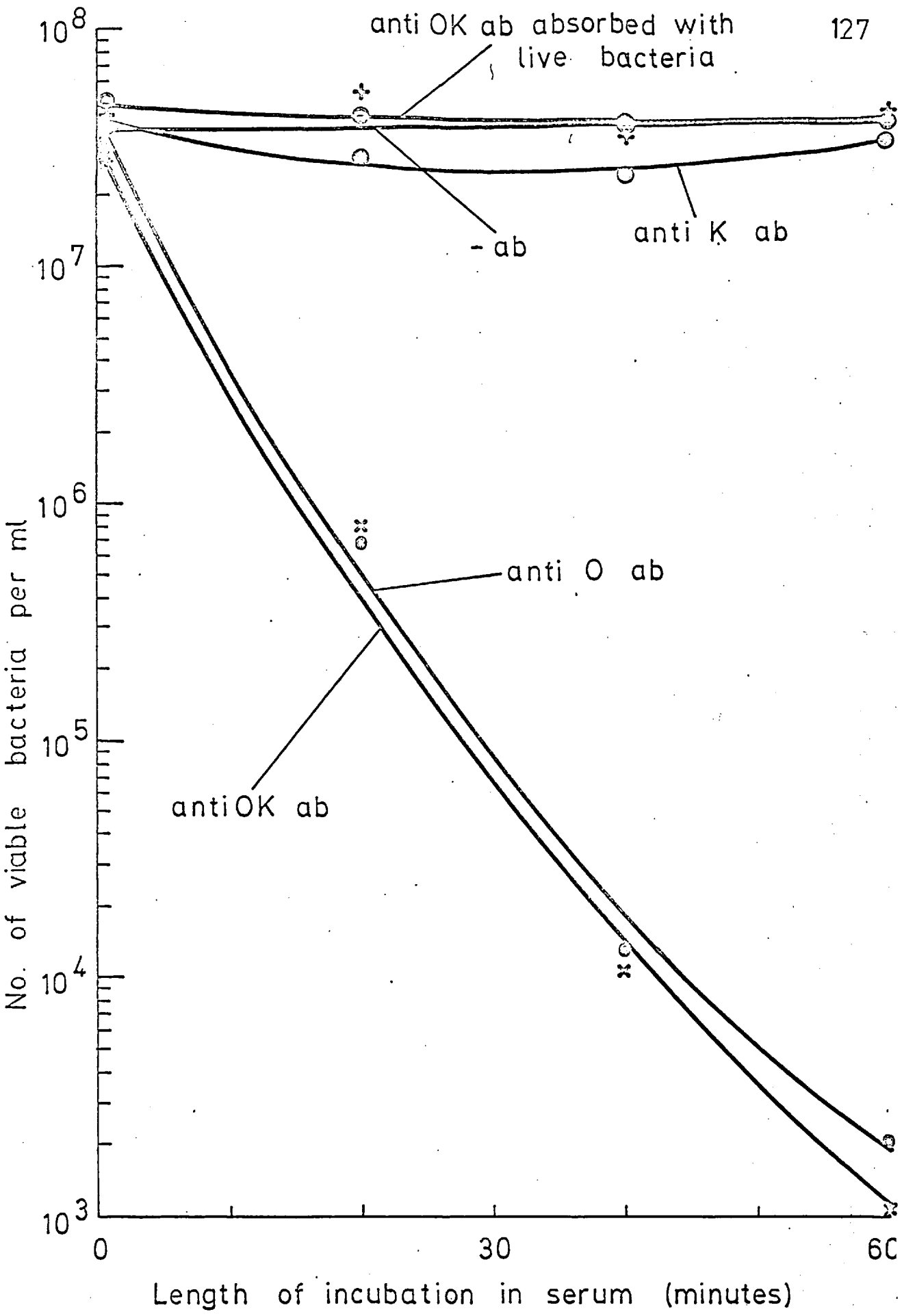

anti OK ab absorbed with live bacteria
- ab
anti K ab
anti O ab
anti OK ab
No. of viable bacteria per ml
10^8
10^7
10^6
10^5
10^4
10^3
0
30
60
Length of incubation in serum (minutes)

Fig. 22. Bactericidal action of piglet complement for *E. coli* WF96 sensitised with anti O and anti K antibody.

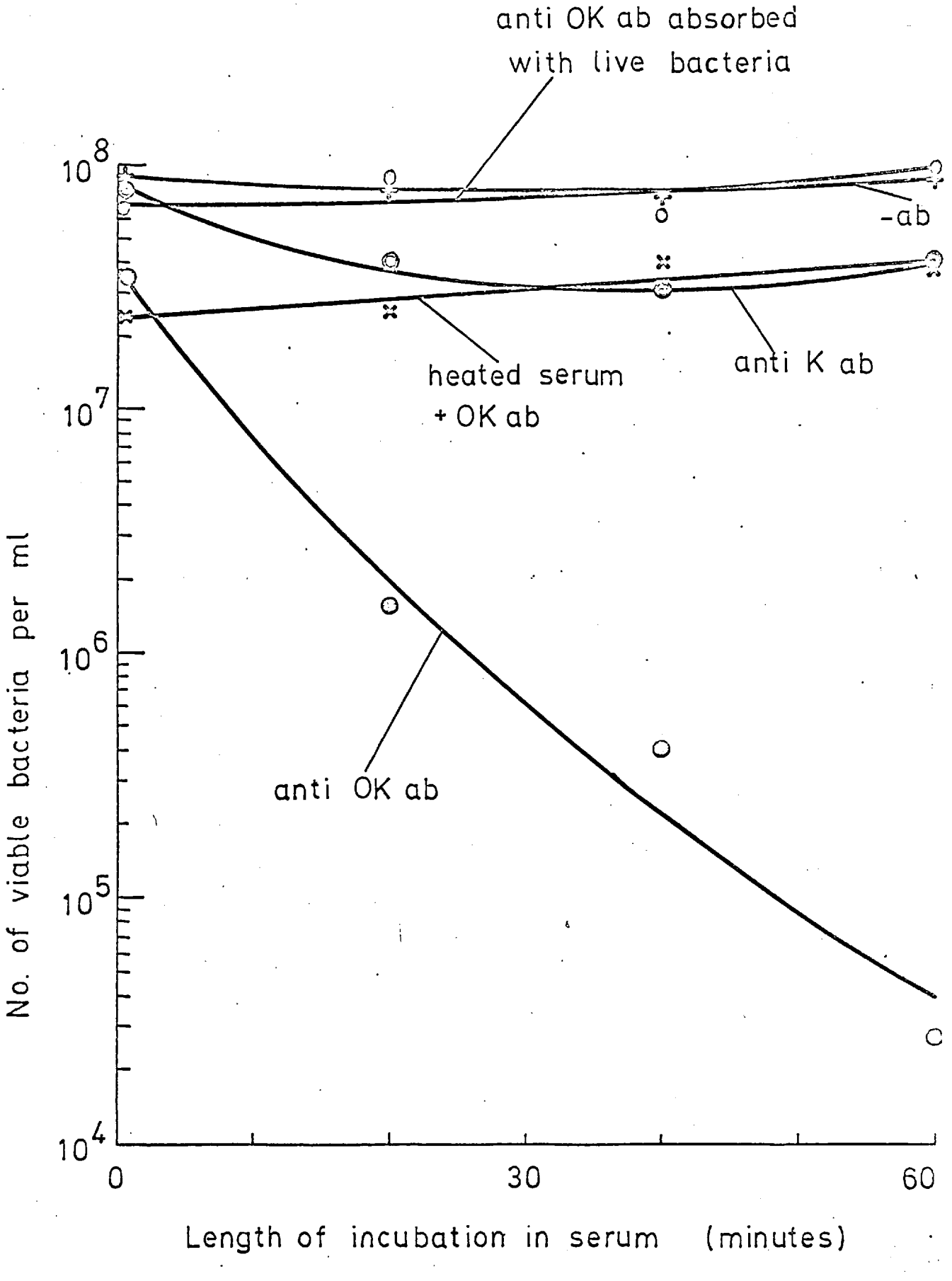

anti OK ab absorbed
with live bacteria
-ab
heated serum
+ OK ab
anti K ab
anti OK ab
No. of viable bacteria per ml
10^8
10^7
10^6
10^5
10^4
0
30
60
Length of incubation in serum (minutes)

Fig. 23. Demonstration of complement fixation by anti K antibody.

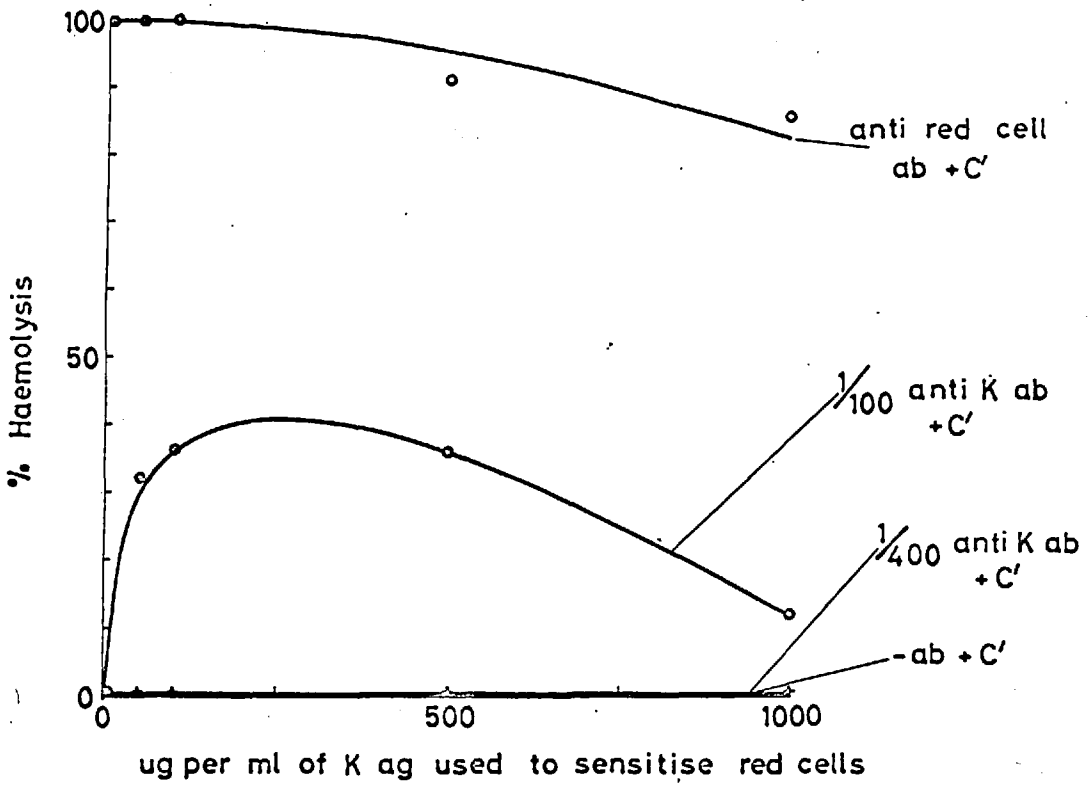
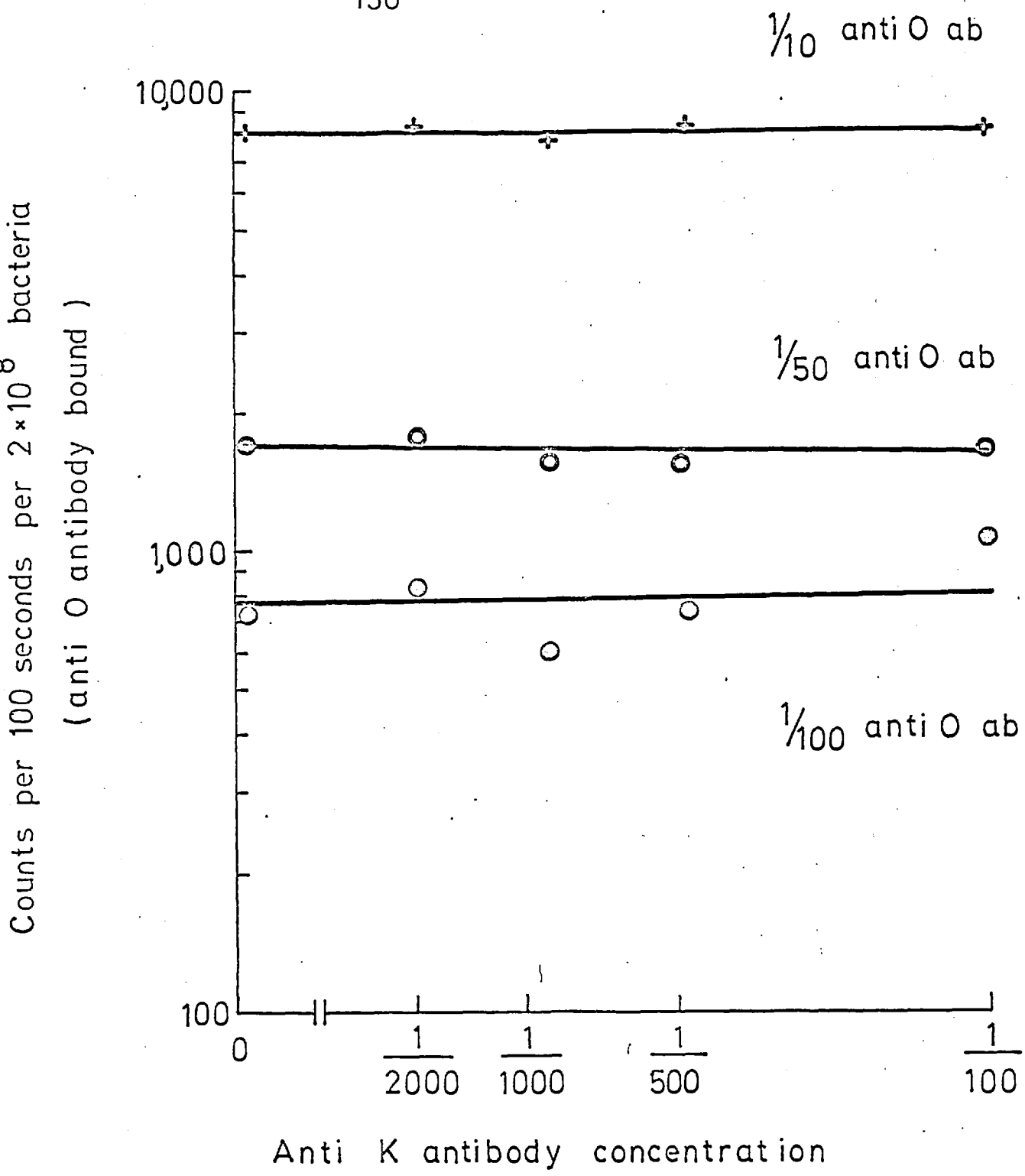

100
50
0
% Haemolysis
0
500
1000
ug per ml of K ag used to sensitise red cells
anti red cell ab +C'
1/100 anti K ab +C'
1/400 anti K ab +C'
-ab +C'

Fig. 24. Effect of anti K antibody on the binding of ^{125}I-anti O antibody to *E. coli* WF82.


1/10 anti O ab
1/50 anti O ab
1/100 anti O ab
10,000
1,000
100
0
1/2000
1/1000
1/500
1/100
Counts per 100 seconds per 2×10^8 bacteria
(anti O antibody bound)
Anti K antibody concentration

DISCUSSION

Four strains of E. coli serotype O6:K13 were compared and increased K antigen content was found to be associated with increased resistance to killing by human serum and to decreased susceptibility to phagocytosis by the reticulo-endothelial system (see Section II and Fig. 13).

Amongst other serotypes of E. coli increased resistance to the bactericidal action of serum was also associated with decreased susceptibility to phagocytosis (Fig. 14).

Strains of E. coli were tested for their mouse virulence by intracerebral challenge (Fig. 15). Although in general the more resistant a strain was to C' the lower the LD_{50} the relation was not absolute. E. coli WF95 for example was more virulent than might have been expected from its C' sensitivity.

In Section III it was suggested that the ability of K antigens to inhibit the binding of non homologous antibody prevented normal anti O antibody from sensitising bacteria to C'.

This property of K antigens could also explain why increased K antigen content was associated with decreased susceptibility to phagocytosis. By inhibiting the binding of anti O antibody K antigens would prevent normal anti O

antibody from opsonising bacteria. As the K antigen content of bacteria increased less anti O antibody would bind to the organisms and bacteria would be phagocytosed less.

If this was the mechanism by which K antigens affected C' sensitivity and susceptibility to phagocytosis then anti O and anti K antibodies would not be expected to be equally effective as opsonins. K antigen should prevent anti O antibody from binding so that strains rich in K antigen would not be opsonised by anti O. Anti K antibody should combine with K antigen on the bacterial cell surface and should be opsonic. C' sensitive strains of E. coli containing both O and K antigens should be opsonised by both anti K and anti O antibodies.

The results obtained when the opsonic effect of anti O and anti K antibodies were examined are given in Table 8. The C' sensitive strain WF96 was being cleared so rapidly that no stimulatory effect could be demonstrated by antibodies directed against either antigen. The other three strains were all more resistant to C' than WF96. Anti O antibody had little effect on the phagocytic index of any of these C' resistant strains. Antisera to the homologous K antigen markedly increased the phagocytosis of all three.

As anti K antibody was bound to C' resistant bacteria when anti O antibody was not, as judged by the opsonic effect

of the two antibodies, it seemed likely that anti K antibody might facilitate the killing of strains that were resistant to the bactericidal action of N.H.S.

The addition of antisera containing anti O and anti K antibody did not augment the bactericidal action of N.H.S. for E. coli strains WF96 and WF26. For E. coli WF98 a marginal increase in killing by N.H.S. was achieved when an antiserum concentration of 1/500 was used. The titre of this antiserum was 2560 against live and heated cells.

The lack of any marked enhancement of killing when antisera containing anti O and anti K antibody had been added to N.H.S. indicated that the availability of antibody was not the factor limiting the extent to which strains of E. coli were killed by N.H.S.

Use was made of precolostral piglet serum as a source of C' which was devoid of normal antibacterial antibody and the ability of anti O and anti K antibody to sensitise bacteria to killing by C' was examined (Figs. 19-22).

Anti O antibody was capable of sensitising cells to C'. The bactericidal activity of an antiserum which contained both anti O and anti K antibody appeared to be due in the main to the anti O antibody it contained. Absorption of anti O antibody from the anti OK serum reduced the extent to which bacteria were killed in 40 minutes from 99.9% of the inoculum

to 40-50%.

This amount of killing achieved using anti K antibody and C' could have been due to incomplete absorption of anti O antibody. However it seems likely that the 40-50% killing was due to anti K antibody since when the anti OK antiserum was absorbed with live bacteria, which removed both anti O and anti K antibody, the absorbed antiserum did not sensitise bacteria to killing at all.

If the 40-50% killing achieved with the anti K serum was due to antibody directed against the K antigen and not due to unabsorbed anti O antibody anti K antibody was at best much less effective than anti O antibody at sensitising bacteria to killing by C'.

The anti K antibody concentration used was 1/100 in the cuvette. The agglutination titre was 5120 against live WF96. Lack of killing would therefore not appear to be due to the lack of availability of anti K antibody in the system used.

There are several ways of explaining why anti K antibody was opsonic but less effective than anti O antibody at sensitising bacteria to C'.

One possibility was that anti K antibody was in fact not itself opsonic but that it "neutralised" the K antigen and so enabled anti O antibody to combine with O antigen and opsonise

the bacteria. Using anti K antibody and ^{125}I labelled anti O antibody this possibility was disproved (Fig. 24). Anti K antibody had no effect on the amount of ^{125}I labelled anti O antibody bound to the bacterial cell.

Another possibility was that anti K antibody did not fix C'. Sheep red cells were sensitised with K antigen and anti K antibody and C' were incubated with the sensitised cells (Fig. 23). Lysis of the sensitised red cells could be demonstrated and therefore the anti K antibody was fixing C'.

The extent of lysis was not as great as might have been expected from the concentrations of C' and antibody used. In the presence of anti sheep red cell antibody the C' concentration added was sufficient to cause 100% lysis. Lysis of the red cells sensitised with K antigen by anti K antibody and C' was not this great. K antigen is capable of inhibiting lysis of red cells by C' but the concentration used was not sufficient to explain the small amount of lysis achieved with K antibody.

Since the agglutination titre of the same anti K antiserum was 1000 against red cells sensitised with K antigen there was apparently no shortage of available antibody. Paucity of antigenic sites did not seem to be a very likely explanation since each acid polysaccharide molecule bearing the K antigen determinant group would be

expected to have many antigenic sites. The explanation may lie in the large size of the antigen which is greater than 10^6 judged by sepharose fractionation (see Section VII). The large size of the attached molecule may mean that C' becomes less effective at lysing the red cells because it is activated further away from its substrate, the red cell membrane in this case.

Humphrey and Dourmashkin (1969) found that when Type 3 pneumococcal capsular polysaccharide was attached to red cells more anti pneumococcal antibody was needed to produce haemolysis by C' than was needed to agglutinate the sensitised cells, although C' was fixed extensively.

Electron microscopy showed that the polysaccharide became aggregated by antibody into an uneven mass which was no longer close to the membrane.

This fits with the third possible explanation of why anti K antibody was less effective than anti O antibody as a "bactericidin". It is possible that anti K antibody activated C' too far from the lipopolysaccharide to be effective.

Experiments reported by Rowley and Turner (1968) support this theory. Proteins of different molecular weights were attached to the surface of a C' sensitive strain of S. adelaide. Antibody directed against the attached protein

and C' were added to the bacteria and it was found that as the size of the attached protein increased the antibody became less effective at sensitising bacteria to killing by C'. Antibody directed against aggregated γ globulin was totally ineffective at sensitising bacteria to killing by C' although it was opsonic (Rowley, Thoni and Isliker, 1965). This situation is analogous to that with K antigens where anti K antibody is opsonic but not bactericidal. There is also a similarity in the molecular weights, aggregated globulin being 1-2 x 10^6 (Rowley and Turner, 1968).

From these results a theory can be suggested on how K antigens affect the virulence of E. coli. Bacteria rich in K antigens will be resistant to two of the hosts defence mechanisms, killing by C' and phagocytosis. They will thus be able to grow in host tissues where strains that possess a small amount of K antigen or none will be killed.

A characteristic of virulent E. coli is their ability to multiply from a small inoculum and to continue to grow until the host dies, presumably from the mass of endotoxin present (Rowley, 1954). Rowley also showed that the resistance to serum of E. coli was associated with increased virulence for mice judged by intraperitoneal challenge in mucin.

Kauffmann (1954) compared a strain of E. coli possessing both O and K antigens with an "acapsular" variant derived from it. The LD_{50} for mice of the K- strain injected

intraperitoneally was greater than that of the original K+ strain. Wolberg and DeWitt (1969) performed similar experiments in which they compared two strains serotype 02:K1 with their K- variants. The K+ strains had LD_{50}'s of <10 when injected intraperitoneally with mucin compared with LD_{50}'s of 10^7 and 10^8 for the K- variants. They also found using an *in vitro* phagocytosis system that the K+ strain was less susceptible to phagocytosis than the K- variant and that an antiserum containing anti K antibody was more effective at opsonising the K+ strain than anti O antibody. Passive administration of antisera containing anti K antibody was found to be protective against challenge with the K+ strain whereas anti O antibody had a minimal effect.

Like the majority of K antigens the Vi antigen of *S. typhi* is an acidic polysaccharide (Luderitz *et al.*, 1968). Kauffmann (1954) stated that its properties were like those of the B type of K antigen. The existence of the Vi antigen was demonstrated by Felix and Pitt (1934b) who also showed that the amount of antigen present in *S. typhi* strains was directly related to their mouse virulence measured by the results of intraperitoneal challenge. Anti Vi antibody administered passively or raised by immunisation with Vi

containing strains of S. typhi protected mice against intraperitoneal challenge with virulent strains.

If mice were injected intracerebrally with S. typhi infections could be established with small numbers of bacteria without it being necessary to use a mucin adjuvant (Landy et al. 1957a). Virulence was dependent on both the O and Vi antigens being present and a characteristic of the virulent strain was its ability to multiply from a small inoculum. Intraperitoneal vaccination with strains containing Vi antigen protected against intracerebral challenge but vaccination with bacteria containing O and not Vi antigen did not. Rabbit anti Vi antibody was protective when given passively but anti O antibody was not.

O inagglutinable strains of S. typhi were characterised by their decreased susceptibility to phagocytosis compared with Vi- avirulent strains (Bhatnager, 1935). Anti Vi antibody has also been found to be more effective at opsonising O inagglutinable strains than anti O antibody (Felix and Bhatnager, 1935). Much evidence has therefore been accumulated associating the Vi and K antigen content of S. typhi and E. coli with virulence as judged by experimental infections induced by various routes. Increased mouse virulence amongst these strains has also been associated with resistance to killing by C^9 and resistance to phagocytosis.

Other reports have related the susceptibility of E. coli to killing in vivo to C⁹ sensitivity in vitro. Roantree and Pappas (1960) injected paired strains of E. coli, one serum resistant and one serum sensitive, into rabbits and measured the disappearance of viable organisms from the blood. The strains could be distinguished by their antibiotic sensitivities. Although the number of viable cells of both strains in the blood rapidly decreased, the strain that was resistant to C⁹ in vitro was killed less rapidly in vivo. No attempt was made to distinguish between killing due to C⁹ or removal of viable bacteria from the circulation due to phagocytosis by the R.E.S.

The killing of paired C⁹ resistant and C⁹ sensitive strains of E. coli in the guinea pig peritoneum was compared by Roantree and Collis (1960). The bacteria were put in chambers which allowed body fluids to diffuse in but not cells. Strains that were resistant to C⁹ in vitro usually multiplied in vivo whereas strains that were sensitive to C⁹ in vitro were killed in the chambers.

Although mouse C⁹ has not been shown to be bactericidal in vitro it is in vivo. The rate at which viable bacteria disappeared from the mouse circulation was compared with the rate at which ^{14}C labelled bacterial cells were removed in normal and C⁹ deficient mice by Glynn and Medhurst (1967). They demonstrated killing of a C⁹ sensitive strain of E. coli, but not of a C⁹ resistant strain, by C⁹ in the mouse circulation.

From the experiments quoted above resistance to C' would be expected to be one of the characteristics of strains of E.coli necessary for the bacterium to survive and grow in the host tissues. Reports have appeared suggesting that resistance to C' is a characteristic of strains of E. coli isolated from human systemic infections. Roantree and Rantz (1960) showed that strains of E. coli that had been isolated from human blood were generally more resistant to C' than strains isolated from faeces. Waisbren et al. (1959) reported 3 cases of generalised E. coli infections in adults. These strains did not belong to any of the enteropathogenic serotypes. Their LD_{50}'s for mice challenged intraperitoneally with mucin adjuvant was lower than that of any of the enteropathogenic serotypes they were compared with and the authors state that the strains were serum resistant.

Jacks and Glantz (1967) found that the mouse virulence of E. coli injected intraperitoneally in mucin was related to the source of the strains. Strains from systemic human and animal infections generally being more mouse virulent than strains from enteric infections.

These papers all support the view that strains from human systemic infections are generally more mouse virulent and more resistant to C' than strains from enteric sources.

Since K antigens affect the C^9 sensitivity and mouse virulence of *E. coli* they are probably one of the characteristics affecting the ability of *E. coli* strains to cause systemic infections in man.

The state of the O antigen also affects the mouse virulence of *E. coli*. Medearis *et al.* (1968) compared a strain of *E. coli* serotype O111:B4 with mutants derived from it that were unable to synthesise the total O antigen polysaccharide present in the original strain. Loss of colitose from the O antigen resulted in loss of virulence as did loss of the total O antigen polysaccharide repeating unit.. Correlated with decreased virulence associated with impairment of O antigen synthesis was an increased susceptibilty to phagocytosis.

Sjostedt (1946) investigated the possibility that anti K antibody might stimulate the bactericidal activity of blood against strains of *E. coli*. The method he used was to add *E. coli* to heparinised blood, the source of C^9, and to estimate the number of viable cells present after 18 hours incubation at 37°C by spreading a loopfull of culture on a plate. He was unable to demonstrate any enhancement of killing when anti K antibody was added. As he found no difference in killing whether he used defibrinated, citrated or heparinised blood he concluded that killing in whole blood was due to

serum factors and not because of phagocytosis.

This qualitative way of testing for serum sensitivity would not be expected to detect small changes in bactericidal activity due to anti K antibody and the C' present could loose its activity over this length of incubation at 37°C. When bacteria are grown in serum instead of broth they may be phenotypically converted to a serum sensitive state (Muschel, 1965; Osawas and Muschel, 1964a).

The physiological state of the organisms after the length of incubation in serum used by Sjostedt may well not have been the same as that of the bacteria used to inoculate the blood. Precise deductions of what was happening in the system used would therefore be difficult to make.

Muschel and Treffers (1956b) examined the bactericidal activity of Vi antibody raised in rabbits against a strain of Paracolobactrum ballerup which contained an antigen serologically identical to the Vi antigen of S. typhi. Anti Vi antibody and guinea pig C' were incubated with the bacteria but no bactericidal activity could be demonstrated.

Osawa and Muschel (1964b) compared anti O and anti Vi antibody for their bactericidal activity against a strain of S. typhi Ty 2. They found that antibody directed against the O antigen was more effective at sensitising bacteria to C' than antibody directed against the Vi antigen. Absorption

of anti O antibody from an antiserum which contained both anti O and anti Vi antibody reduced the bactericidal antibody titre of the antiserum by 90%.

Nagington (1956) found that both anti O and anti Vi antibodies were capable of sensitising S. typhi to C' and that as the Vi antigen content of strains increased there was an associated decrease in the ability of both the O and Vi antibody to sensitise bacteria to C'.

A hypothesis on the mechanism by which K antigens affect the properties of the bacterial cell can be suggested from these results.

Differences in the composition of the bacterial cell will affect where antibodies are bound and whether they are bound at all. If a bacterium does not contain any K antigen anti O antibody would be expected to combine with its substrate and sensitise bacteria to C'. Loss of the specific polysaccharide side chains responsible for O antigenicity may well result in increased C' sensitivity because rough antigenic determinants are nearer the C' substrate in the lipopolysaccharide than the O specific side chains so C' is activated nearer its substrate and is more effective. Bacteria which contain less endotoxin may be more sensitive to C' because there is less C' substrate to be digested before the bacterium is damaged (Wardlaw, 1964).

As the K antigen content of bacteria increases the amount of anti O antibody which binds to the organism decreases. K antigens therefore prevent anti O antibody from sensitising bacteria to C^{9} or from opsonising them. The amount of K antigen present is the property controlling the degree of C^{9} sensitivity and susceptibility to phagocytosis.

If bacteria contain sufficient K antigen to prevent killing by anti O antibody and C^{9} then anti K antibody is also ineffective because C^{9} is not activated in a suitable situation.

If the organisms contain a small amount of K antigen anti K antibody, if effective at all, is much less effective than anti O antibody at sensitising bacteria to C^{9}. This probably reflects the site at which anti K antibody is bound and the distance from the lipopolysaccharide at which C^{9} is activated by the anti K antibody K antigen complex.

Anti K antibody opsonises bacteria containing K antigens which are not opsonised by anti O antibody since this reaction occurs on the surface of the organism. Bacteria which contain K antigens but which are C^{9} sensitive and are opsonised by anti O antibody would also be expected to be opsonised by anti K antibody.

K antigens will thus enable bacteria to persist and multiply in the host tissues and affect bacterial virulence in this way.

V. K ANTIGENS AND URINARY TRACT INFECTIONS

INTRODUCTION

The urinary tract is considered to be infected when the number of bacteria per ml. of urine is more than 10^5. This figure was proposed by Kass (Norden and Kass, 1968). If the urine contains this number of organisms the patient is said to have significant bacteriuria. When the concentration of bacteria is below this level the organisms present are considered to be due to urethral contamination of the urine.

Gould (1968) stated that 91% of patients presenting with significant bacteriuria who had no previous history of urinary tract infections were infected with Escherichia coli. 38% of patients who had previously suffered a urinary tract infection had only E. coli present in the urine. 47% of patients with a past history of infection who had mixed infections had E. coli present. These figures demonstrate that in the majority of cases human urinary tract infections are caused by E. coli.

Depending on their social class between 2 and 13% of pregnant women have been found to become infected (Norden and Kass, 1968).

Pregnant women with significant bacteriuria but no symptoms of pyelonephritis were divided into two groups. One group was treated with antibiotics to free them from their

bacteriuria the other group serving as controls. Although 20% of the control group developed symptomatic pyelonephritis there was virtually no risk of developing pyelonephritis of pregnancy amongst women who had been freed of their infection (Norden and Kass, 1968). It therefore appears that pyelonephritis may develop from a urine infection.

The source of the infecting bacteria is considered to be the faeces (Gruneberg, et al., 1968). Patients with significant bacteriuria may have an infection that is confined to the bladder or the renal tissues may be involved.

Since the K antigens of E. coli had been shown to affect the virulence of bacteria in experimental infections the possibility was examined that these antigens might be of importance in determining whether or not bacteria were able to invade and colonise the renal tissue.

Strains of E. coli that had been isolated from pregnant women with significant bacteriuria were examined for their K antigen content. Bacteria were divided into two groups one of which consisted of bacteria considered to be confined to the bladder, a lower urinary tract infection, the other group consisted of bacteria considered to have invaded the renal tissue, an upper urinary tract infection.

Bacteria were divided into these two groups on the basis of the anti O antibody titre of the patient from whom they had

been isolated (Percival et al., 1964) or on the basis of the results of the technique proposed by Fairley et al. (1967)

The K antigen content of bacteria causing upper or lower urinary tract infections and of faecal isolates was compared to see if evidence could be obtained that K antigens affected the pathogenesis of renal infection by E. coli.

MATERIALS AND METHODS

The K antigen content of urinary strains of E. coli was estimated using the method described in Section II. By measuring the agglutination inhibiting activity of bacterial extracts, which was dependent on the amount of K antigen bacteria contained, it was possible to compare the K antigen content of strains with different serotypes.

Bacteria

Strains of E. coli isolated from patients with significant bacteriuria were provided by Dr. W. Brumfitt from Edgware General Hospital. The agglutination inhibiting activity of these strains was compared with that of strains of E. coli isolated from rectal swabs at the routine bacteriology department of St. Mary's Hospital.

Bacteria were grown overnight on nutrient agar at 37°C and acetone dried. Dried cells were suspended in 0.15 M saline to give a final concentration of 10 mg/ml and the

suspension was homogenised for three minutes on ice using an "Ultra Turrax" homogeniser. After centrifugation the supernatant was collected and kept frozen at -20°C until it was required to measure the agglutination inhibiting activity.

Measuring agglutination inhibiting activity

The method used was a slight modification of the procedure recorded in Section II. Doubling dilutions of the bacterial extracts were made in Dulbecco A and these were incubated with an equal volume of 1% v/v sheep red cells in the same buffer at 37°C for 30 minutes.

Doubling dilutions of an anti sheep red cell serum, obtained from Staynes Laboratories Ltd., were made up in the same buffer and 0.1 ml volumes added to a WHO haemagglutination tray so that antibody concentration decreased from left to right. 0.1 ml of extract treated red cells together with control cells that had been incubated with buffer alone were mixed with the antiserum so that the extract concentration decreased from the top to the bottom of the tray. The trays were incubated for 1 hr. at 37°C and overnight at 4°C.

The titre of the anti sheep red cell serum against untreated red cells was compared with the titre against extract treated cells. The activity of the bacterial extract

was recorded as the reciprocal of the highest dilution of the extract that would reduce the haemagglutinating activity of the antiserum by 50%. The activity was converted into $\log_2$ units to simplify statistical analyses performed on the results.

RESULTS

The patient from whom the strains were isolated, together with the code number of the strain and the O serotype are given in Table 9. The results of the Fairley test (Fairley et al., 1967) are recorded as L, indicating bacteria were confined to the bladder causing a lower urinary tract infection, or as U meaning bacteria were present in the uteric urine indicating an upper urinary tract infection. The anti O agglutination titre of the patients serum determined by bacterial agglutination is also recorded (Percival et al., 1964). Bacteria, O serotyping, the results of the Fairley test and the anti O titre were provided by Dr. W. Brumfitt of the Edgware General Hospital, Edgware, Middlesex. The final column in Table 9 records the agglutination inhibiting activity of the various strains which represents the K antigen content of the bacteria.

Table 10 shows the agglutination inhibiting activity of 20 strains of E. coli isolated from faecal samples. These

bacteria were examined as a control group providing a value for the K antigen content of faecal strains of E. coli.

Relation between K antigen content of strains of E. coli and ability to cause upper or lower urinary tract infections as judged by the patients anti O antibody response

Fig. 25 shows the relation between the patients anti O antibody titre and the agglutination inhibiting activity of the corresponding bacteria. An anti O titre of $\geqslant 320$ is considered to indicate that the renal tissue has been infected (Percival et al., 1964). From Fig. 25 it can be seen that strains which had invaded the renal tissue had on average a higher K antigen content than strains which were confined to the bladder.

In Fig. 26 the K antigen content of faecal strains of E. coli and of strains that were confined to the bladder or had invaded the renal tissue, grouped on the basis of the patients anti O response, have been compared. The similarity in K antigen content between faecal strains and strains confined to the bladder is evident. Strains causing renal involvement contained more K antigen than either of the other two groups.

The agglutination inhibiting activity of strains of E. coli isolated from faeces or causing an upper or lower urinary tract

infection, as judged by the patients anti O antibody response, were compared using Student's t test (Hill, 1961) and the results are given in Table 11.

Strains isolated from patients with an anti O antibody titre of $\geqslant 320$ contained significantly more K antigen than strains isolated from patients with an antibody titre of $\leqslant 160$, $p < 0.01$.

There was no significant different between the K antigen content of faecal strains and of strains from patients with lower urinary tract infections, $p > 0.5$. Strains causing upper urinary tract infections contained more K antigen than faecal strains, $p < 0.05$.

Relation between K antigen content of strains of *E. coli* and ability to cause upper and lower urinary tract infections as judged by the results of the Fairley technique

In Fig. 27 smooth strains of *E. coli* isolated from urine have been divided into two groups, one of which was composed of bacteria that were confined to the bladder and the other was composed of bacteria which were found in the uteric urine, indicating renal involvement, according to the test of Fairley *et al.* (1967). There was no significant difference between the K antigen content of these two groups, $p < 0.2$ or between the K antigen content of these two groups and that of faecal strains (Table 11).

Relation between K antigen content and ability to cause upper or lower urinary tract infections judged by the antibody response and results of the Fairley test

In Table 12 the agglutination inhibiting activity of strains of E. coli, the patients anti O antibody titre and the results of the Fairley test have been compared. Eight out of twenty strains designated as having invaded renal tissue on the basis of the antibody response were designated as being confined to the bladder based on the results of the Fairley test. When the two techniques used to localise the site of infection disagreed deductions about the importance of the K antigen could not be made.

If strains for which the antibody response and Fairley test agreed were compared, i.e. there were two independent means of localising the site of infection (Table 12) then bacteria which had invaded renal tissue contained significantly more K antigen than bacteria which were confined to the bladder, $p < 0.01$ (Table 11). A comparison of these two groups with faecal strains showed there was no significant difference between the K antigen content of bladder inhabitants and faecal E. coli, $p < 0.4$. Bacteria that had caused renal involvement contained more K antigen than faecal strains, $p < 0.05$.

Relation between O serotype, K antigen content and ability to invade renal tissue

Different O serotypes were compared for their K antigen content and ability to invade renal tissue (Table 13). The ability of any O serotype to invade renal tissue was explicable in terms of the K antigen content of the bacteria.

Table 9. O serotype, Fairley test, patients anti O titre and agglutination inhibiting activity of strains of *E. coli* from urinary tract infections

Patient	Strain No.	O Serotype	Fairley	Anti O titre	AIA
Shepperd	B189	01	L	320	128
Wilson, C.	L189	01	U	320	128
Riddington	B61	01		640	256
Nicola	B180	01		1280	128
Leggatt	L157	01	U	5000	32
McBride	B72	01	L	5000	256
Jones	B78	02	L	1280	256
Thackeray	B88	02	U	1280	128
Crozier	L165	02	L	2560	128
Scully	B163	04	L	40	0
Seth	B108	04		1280	0
Inglis	B154	05	L	20	0
Toole	B151	05	L	40	0
Bentley	L198	06	L	20	0
Bedessee	B101	06		80	0
Richards	B24	06		80	8
Millington	B148	06	L	160	0
Koo	B122	06		320	0
O'Brien	B87	06		640	16
Mallon	B58	06		640	256
Sanger	B155	06	U	2560	128
Langston	B191	06	U	>5000	64
Nicoll	L168	06	U	>5000	256
Allcock	B160	06		160 → 5000	256
Stevens	B144	07		1280	16
Quarm	B115	011		1280	512
Davidson	B2	039		80	8
Binnie	L190	039	U	80	0
Wilkinson	B171	039	L	640	64
Hall	B188	039		1280	128

Continued over

Table 9 contd.

Patient	Strain No.	O Serotype	Fairley	Anti O titre	AIA
Salter	B146	075	L	20	128
Rawlinson	B109	075		40	0
Stewart	B168	075	L	80	8
Lynch	B143	075		160	512
Vaid	B172	075	L	160	8
Griffiths	L57	075	U	320	256
Patel	B145	075	U	640	128
Egan	B100	075		640	0
Phillips	L194	075	L	1280	128
Stillwell	L156	075	U	1280	32
Cox	B194	075	U	>5000	64
Ryan	B106	075		>5000	0
Gaind	B129	N.T.		20	64
Price	B120	N.T.		20	64
Duggan	B165	N.T.	L	20	128
Johnson	B149	N.T.	L	20	0
Luck	L163	N.T.	L	80	128
Welsh	B167	N.T.	L	160	4
Fisher	B187	N.T.	L	320	0
Green	B123	N.T.		1280	512
Rook	B71	N.T.	U	1280	0
Sebesteny	L166	N.T.	L	1280	0
Edwards	L159	N.D.	U	2560	8
Rossi	B153	A.A.	L	A.A.	0
Crane	B176	A.A.	L	A.A.	0
Sanders	L49	A.A.	U	A.A.	0
Adams	L148	A.A.	U	A.A.	0
Saunders	L150	A.A.	L	A.A.	0
Bainbridge	L154	A.A.	U	A.A.	0
Ubale	L164	A.A.	L	A.A.	0
Jones	L167	A.A.	L	A.A.	128
Casey	B132	A.A.	U	A.A.	>256

N.T. - bacteria not typable with the antisera used.
A.A. - autoagglutinable bacteria.
AIA - agglutination inhibiting activity.
N.D. - bacteria not serotyped.
L or U - bacteria confined to the bladder (L) or found in the uteric urine (U) according to the technique of Fairley et al. (1967).

Table 10. Agglutination inhibiting activity and O serotype of strains of *E. coli* isolated from faecal specimens

Strain	O serotype	Agglutination inhibiting activity
FS16	05	0
FS2	05	64
FS8	06	16
FS4	06	64
FS9	06	128
FS18	06	512
FS1	07	256
FS12	011	0
FS10	039	64
FS19	075	256
FS5	N.T.	0
FS6	N.T.	0
FS11	N.T.	0
FS13	N.T.	0
FS15	N.T.	0
FS17	N.T.	0
FS7	A.A.	0
FS14	N.D.	32
FS20	N.D.	64

A.A. - autoagglutinable bacteria.

N.D. - bacteria not serotyped.

N.T. - bacteria not typable with the antisera used.

Table 11. Differences in the K antigen content of E. coli related to their source

Source of strain	Patients ab. titre	Fairley	No. of strains	Mean AIA of coli	p.
Urine	⩽160		20	4	)
Urine	⩾320		33	32	) < 0.01
Faeces			20	8	)
Urine	⩽160		20	4	) > 0.5
Faeces			20	8	)
Urine	⩾320		33	32	) < 0.05
Urine		L	19	8	)
Urine		U	13	32	) < 0.2
Faeces			20	8	)
Urine		L	19	8	) > 0.5
Faeces			20	8	)
Urine		U	13	32	) < 0.2
Urine	⩽160	L	12	8	)
Urine	⩾320	U	12	64	) < 0.01
Urine	⩽160	L	12	8	)
Faeces			20	8	) < 0.4
Urine	⩾320	U	12	64	)
Faeces			20	8	) < 0.05

AIA = agglutination inhibiting activity.

U or L = bacteria found in the uteric urine or bladder according to the technique of Fairley et al. (1967).

Table 12. Comparison of agglutination inhibiting activity, patients anti O titre and the results of the Fairley test

AIA	Ab ⩽ 160 Fairley L	Ab ⩽ 160 Fairley U	Ab > 160 Fairley L	Ab > 160 Fairley U	Total
< 16	9	1	2	2	14
⩾16	3	0	6	10	19
Total	12	1	8	12	33

AIA - agglutination inhibiting activity

Ab⩽160 - patients anti O antibody titre

Fairley U or L - upper or lower urinary tract infection according to the results of the test of Fairley et al. (1967).

Table 13. Relation between O serotype, K antigen content and ability to invade renal tissue

(a) Strains divided into two groups causing upper or lower urinary tract infections, based on the patients anti O antibody titre.

Agglutination Inhibition Titre	Serotype					
	01		06		075	
	Upper	Lower	Upper	Lower	Upper	Lower
<16			1	4	2	3
⩾16	6		6		4	2

(b) Strains divided into two groups causing upper or lower urinary tract infections, based on the results of the Fairley test.

Agglutination Inhibition Titre	Serotype					
	01		06		075	
	Upper	Lower	Upper	Lower	Upper	Lower
<16				2		2
⩾16	2	2	3		3	2

The figures in the Tables represent the number of strains in each group.
The designation upper or lower relates to whether the bacteria had invaded the renal tissue or not based on either the patients anti O antibody response or the results of the Fairley test.

Fig. 25. Relation between agglutination inhibiting activity and patients anti O titre for strains of *E. coli* from urinary tract infections.

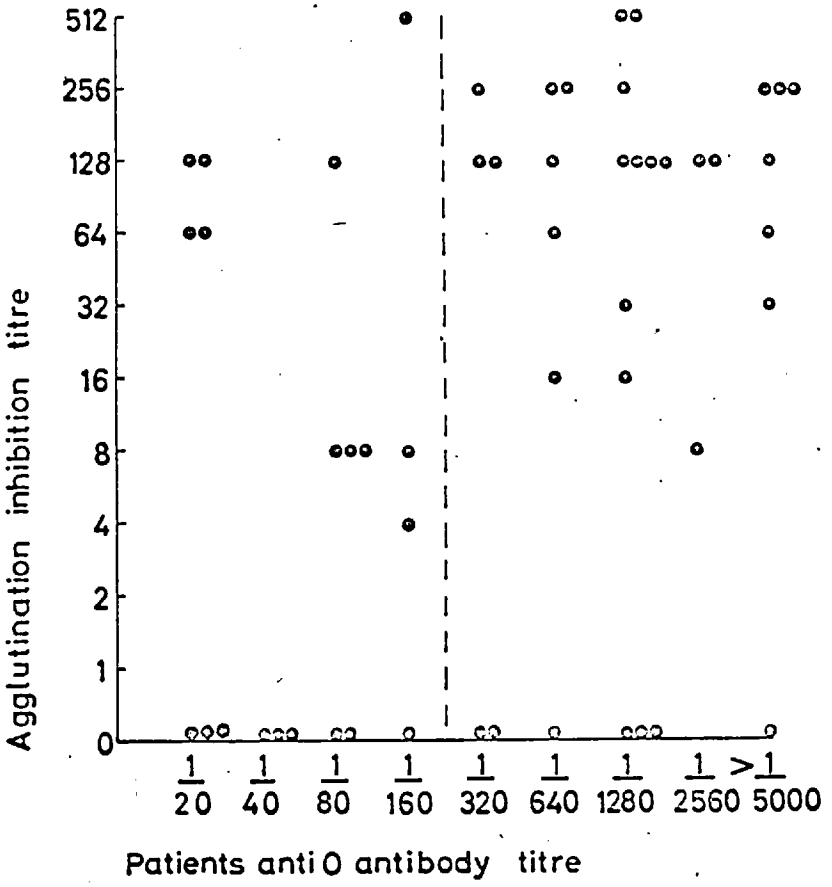

512
256
128
64
32
16
8
4
2
1
0
Agglutination inhibition titre
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
>1/5000
Patients anti O antibody titre

Fig. 26. Comparison between the agglutination inhibiting activity of strains of *E. coli* from faeces and upper or lower urinary tract infections as judged by patients anti O antibody titre.

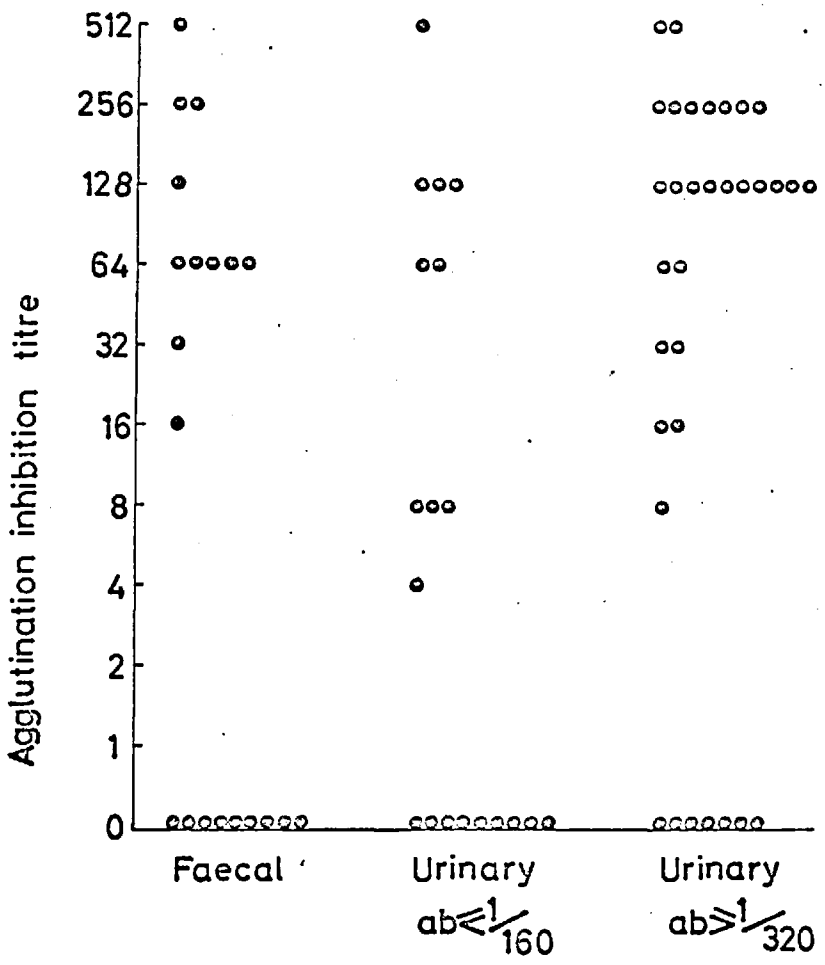
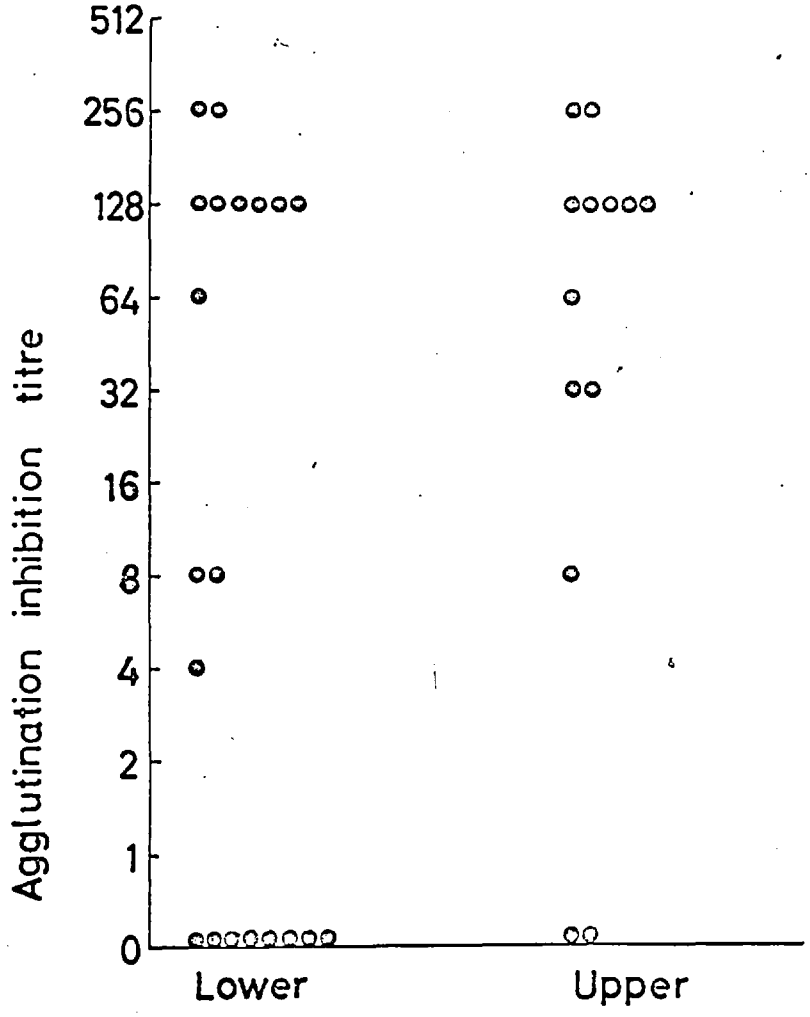

Agglutination inhibition titre
512
256
128
64
32
16
8
4
2
1
0
Faecal
Urinary
ab≤1/160
Urinary
ab≥1/320

Fig. 27. Relation between agglutination inhibiting activity of strains of *E. coli* and site of urinary tract infection based on the results of the Fairley test.


512
256
128
64
32
16
8
4
2
1
0
Agglutination inhibition titre
Lower
Upper

DISCUSSION

It is evident that there was no difference between the K antigen content of strains of E. coli which were confined to the bladder and strains which were found in faecal specimens. Bacteria which were able to invade the renal tissue, as judged by the patients anti O antibody response or both the antibody response and the results of the Fairley technique, contained significantly more K antigen than bacteria that had been isolated from faeces or were confined to the bladder. The results therefore indicate that the K antigens are involved as factors influencing the ability of E. coli to cause tissue invasion in human urinary tract infections.

Two theories have been suggested to explain the mechanism of infection of the urinary tract. One is called the "special pathogenicity theory" which depends on the bacteria having particular properties which enable them to colonise the urinary tract. The other theory, called the "prevalence theory", postulates that the infecting bacteria have no peculiar properties but rather that infection depends on what organism is introduced into the urinary tract from the gut.

Gruneberg et al. (1968) sought to find which theory was correct by comparing the extent to which the serotype infecting the urinary tract was present amongst smooth E. coli isolated from a rectal swab. Since faecal matter was not

homogeneous with respect to the O serotypes present 10 colonies from each swab were serotyped. 23 patients were studied and only in one case was the urinary serotype not present amongst the 10 faecal colonies examined. Of the remaining patients in 18 out of 23 cases more than half the faecal isolates were of the same serotype as the urinary strain. They therefore conclude that in domiciliary practice although urinary tract infections were usually caused by only a few O serotypes this was not because they were particularly pathogenic but because they were the most common types in faeces. In 1863 Pasteur observed that human urine could support bacterial growth (Wilson and Miles, 1964). Once bacteria of faecal origin reach the bladder they are therefore likely to persist since urine is a suitable culture medium.

The K antigen content of bacteria confined to the bladder was no different to that of faecal isolates. These findings fit the hypothesis that urinary infections have faecal origins.

Norden and Kass (1968) found that 20% of women with significant bacteriuria, but no symptoms of pyelonephritis, later developed symptomatic pyelonephritis of pregnancy if the infecting bacteria were not removed by antibiotic treatment. It therefore seems likely that when bacteria of faecal origin infect the bladder urine infection may be confined to this region thus causing a lower urinary tract infection or bacteria

may infect the renal tissue from the bladder.

Since the K antigen content of strains that had infected the renal tissue was significantly higher than that of bacteria confined to the bladder it is likely that a selection pressure is applied to the organisms infecting the bladder and only those possessing sufficient K antigen to protect them from host defence mechanisms, killing by C^9 and phagocytosis, will be able to invade and colonise the renal tissues.

These results therefore support the special pathogenicity theory. Pathogenicity is not related to the serotype of the bacteria but to the amount of K antigen it contains.

Vahlne (1945) compared bacteria that had been isolated from urine with bacteria that had been isolated from faecal material. He found 65% of the faecal strains were O inagglutinable compared with 82% of the urinary strains. Since O inagglutination was due to K antigens (Kauffmann, 1954) his findings suggested that K antigens were involved in the pathogenesis of urinary tract infections.

The results of Roantree and Rantz (1960) conflicted with Vahlne's findings. They examined the C^9 sensitivity of strains of E. coli isolated from urine and faeces and were unable to demonstrate any differences.

Beeson and Rowley (1959) suggested that the ability of strains of E. coli to infect the kidney was not because of the

enhanced virulence of the strain but because of the "anticomplementary" effect of kidney tissue which could be demonstrated *in vitro*. This effect could prevent the host defence mechanisms from being effective in this organ.

Besides the suggestion put forward here that K antigens affect the ability of *E. coli* to invade renal tissue other reports have suggested they may affect the ability of the organism to cause generalised systemic infections in humans.

Roantree and Rantz (1960) showed that strains of *E. coli* isolated from the blood were generally more resistant to killing by C^9 than strains isolated from faeces.

Strains isolated from pathological material which included peritoneal pus, appendicitic material, urine and bile were more likely to be O inagglutinable than strains isolated from faecal material (Kauffmann, 1954).

Jacks and Glantz (1967) compared various serotypes of *E. coli* to see if mouse virulence was related to source. They found that strains from systemic infections were generally more virulent than strains from an enteric source.

Muschel (1960) was unable to demonstrate any difference between the C^9 sensitivity of strains of *E. coli* isolated from normal children and strains isolated from children with diarrhoea. K antigens therefore do not appear to be involved in the pathogenesis of this disease.

Taylor et al. (1961) found that the enteropathogenicity of E. coli was related to the ability of strains to cause dilation of ligated rabbit intestine. Urinary strains of E. coli did not possess this property. This evidence further supports the hypothesis that the mechanisms of pathogenicity in enteric and urinary infections are different.

The general conclusions that can be drawn from these results and from those of other authors is that K antigens are one of the factors affecting the human pathogenicity of E. coli. It is likely that human pathogenicity is affected in the same way as is proposed for animal virulence. This is because K antigens protect bacteria from two of the host's defence mechanisms, killing by C^{9} and phagocytosis thus enabling bacteria to persist in host tissue once it has been invaded.

VI. AN INVESTIGATION OF A PROPOSED MECHANISM FOR NATURAL IMMUNITY TO ENTEROBACTERIAL PATHOGENS

INTRODUCTION

Chedid, Parant, Parant and Boyer (1968) proposed a mechanism by which the host may be capable of coping rapidly with a spectrum of gram negative pathogens. They suggested the host may phenotypically modify smooth virulent bacteria in such a manner that they were made more sensitive to serum factors which reacted with rough antigens. This mechanism would make it possible for a limited number of antibodies to deal with a great variety of strains with unrelated O antigens but related rough antigens.

Chedid et al. (1968) worked with a smooth strain of Klebsiella pneumoniae which they had previously found to be phenotypically changed to a rough state when recovered from animal tissues after infection.

Using a system to which C^7 was not added they found that the "bactericidal" action of a homologous antiserum was increased if bacteria were incubated in mouse serum prior to exposure to the anti bacterial antiserum.

They also found that an antiserum raised against a rough strain of Salmonella typhimurium killed the Klebsiella strain after it had been incubated in mouse serum but not otherwise. The bactericidal action of the anti rough Salmonella serum

could be partially removed by absorption with rough but not smooth strains of S. typhimurium. On the basis of these findings it was suggested that incubation in mouse serum exposed rough antigenic determinants on the bacterium.

Exposure of rough sites could have been because growth in mouse serum caused the bacterial cell wall to be synthesised in a different way to when bacteria grew in broth.

Chedid et al. (1968) put forward another possible explanation. They suggested that if serum contained enzymes capable of breaking down the bacterial cell wall their action might expose rough antigens present in the cell wall making the bacteria sensitive to antibody directed against these rough determinants.

The experiments recorded here were to examine the possible existence of a non specific mechanism of immunity due in part to the digestion of the bacterial cell wall by host enzymes.

Bacteria were incubated in mouse serum and intestinal juice to see if this caused them to become more sensitive to the bactericidal action of human serum. Isolated K antigen was incubated with intestinal juice to see if enzymes were present capable of digesting it.

MATERIALS AND METHODS

Intestinal juice

The intestinal secretions of humans were collected from

the duodenal jejuno flexure by means of a ryles tube. Intestinal juice collected in this way is mainly pancreatic juice which contains enzymes capable of digesting proteins, fats and carbohydrates. Duodenal juice was provided by Dr. B. Drasar of the Wright-Fleming Institute and stored at -20°C until required.

Mouse serum

Mice were bled from the retro-orbital plexus and the blood was allowed to clot on ice. The serum was collected after centrifugation, kept on ice and used within 1 hour.

Complement

N.H.S. was collected as described in Section II and used as the source of both antibody and C'.

Bacteria

The strains of E. coli used were grown into the log phase in nutrient broth at 37°C, washed and suspended in 0.15M saline to give the required bacterial concentration as judged by optical density.

Effect of intestinal juice on E. coli

Intestinal juice was examined to see if it was bactericidal. 0.1 ml of a bacterial suspension containing 10^6

cells per ml. was added to 0.9 ml of intestinal juice in an L tube. The bacteria were incubated at 37°C, 0.1 ml samples removed at timed intervals diluted in nutrient broth and the number of viable cells estimated by plate counts.

Effect of incubation in intestinal juice on the C' sensitivity of strains of E. coli

E. coli strains WF96, WF82, WF98 and WF26 were grown into the log phase (see Section II). Bacteria were harvested by centrifugation, washed and suspended in saline to give 2×10^9 cells per ml as judged by optical density.

0.1 ml of bacteria were added to 0.9 ml of intestinal juice in L tubes and incubated at 37°C for 1 hour. Control cells were incubated in 0.9 ml of saline of the same ionic strength and pH as the intestinal juice.

Bacteria were harvested by centrifugation, washed in 0.15M saline and made up in saline to give 1×10^7 cells per ml. Bacteria that had been incubated in intestinal juice were compared with bacteria that had been incubated in saline to see if there was any difference in C' sensitivity.

Measuring C' sensitivity

For strains WF82, WF26 and WF98 0.1 ml of bacteria that had been incubated with intestinal juice or saline were added to 0.9 ml of N.H.S. at 37°C in L tubes. Samples were taken at

timed intervals into nutrient broth and the number of viable cells estimated by plate counts. For strain WF96 0.1 ml of bacteria were added to a mixture of 0.1 ml of N.H.S. and 0.8 ml of heated serum to give a final C' concentration of 3.5 $C'H_{50}$ units per ml. Control tubes contained heated serum only.

Effect of mouse serum on the C' sensitivity of E. coli

Mouse serum was examined for enzymic activity in the same way as the intestinal juice. Bacteria that had been grown in nutrient broth were suspended in saline and incubated with pooled mouse serum for 1 hr. After washing, bacteria that had been exposed to mouse serum were compared with bacteria harvested from nutrient broth to see if there was any difference in C' sensitivity. To examine the sensitivity of the strains to C' 0.1 ml of mouse serum treated and control bacteria were incubated with N.H.S. as described above.

Attempt to digest K antigen with intestinal juice

Antiserum. An antiserum containing anti K antibody was raised in a rabbit against live E. coli WF82 (Edwards and Ewing, 1962).

K antigen. The K antigen from E. coli WF82 was isolated from the aqueous phase of a phenol-water extract of the bacteria by fractional precipitation with cetavlon (see Section VII).

Antigen measurement. The concentration of antigens in solutions was measured by radial immunodiffusion using purified K antigen as a standard (see Section II).

Treatment of antigen with intestinal juice

K antigen was dissolved in 0.15M NaCl and the solution diluted to give a final concentration of 500 µg per ml. Equal volumes of antigen solution and different intestinal juice samples were incubated together for 3 hours at 37°C. The amount of antigen remaining in the antigen-intestinal juice mixture was measured by radial immunodiffusion to see if there had been any digestion of the antigen.

RESULTS

Effect of intestinal juice on the viability of E. coli

Two strains of E. coli were added to intestinal juice to give a final concentration of 1×10^5 bacteria per ml. After one hour's incubation at 37°C the bacterial concentration was 4×10^5 per ml. The intestinal secretions examined supported the growth of the bacteria.

Effect of exposure to intestinal juice on the C' sensitivity of E. coli

Incubation in intestinal juice did not cause any of the

E. coli strains examined to become more sensitive to the bactericidal action of human serum (Figs. 28 and 29). E. coli WF98 appeared to be less sensitive to serum killing after exposure to intestinal juice than control cells (Fig. 29). Possibly cell wall structure was modified by growth in intestinal juice instead of broth and this could have caused the organisms sensitivity to serum to be changed. Maaloe (1948b) showed that by increasing the concentration of the carbon source in a synthetic medium the sensitivity of S. typhimurium to C⁰ was increased.

Effect of exposure to mouse serum on the sensitivity of E. coli to complement

E. coli strains WF96, WF26 and WF82 that had been exposed to mouse serum were no more sensitive to the bactericidal action of human serum than bacteria taken straight from broth (Figs. 30 and 31).

Inability of intestinal juice to digest K antigen

The K antigen from E. coli WF82 was incubated with intestinal juices from five people. None of the samples tested broke down the bacterial antigen as judged by radial immunodiffusion.

Fig. 28. Effect of incubation in intestinal juice on the sensitivity to complement of *E. coli* WF96.

IJa - bacteria incubated with intestinal juice sample a

IJb - bacteria incubated with intestinal juice sample b

-IJ - bacteria not exposed to intestinal juice

+H.S. - bacteria incubated with heated serum

+C' - bacteria incubated with normal human serum.

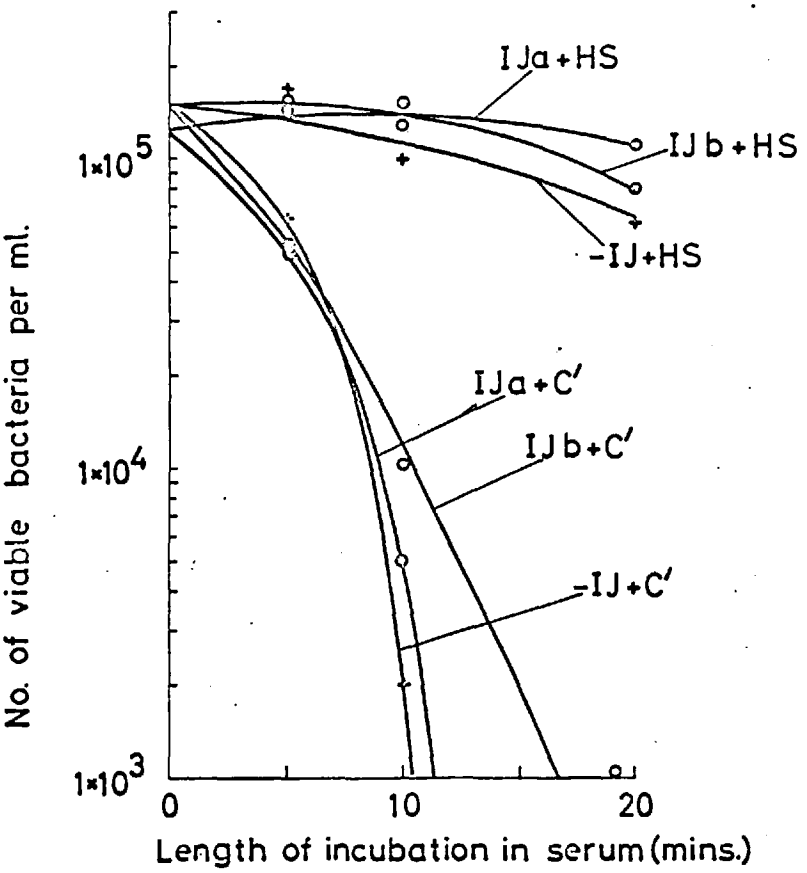

IJa+HS
IJb+HS
-IJ+HS
IJa+C′
IJb+C′
-IJ+C′
1×10^5
1×10^4
1×10^3
No. of viable bacteria per ml.
0
10
20
Length of incubation in serum (mins.)

Fig. 29. Effect of incubation in intestinal juice on the complement sensitivity of *E. coli* strains WF82, WF98, WF26.

——+ - bacteria which had been incubated in saline and exposed to C'

——● - bacteria which had been incubated with intestinal juice prior to exposure to C'.

——o - bacteria which had been incubated in saline and exposed to heated serum

——x - bacteria which had been incubated in intestinal juice prior to exposure to heated serum.

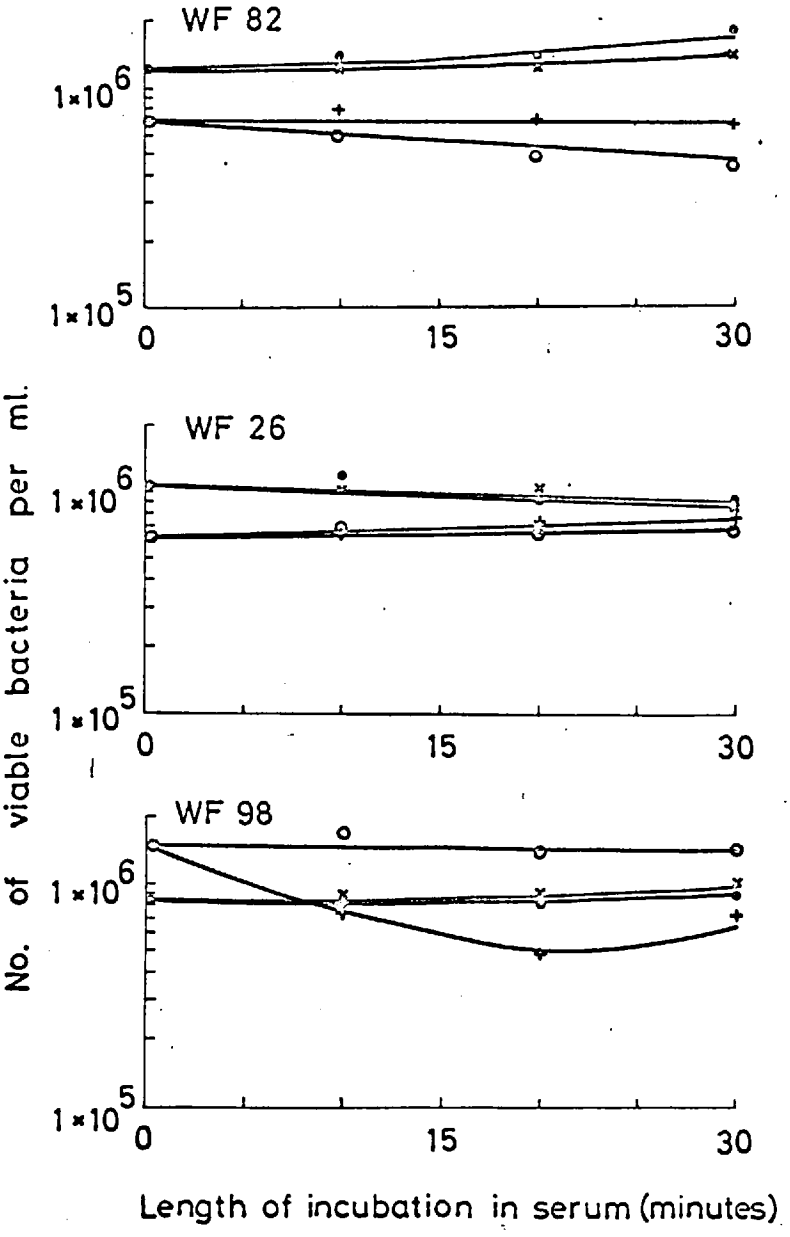

WF 82
WF 26
WF 98
1×10^6
1×10^5
0
15
30
No. of viable bacteria per ml.
Length of incubation in serum (minutes)

Fig. 30. Effect of incubation in mouse serum on the sensitivity to C° of *E. coli* WF96.

N.B. + H.S. - bacteria grown in nutrient broth and exposed to heated serum

M.S. + H.S. - bacteria incubated in mouse serum and exposed to heated serum

N.B. + C° - bacteria grown in nutrient broth and exposed to C°

M.S. + C° - bacteria incubated in mouse serum and exposed to C°.

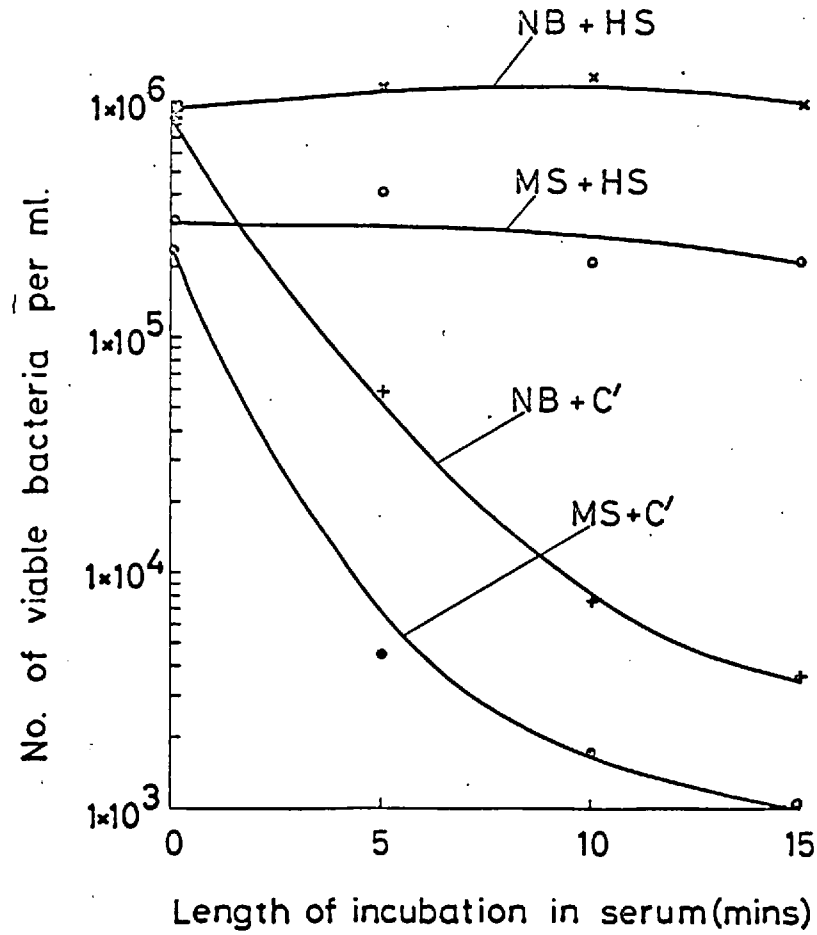

NB + HS
MS + HS
NB + C′
MS + C′
1×10^6
1×10^5
1×10^4
1×10^3
No. of viable bacteria per ml.
0
5
10
15
Length of incubation in serum (mins)

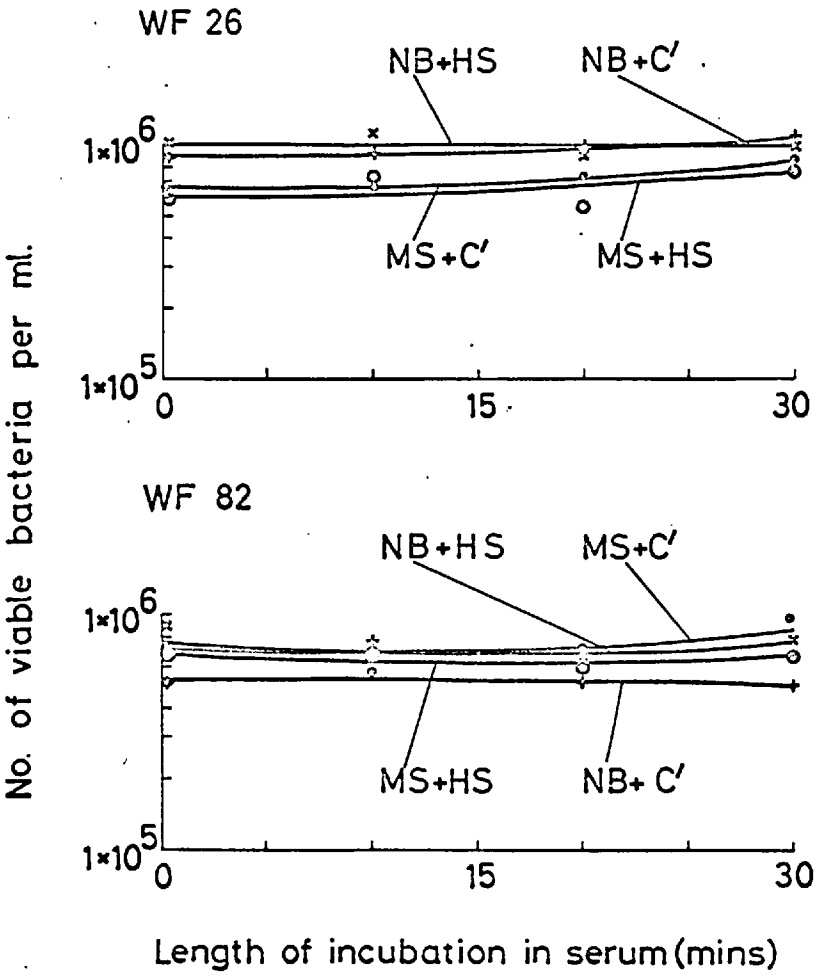
Fig. 31. Effect of incubation in mouse serum on the sensitivity to C° of *E. coli* strains WF26 and 82.

N.B. + H.S. - bacteria grown in nutrient broth and exposed to heated serum

M.S. + H.S. - bacteria incubated in mouse serum and exposed to heated serum

N.B. + C° - bacteria grown in nutrient broth and exposed to C°

M.S. + C° - bacteria incubated in mouse serum and exposed to C°.


WF 26
NB+HS
NB+C′
MS+C′
MS+HS
1×10^6
1×10^5
0
15
30
WF 82
NB+HS
MS+C′
MS+HS
NB+ C′
1×10^6
1×10^5
0
15
30
No. of viable bacteria per ml.
Length of incubation in serum (mins)

DISCUSSION

Exposure to human intestinal juice or mouse serum did not cause any of the strains of E. coli examined to become more sensitive to the bactericidal action of human serum. Neither was there any demonstrable digestion of isolated bacterial antigen.

No evidence was obtained in support of the attractive theory of Chedid et al. (1968) that enzymic digestion of the cell wall of smooth bacteria may expose rough antigenic determinants making the bacteria susceptible to serum factors directed against these rough antigens. If this theory was true bacteria that had been exposed to host enzymes capable of digesting the bacterial cell wall would be expected to have an enhanced sensitivity to C°. This would be so whether enzymic digestion removed the O specific polysaccharide side chains present in the bacterial cell wall or if these enzymes digested K antigens since both would affect the sensitivity of the bacterium to C° (Nelson and Roantree, 1967, and Section II).

The bactericidal effect noted by Chedid et al. (1968) was considered to be due to antibody directed against rough determinants since the cidal effect of the antiserum used was reduced after absorption with rough but not smooth bacteria. Whether enhanced sensitivity to the bactericidal action of the antiserum used was due to some enzymic action of the mouse

serum or whether it was because incubation in a different growth medium was causing differences in the synthesis and structure of the cell wall was not shown.

Differences in the sensitivity of bacteria to the bactericidal action of serum due to growth in different culture media has been demonstrated. Maaloe (1948b) found that when the concentration of the carbon source in the culture medium was increased S. typhimurium became more sensitive to C^9. Bacteria also became more sensitive to C^9 if the nitrogen source was changed from being organic to inorganic.

Osawa and Muschel (1964a) reported that when a C^9 resistant strain of Paracolobactrum ballerup, which contained the Vi antigen, was grown in inactivated guinea pig serum instead of broth it became sensitive to killing by C^9. Presumably changing the "culture medium" was affecting the final C^9 sensitivity of the organism.

In the experiments that have been recorded here there was no increased sensitivity to the bactericidal action of C^9 demonstrable due to exposure of the bacterial cells to either mouse serum or human digestive juices. The only conclusion that can be reached, therefore, is that the mechanism of non specific immunity based on enzymic digestion of the bacterial cell wall and exposure of rough antigenic determinants, if true,

is not a general phenomenon. The changes in susceptibility to anti rough antibody observed by Chedid et al. (1968) may merely reflect differences in bacterial metabolism and cell wall synthesis due to growth in different media.

VII. PHYSICAL PROPERTIES OF K ANTIGENS RELATED TO THEIR BIOLOGICAL ACTIVITY

INTRODUCTION

In Section II the extent to which bacterial extracts inhibited the agglutination of sheep red cells by an anti sheep red cell serum was related to the sensitivity of the strain to the bactericidal action of human serum. When K antigens were isolated from different strains of *E. coli* and compared on a weight for weight basis for their ability to inhibit red cell agglutination they were found to differ. Since the inhibiting activity of the bacterial extracts was related to the C^9 sensitivity of the bacterium it seemed possible that the differences in inhibiting agglutination *in vitro* might reflect the activity of the antigen on the bacterial surface. The isolated antigens were examined to see if their differing abilities to inhibit agglutination could be related to either the charge or the size of the antigen molecule.

Since sodium hydroxide had been reported to break down K antigen (Hungerer *et al.*, 1967) the effect of sodium hydroxide hydrolysis on the agglutination inhibiting activity and serological specificity of the antigens was examined.

K antigens were reported by Kauffmann (1947) to be heat labile although this has been disputed (Ørskov and Ørskov,

1968). The effect of temperature on the agglutination inhibiting activity and serological properties of K antigens was also investigated.

MATERIALS AND METHODS

Bacteria

E. coli strains WF82, WF96 and WF26 were grown by M.R.E. Porton, supplied as frozen wet cells and stored at -20°C. Samples were extracted as required.

Isolation of K antigens

K antigens were purified by the method of Hungerer *et al.* (1967). Bacteria were extracted with 45% phenol at 65°C and lipopolysaccharide was removed by centrifugation at 100,000 g for 2 hours. K antigen was isolated from the phenol-water extract by fractional precipitation with cetavlon.

Residual ribonucleic acid was removed by digestion with ribonuclease (Koch-light Ltd.). K antigen was dissolved in 0.02M phosphate buffer pH 7.2 to give a final concentration of 20 mg per ml. Ribonuclease was added to give a final concentration of 0.1% and incubated with antigen overnight at 37°C (McDonald, 1955).

To remove the enzyme phenol was added at 65°C to give a

final concentration of 45%. The aqueous phase was collected, dialysed against saline, the K antigen precipitated in the cold by the addition of 4 volumes of ethanol and dried from ether (Bolanos and DeWitt, 1966). The purified antigen contained less than 1% protein by the method of Lowry et al. (1951) and less than 5% nucleic acid by u.v. absorption (Mach, 1968). No O antigen was demonstrated by immunodiffusion. The acidic nature of the isolated antigens was demonstrated by immunoelectrophoresis.

Agglutination inhibiting activity of K antigens

K antigens isolated from different strains of E. coli were compared on a weight for weight basis for their ability to inhibit the agglutination of sheep red cells by anti sheep red cell serum. The method used was the same as described in Section V except that different concentrations of antigens were incubated with the red cells instead of the bacterial extracts. The extent to which the titre of the haemolysin was reduced was recorded as the amount of inhibition of agglutination.

Immunodiffusion

Double diffusion studies were carried out in 1% w/v Oxoid Ion Agar No. 2 in 0.05M veronal buffer pH 8.2 (Grabar and Burtin, 1964). Ouchterlony plates were incubated at room

temperature.

Immunoelectrophoresis

The method used was as described in Section III. Samples were electrophoresed in the same strength agar and buffer used for immunodiffusion studies.

Ion exchange chromatography

Ion exchange chromatography was used to compare the charges on different antigens and examined as a means of purifying K antigens.

Whatman "D.E.A.E. 52" was equilibrated with 0.01M phosphate buffer pH 7.2 and used to pack columns. In order to find the molarity of the buffer needed to elute different K antigens from the resin 25 mg of a phenol-water extract of the strain was dissolved in the 0.01M buffer and pipetted onto a column 15 cm long and 1 cm diameter. After washing the column through with the 0.01M buffer a sodium chloride gradient was used to elute carbohydrate from the resin. 2.0 ml fractions were collected and the carbohydrate in the fractions measured by the method of Dubois et al. (1956). By measuring the conductivity of the fractions with a conductivity meter and comparing the value obtained with a standard curve of molarity against conductivity the molarity of the different fractions could be calculated. The

carbohydrate peak representing K antigen was identified by immunodiffusion using an antiserum raised against the strain being examined.

The possibility that ion exchange chromatography could be used as an alternative method of purifying K antigens to cetavlon fractionation was examined. The buffer molarities chosen to separate the K antigen from other components of a phenol-water extract of the bacteria were based on that found to elute the antigen from D.E.A.E. cellulose using a molarity gradient. The molarities chosen were tried on a small scale before a preparative attempt was made.

500 mg of a phenol-water extract of E. coli WF82 was dissolved in 0.01M buffer and pipetted onto a D.E.A.E. cellulose column 2.5 cm x 40 cm. Four carbohydrate fractions were obtained using 0.01M, 0.16M, 0.25M and 0.35M phosphate buffered saline pH 7.2. 3.0 ml fractions were collected at a flow rate of 60 ml per hour. Fractions were pooled, concentrated by negative pressure dialysis against 0.15M saline and the carbohydrate precipitated with 4 volumes of alcohol. The precipitate was washed with acetone and dried from ether. The antigen content of the different fractions was examined by immunodiffusion.

Sepharose chromatography

In order to compare the sizes of different K antigens and

of K antigens that had been treated with sodium hydroxide use was made of Sepharose chromatography.

Sepharose (Pharmacia Ltd.) separates molecules according to their size. According to the manufacturers Sepharose 6B excludes molecules that have a molecule weight of more than 1×10^6. Sepharose 4B has an exclusion limit of 5×10^6 and Sepharose 2B has an exclusion limit of 2×10^7. These values were based on studies using dextrans of various molecular weights (Pharmacia Bulletin July 1969).

Sepharose was equilibrated with 0.15M phosphate buffered saline pH 7.2 and columns were packed after the gel had been deaerated. The dimensions of the columns used to compare the sizes of different antigens were 1 x 50 cm. The void volumes of the columns were determined using Blue Dextran T2000 (Pharmacia Ltd.), part of which is large enough to be excluded from the gels. To compare the sizes of different antigens 0.2 ml of an antigen solution containing 10 mg of polysaccharide per ml was put onto a column. 0.8 ml fractions were collected at a flow rate of 10 ml per hour and their carbohydrate content measured.

The K antigen from E. coli WF82 was separated into two fractions on Sepharose 2B. 5 mg of antigen in 0.5 ml of buffer was put onto a 2B column. The two fractions were collected, concentrated by negative pressure dialysis against 0.15M saline and rerun on the same column to ensure they were

not artifacts.

Effect of sodium hydroxide on K antigen

K antigens were dissolved in water to give a final concentration of 4 mg/ml. Sodium hydroxide was added to give final concentrations of 0.005N, 0.01N, 0.05N and 0.1N. The mixtures were incubated overnight at 37°C, neutralised by adding the required concentration of hydrochloric acid and the pH tested with indicator paper. The alkali treated antigens were tested for their ability to inhibit agglutination and to precipitate with specific antiserum. The effect of NaOH on molecular weight was examined by comparing the elution patterns of untreated and NaOH treated K antigens on Sepharose 6B.

Effect of temperature on K antigen

K antigens were dissolved in 0.15M saline to give a concentration of approximately 2 mg per ml and steamed for 2½ hours. This length of time is about twice as long as that recommended to "destroy" K antigens (Edwards and Ewing, 1962). After cooling the antigens were examined for their ability to inhibit agglutination and for their serological activity.

Passive haemagglutination

A range of concentrations of the K antigen from E. coli WF82 and K antigen that had been hydrolysed with 0.05N sodium hydroxide were made in 0.15M phosphate buffered saline pH 7.2. 0.5 ml of a 1% v/v suspension of sheep red cells in the same buffer was added to the antigen solutions. Red cells and antigen were incubated together for 30 minutes at 37°C, the cells washed and resuspended to give a 0.5% v/v suspension. 0.1 ml of antigen treated and untreated red cells were added in a haemagglutination tray to 0.1 ml of doubling dilutions of an antiserum raised against live E. coli WF82 (see Section II). Prior to use the antiserum was absorbed on ice with packed sheep red cells. Haemagglutination trays were incubated at 37°C for 1 hour and overnight at 4°C after which the agglutination titre of the antiserum against sensitised red cells was read.

RESULTS

Purification of K antigen by ion exchange chromatography

Since the K antigen from E. coli WF82 was an acid polysaccharide the possibility that it could be purified by ion exchange chromatography was explored. A phenol-water extract of WF82 was fractionated on D.E.A.E. cellulose and the K antigen was separated from the other components of the bacterial extract.

Fig. 32 shows the separation of the extract into 4 fractions using different strength phosphate buffered saline to elute the different samples.

The antigens present in the fractions were identified by immunodiffusion (Fig. 33). The 0.01M and 0.16M fractions contained an unidentified antigen and the O antigen. K antigen was found in the 0.25M and 0.35M fractions (Fig. 34).

Differences in the extent to which agglutination was inhibited by different K antigens

In Fig. 35 three K antigens have been compared for their ability to inhibit agglutination. The ordinate is the extent to which agglutination was inhibited on a $\log_2$ scale and represents the reduction in titre of the anti sheep red cell serum due to K antigen. The abscissa is the K antigen concentration used to treat the red cells.

For all the antigens the extent of inhibition was dependent on the concentration used. Besides this antigens differed in their ability to inhibit agglutination. The antigen from WF82 was the most effective, a concentration of 3 µg per ml reduced the haemagglutinating activity of the anti sheep red cell serum by half. 7 µg of the antigen from strain WF26 and 11 µg of the antigen from strain WF96

were required to inhibit agglutination to the same extent.

Relation between charge and agglutination inhibiting activities of the antigens

The electrophoretic mobilities of the K antigens from strains WF96 and WF82 were compared (Fig. 36). The acidic nature of both antigens was demonstrated by the finding that both migrated towards the anode. Although less inhibitory the K antigen from strain WF96 moved slightly faster than the antigen from strain WF82. Since mobility in agar depends on the shape and size of the molecule besides the charge it carries (Block et al., 1958) interpretation of this result was difficult and the possibility that inhibitory activity was charge dependent could not be excluded on the basis of this experiment.

The negative charge on different antigens was also compared by finding the sodium chloride molarity needed to elute them from D.E.A.E. cellulose. The higher the negative charge on the molecule the higher the sodium chloride concentration required.

The elution of K antigens from D.E.A.E. cellulose is shown in Figs. 37-39. In Table 14 the molarity needed has been compared with the inhibitory activity of the antigen. There was no relation between the negative charge on the antigen and its inhibitory activity.

Comparison between the molecular weights of different K antigens using Sepharose

The three K antigens were put onto Sepharose 6B and their elution patterns compared (Fig. 40). The antigens from strains WF82 and WF26 were both excluded from the Sepharose gel indicating molecular weights of more than 1×10^6. The antigen from strain WF96 was held up by the gel indicating a molecular weight of less than 1×10^6. Thus the antigen from strain WF96 was the smallest of the three, it was also the least active at inhibiting agglutination *in vitro* (Fig. 35).

The antigens from strains WF82 and WF26 were put onto Sepharose 4B (fig. 41). Both antigens were separated into two fractions. One fraction was held up by the gel and the other was found in the void volume.

The isolated molecules were therefore not homogeneous with respect to molecular weight. The elution patterns of the acid polysaccharides from strains WF82 and WF26 on Sepharose 6B and 4B indicated that the isolated antigens were composed of one fraction which had a molecular weight of $1\text{-}5 \times 10^6$ and another fraction which contained larger molecules.

The cetavlon precipitated K antigen from *E. coli* WF82 was fractionated on Sepharose 2B (Fig. 42). The two fractions

were collected, concentrated by negative pressure dialysis against 0.15M saline and rerun on the same 2B column to ensure they were genuinely different fractions and not artifacts (Fig. 43).

When the two fractions of the antigen separated on 2B were compared by immunoelectrophoresis they were not identical (Fig. 45). The antigen molecules which were excluded from the 2B gel contained both the slow and fast migrating fractions present in the original. The K antigen fraction with a molecular weight of 1-5 x 10^6 which was held up by Sepharose 2B had only the faster migrating portion of the original sample.

The two fractions of the K antigen obtained from Sepharose 2B were compared for their ability to inhibit agglutination. The fraction containing the larger molecules was most effective, 0.3 µg of this reduced the haemagglutination titre of the anti red cell serum by half. 6.0 µg of the K antigen fraction which had a molecular weight of 1-5 x 10^6 were needed to inhibit red cell agglutination to the same extent.

The two K antigen fractions obtained by D.E.A.E. chromatography were compared for their elution patterns on Sepharose 2B (Fig. 44).

The antigen fraction which was eluted by 0.25M buffer from D.E.A.E. was the same as the fraction which was held up

by the 2B gel. The antigen fraction which was eluted by 0.35M buffer from D.E.A.E. was composed of those molecules which were excluded from Sepharose 2B.

The effect of sodium hydroxide on the agglutination inhibiting activity of K antigens

The alkali concentration required to destroy the inhibiting activity of the isolated molecules was similar for all three antigens suggesting a similarity in the mode of action of sodium hydroxide in each case (Table 15).

Sodium hydroxide treated and untreated antigen from E. coli WF82 were compared on Sepharose 6B to see if any changes in size were associated with loss of inhibitory activity. 0.005N alkali did not affect the size of the molecule or its inhibitory properties. When the antigen was incubated with 0.05N alkali it lost its inhibiting activity (Table 15). Coupled with this loss was a reduction in the size of the molecule indicated by the finding that it was held up by the 6B gel unlike untreated antigen or antigen that had been incubated with 0.005N alkali (Fig. 46).

The K antigen from E. coli WF26 was also broken down by 0.05N alkali. This was indicated by the finding that alkali treated antigen, unlike untreated antigen was held up by Sepharose 6B (Fig. 47). Associated with this reduction in

size of the molecule was a loss of agglutination inhibiting activity (Table 15).

The inhibitory activity of these antigens therefore depended on their structural integrity. When the size of the isolated molecule was reduced using sodium hydroxide the activity was lost.

Although the antigen from WF82 lost its ability to inhibit red cell agglutination after it had been incubated with sodium hydroxide it was still able to sensitise red cells to agglutination by anti K antibody. A concentration of 500 µg per ml of sodium hydroxide treated antigen did not have any inhibitory activity but a concentration of 1 µg per ml was sufficient to sensitise red cells to agglutination by anti K antibody. Alkali treated antigen was therefore being attached to red cells.

Effect of sodium hydroxide on the serological specificity of K antigens

The effect of alkali on serological specificity varied for different antigens (Table 15). The serological specificity of the antigen from WF82 was altered by an alkali concentration of 0.005N (Fig. 48). An alkali concentration of 0.05N was needed to affect the antigen from WF26 (Fig. 49) and the antigen from WF96 was unaffected by 0.1N alkali (Fig. 50).

The effect of sodium hydroxide on the serological reactivity of the molecule was therefore independent of its effect on agglutination inhibiting activity (Table 15) and was not related to the reduction in size of the molecule caused by alkali.

Sodium hydroxide caused a change in the precipitin line produced by the antigen from strain WF82 (Fig. 48). In Fig. 51 untreated antigen and NaOH treated antigen were compared for their ability to remove antibody directed against the K antigen. When untreated antigen was incubated with anti K antiserum antibodies which gave a line against untreated and NaOH treated antigen were removed. When the anti K antiserum was incubated with NaOH treated antigen only the line against NaOH treated antigen disappeared.

A phenol-water extract of E. coli WF82 was treated with 0.05N sodium hydroxide and then incubated with an antiserum raised against live E. coli WF82 (Fig. 52). Antiserum treated in this way only gave a precipitin reaction with K antigen and was used as the source of anti K antibody for the estimation of K antigens by radial immunodiffusion in Section VIII.

Effect of steam on K antigens

K antigens were steamed for 2½ hours and examined for the

effect of this on their agglutination inhibiting activity and their serological specificity. Heating at this temperature had no effect on either (Fig. 53). The antigen from strain WF82 (O117:K?) was an L type by Kauffmann's definition (Kauffmann, 1947). The antigen from strain WF26 was either an L or B type. Both antigens would therefore be expected to be affected by heating at this temperature (Edwards and Ewing, 1962).

Table 14. Relation between charge and agglutination inhibiting activity of K antigens

Source of K antigen	K antigen concentration reducing the agglutination titre of haemolysin by 50%	Molarity at which K antigen was eluted from D.E.A.E.
E. coli WF82 0117:K?	3 µg per ml	0.23
E. coli WF26 06:K13	7 µg per ml	0.37
E. coli WF96 07:K1	11 µg per ml	0.28

Table 15. Effect of sodium hydroxide on K antigens

NaOH concentration	Agglutination inhibiting activity			Serological specificity		
	Kag from *E. coli* WF82	Kag from *E. coli* WF26	Kag from *E. coli* WF96	Kag from *E. coli* WF82	Kag from *E. coli* WF26	Kag from *E. coli* WF96
0	+	+	+	+	+	+
0.005N	+	+	+	±	+	+
0.01N	+	0	+	±	+	+
0.05N	0	0	0	±	0	+
0.1N	0	0	0	±	0	+

+ = property retained

± = partial destruction

0 = property lost

Fig. 32. Fractionation of a phenol-water extract of *E. coli* WF82 on DEAE cellulose.

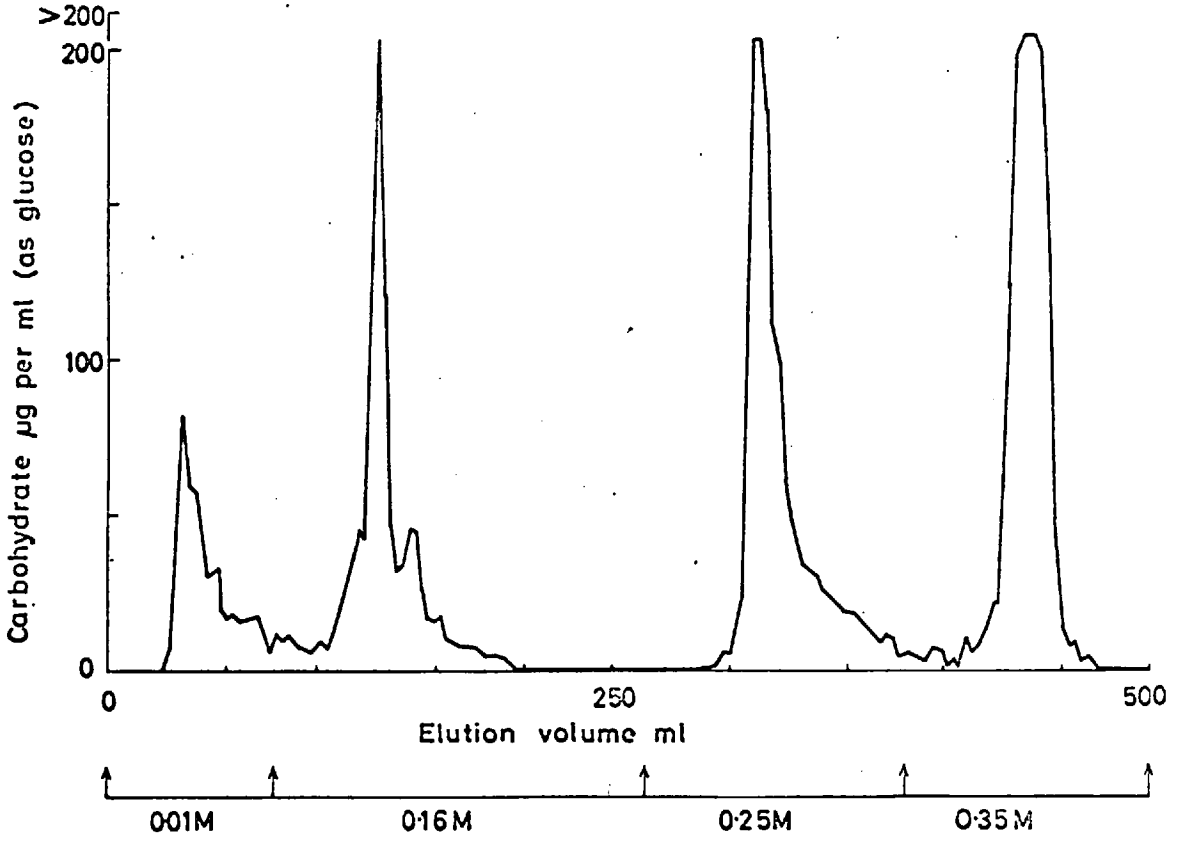
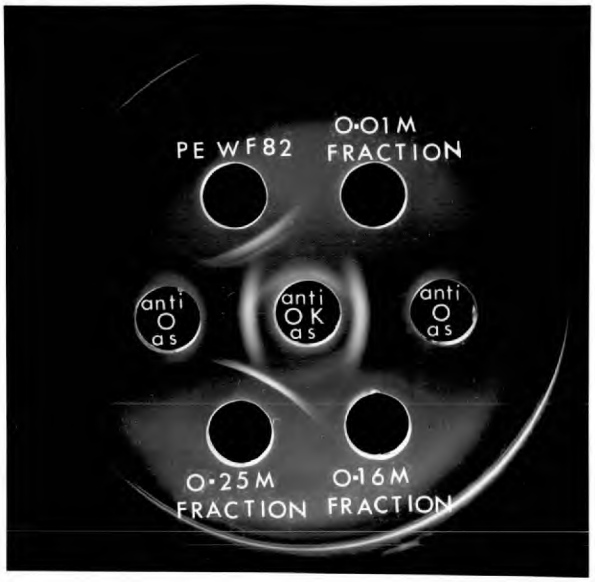

>200
200
100
0
Carbohydrate µg per ml (as glucose)
0
250
500
Elution volume ml
0·01M
0·16M
0·25M
0·35M

Fig. 33. Identification of antigens in the carbohydrate fractions eluted from DEAE cellulose.
The antisera used were, anti *E. coli* WF82 O and anti WF82 OK sera.
The other wells contained: PE WF82 - a phenol-water extract of *E. coli* WF82.
0.01 M, 0.16 M, 0.25 M fractions - carbohydrate fractions eluted from DEAE cellulose with buffer of these molarities.

Fig. 34. An ouchterlony plate of the two fractions of K antigen from DEAE cellulose.
The wells contained: anti OK antiserum - anti WF82 OK antiserum. 0.25M, 0.35M - the two fractions of the extract of *E. coli* WF82 eluted from DEAE cellulose with buffer of these molarities.


PE WF82
0·01M FRACTION
anti O as
anti OK as
anti O as
0·25M FRACTION
0·16M FRACTION


0·25M
0·35M
anti OKas

Fig. 35. Inhibition of red cell agglutination by the K antigens from *E. coli* strains WF82, WF26 and WF96.

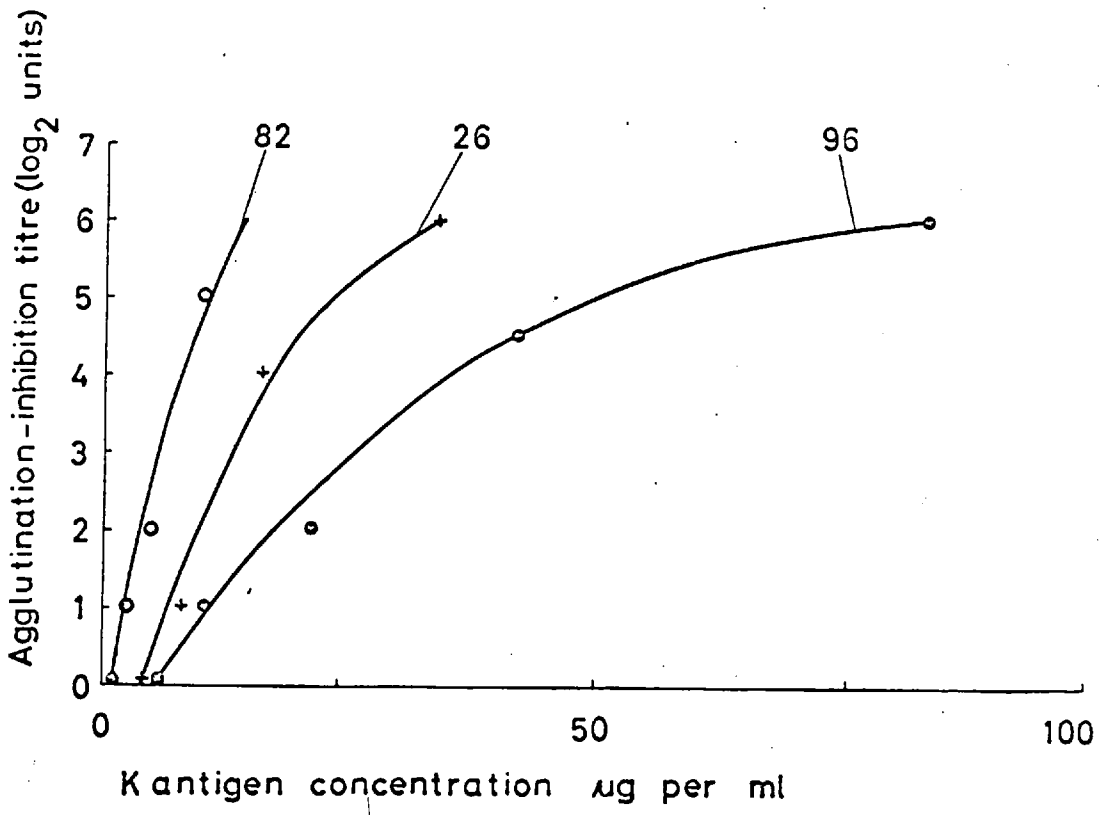

Agglutination-inhibition titre (log_2 units)
0
1
2
3
4
5
6
7
82
26
96
0
50
100
K antigen concentration µg per ml

Fig. 36. Comparative electrophoretic mobilities of the K antigens from WF96 and WF82.
The troughs contained anti *E. coli* WF82 OK serum and anti *E. coli* WF96 OK serum.
The wells contained: 82, K antigen from *E. coli* WF82. 96, K antigen from *E. coli* WF96.

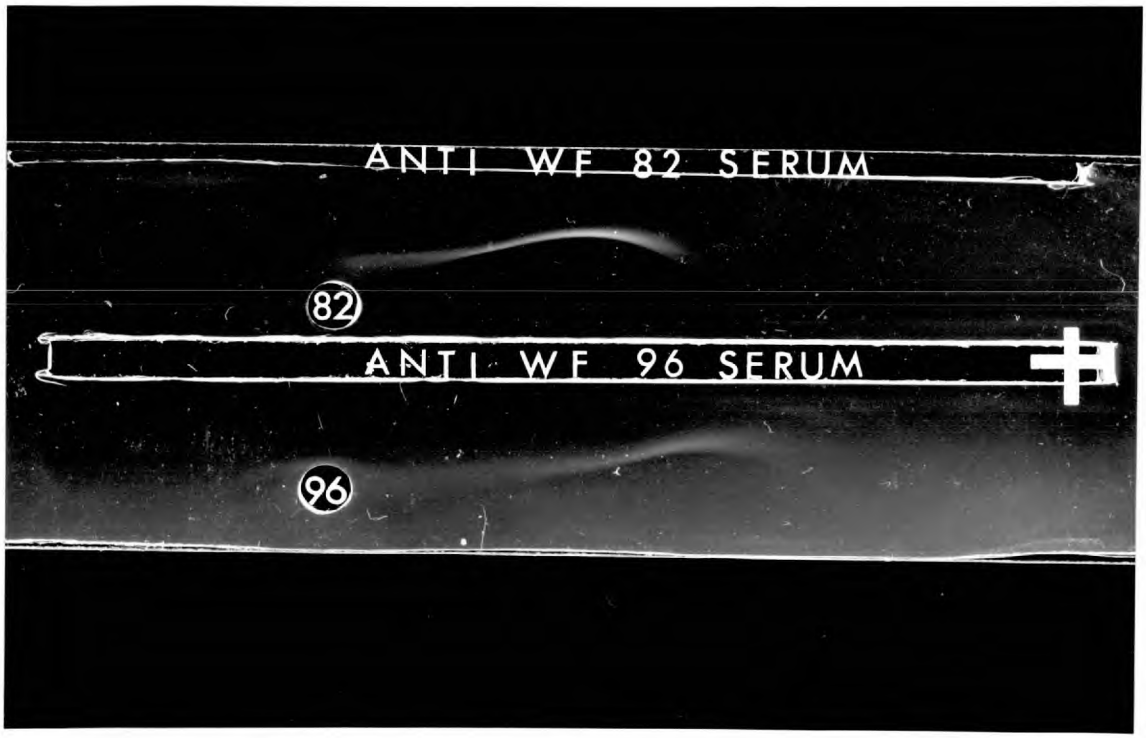
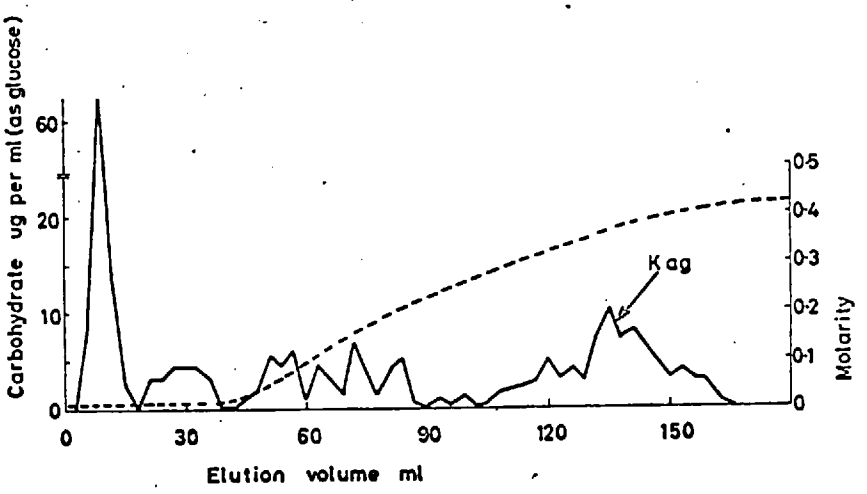

ANTI WF 82 SERUM
82
ANTI WF 96 SERUM
96

Fig. 37. Elution of the K antigen of *E. coli* WF26 from DEAE cellulose.

Fig. 38. Elution of the K antigen of *E. coli* WF96 from DEAE cellulose.


Carbohydrate ug per ml (as glucose)
60
20
10
0
0·5
0·4
0·3
0·2
0·1
0
Molarity
Kag
0
30
60
90
120
150
Elution volume ml

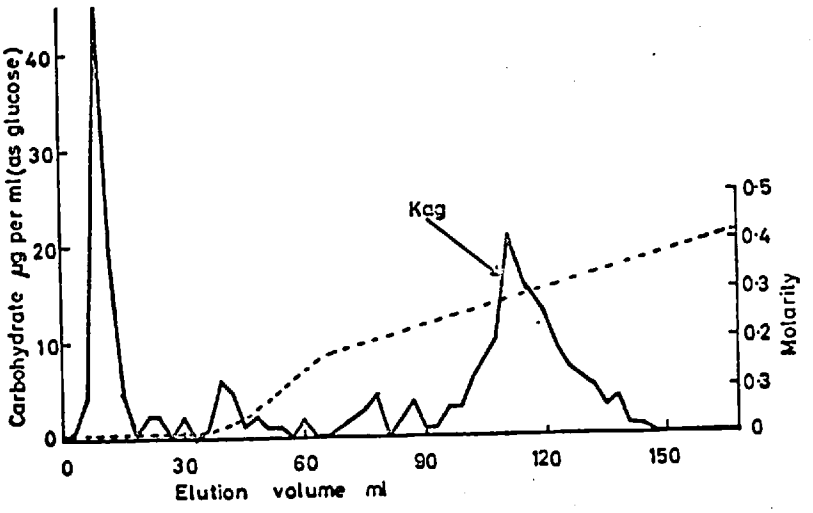

Carbohydrate µg per ml (as glucose)
40
30
20
10
0
0·5
0·4
0·3
0·2
0·3
0
Molarity
Kag
0
30
60
90
120
150
Elution volume ml

Fig. 39. Elution of the K antigen of *E. coli* WF82 from DEAE cellulose.

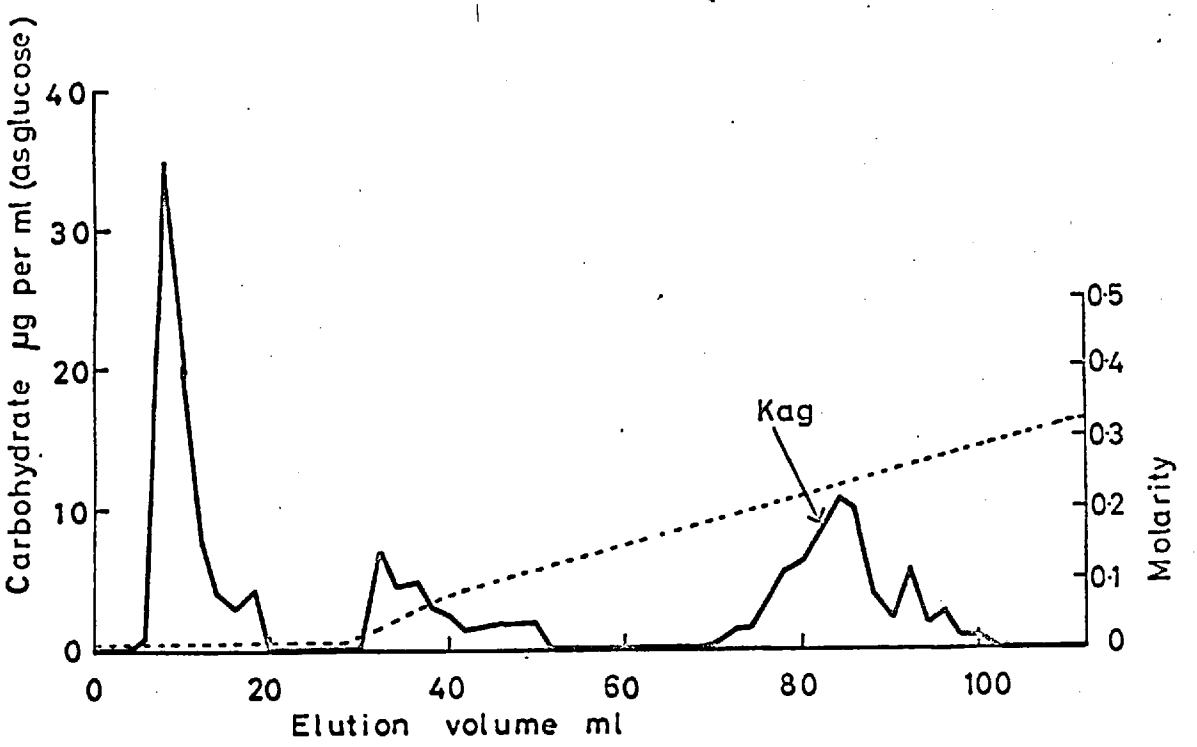

Carbohydrate µg per ml (as glucose)
40
30
20
10
0
Kag
0·5
0·4
0·3
0·2
0·1
0
Molarity
0
20
40
60
80
100
Elution volume ml

Fig. 40. Elution patterns of the K antigens from E. coli strains WF82, WF26 and WF96 on Sepharose 6B.

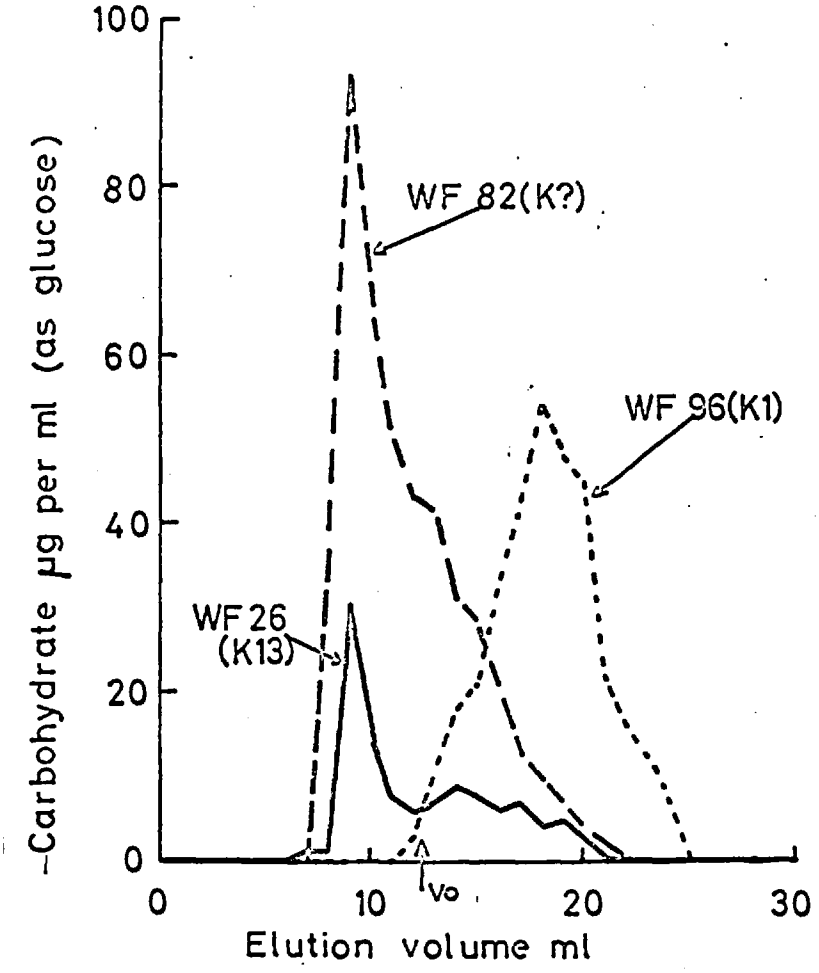

100
80
60
40
20
0
Carbohydrate μg per ml (as glucose)
WF 82(K?)
WF 96(K1)
WF 26
(K13)
Vo
0
10
20
30
Elution volume ml

Fig. 41. Elution patterns of the K antigens from E. coli strains WF82 and WF26 on Sepharose 4B.

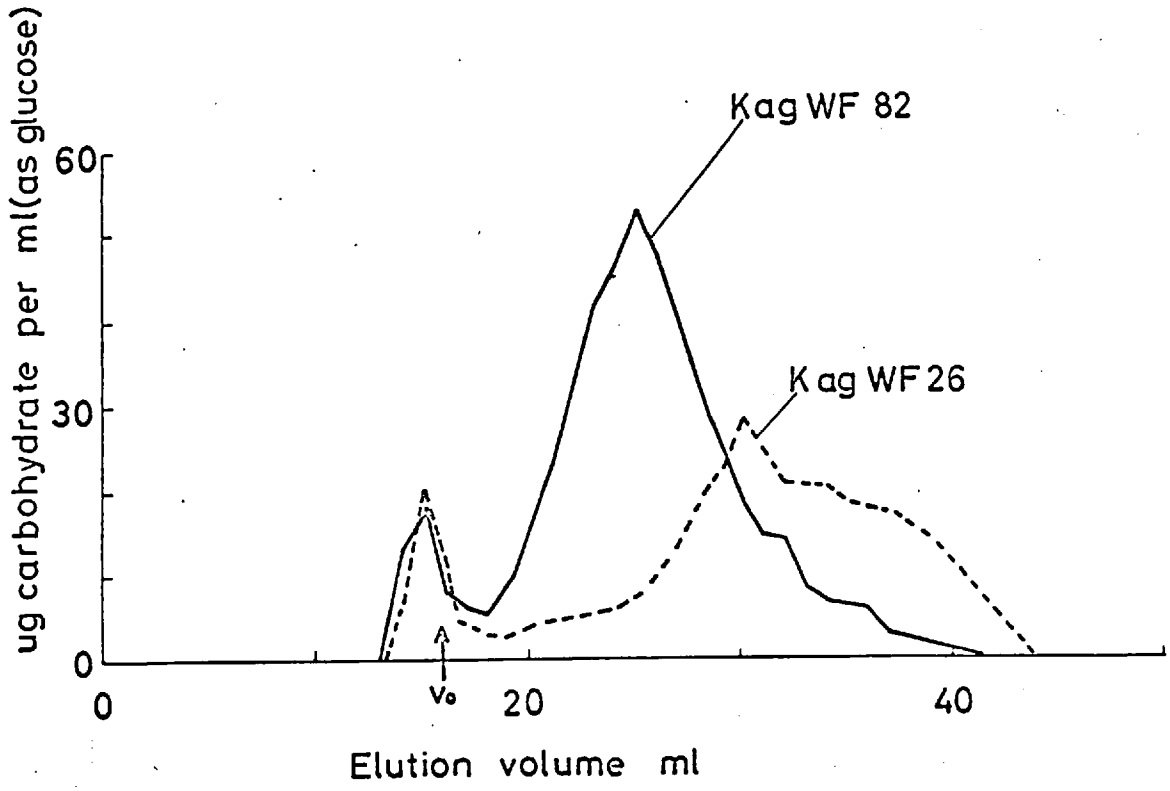
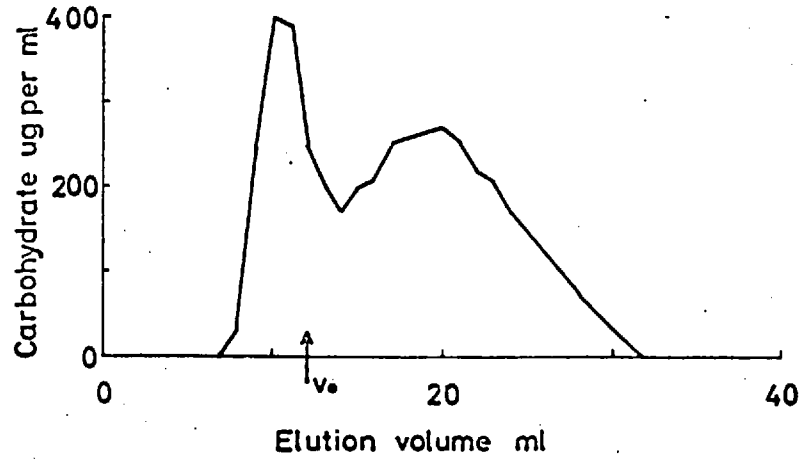

ug carbohydrate per ml(as glucose)
60
30
0
0
Vo
20
40
Elution volume ml
Kag WF 82
Kag WF 26

Fig. 42. Fractionation of the K antigen from E. coli WF82 on Sepharose 2B.

Fig. 43. Elution pattern of the two fractions of the K antigen from E. coli WF82 obtained from Sepharose 2B rerun on 2B.


400
200
0
Carbohydrate ug per ml
0
Ve
20
40
Elution volume ml

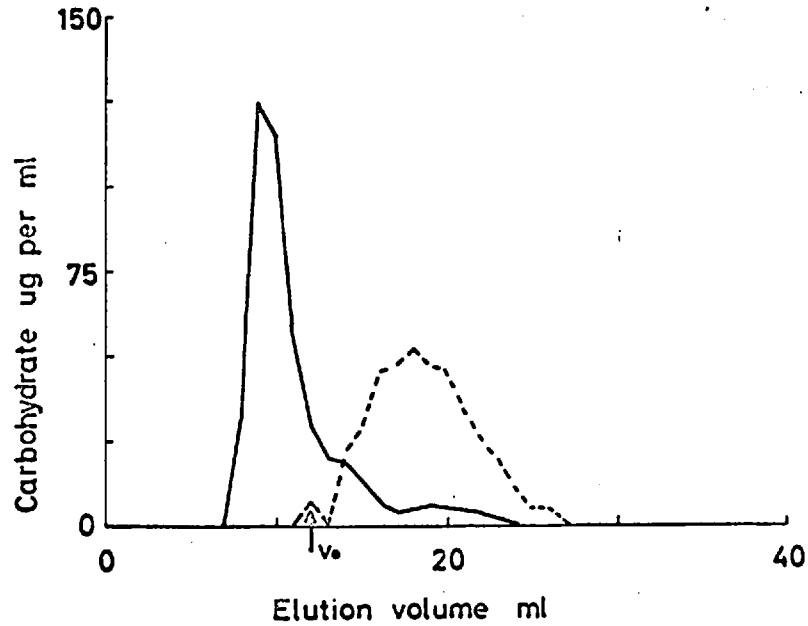

150
75
0
Carbohydrate ug per ml
0
Ve
20
40
Elution volume ml

Fig. 44. Elution pattern on Sepharose 2B of the 0.25 M and 0.35 M DEAE fractions of the K antigen from E. coli WF82.

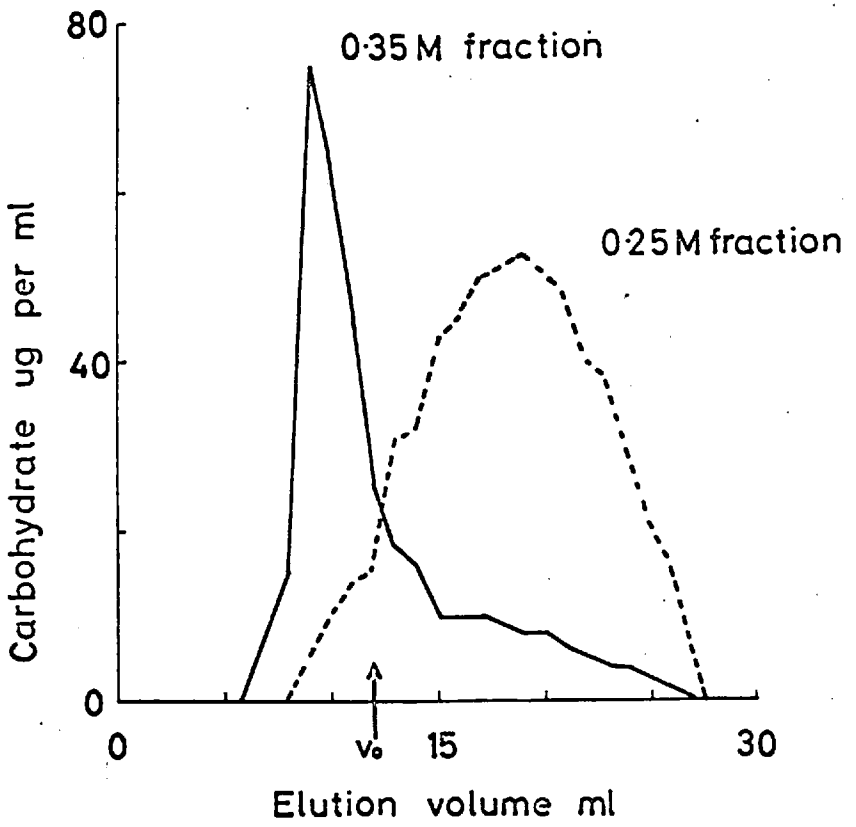

80
40
0
0·35 M fraction
0·25 M fraction
Carbohydrate ug per ml
0
V_o
15
30
Elution volume ml

Fig. 45. Immunoelectrophoresis of the Sepharose 2B fractions of the K antigen from E. coli WF82. The wells contained: Or - K antigen from E. coli WF82 purified by cetavlon fractionation; L - the large fraction of the K antigen from WF82 which was excluded from Sepharose 2B; S - the fraction of the K antigen from WF82 with a molecular weight of 1-5 x 10^6 which was held up by the 2B gel.

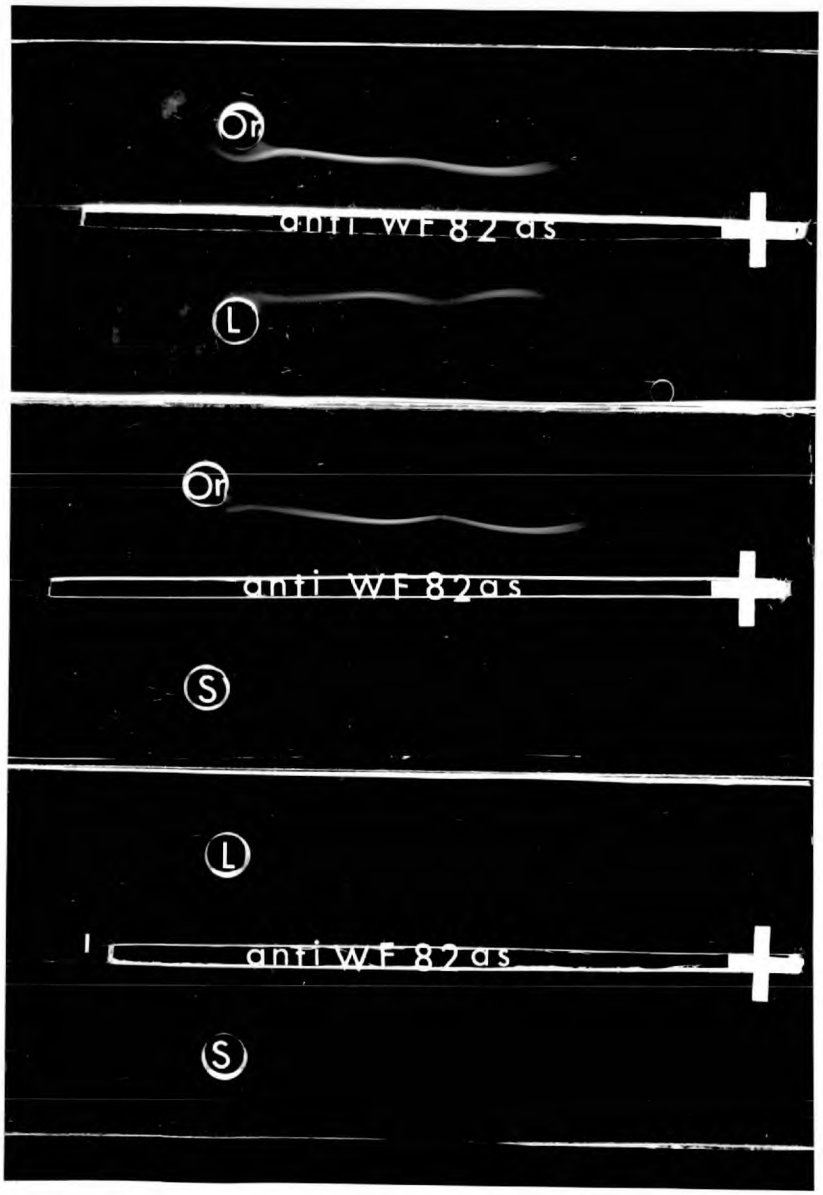

Or
anti WF 82 as
L
Or
anti WF 82as
S
L
anti WF 82as
S

Fig. 46. Fractionation on Sepharose 6B of the K antigen from WF82 before and after sodium hydroxide treatment.

Untreated antigen and antigen that had been hydrolysed overnight at 37°C with 0.005 N or 0.05 N sodium hydroxide were compared.

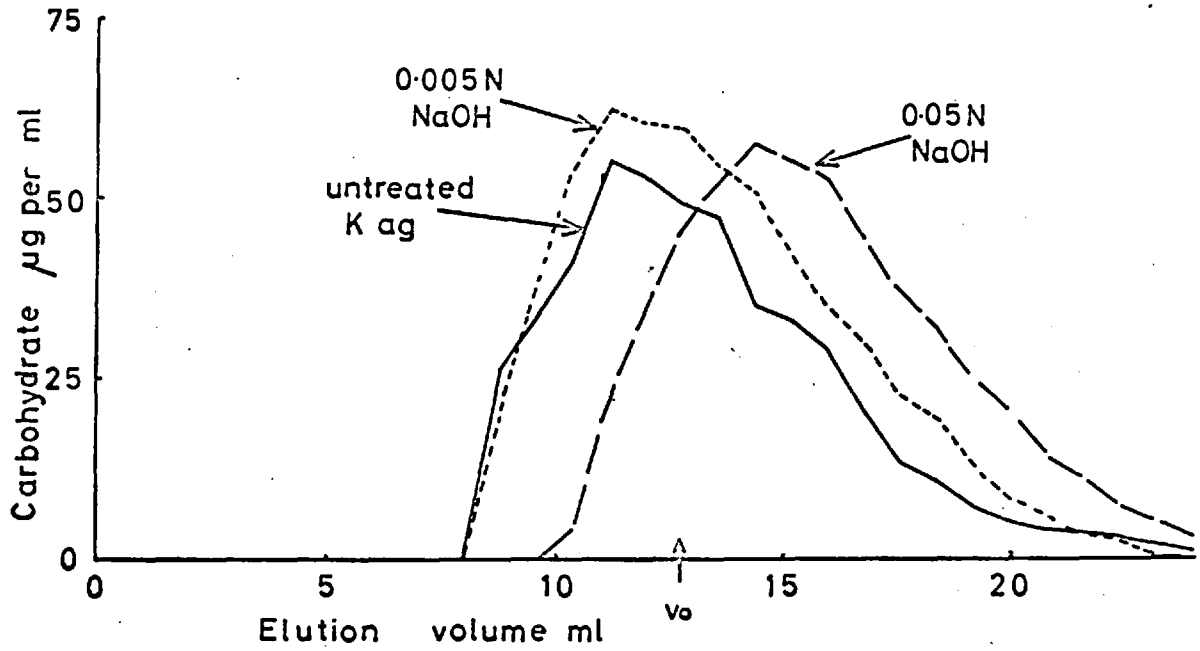

75
50
25
0
Carbohydrate µg per ml
0·005N
NaOH
untreated
K ag
0·05N
NaOH
0
5
10
15
20
Vo
Elution volume ml

Fig. 47. Fractionation on Sepharose 6B of the K antigen from *E. coli* WF26 before and after hydrolysis with sodium hydroxide.

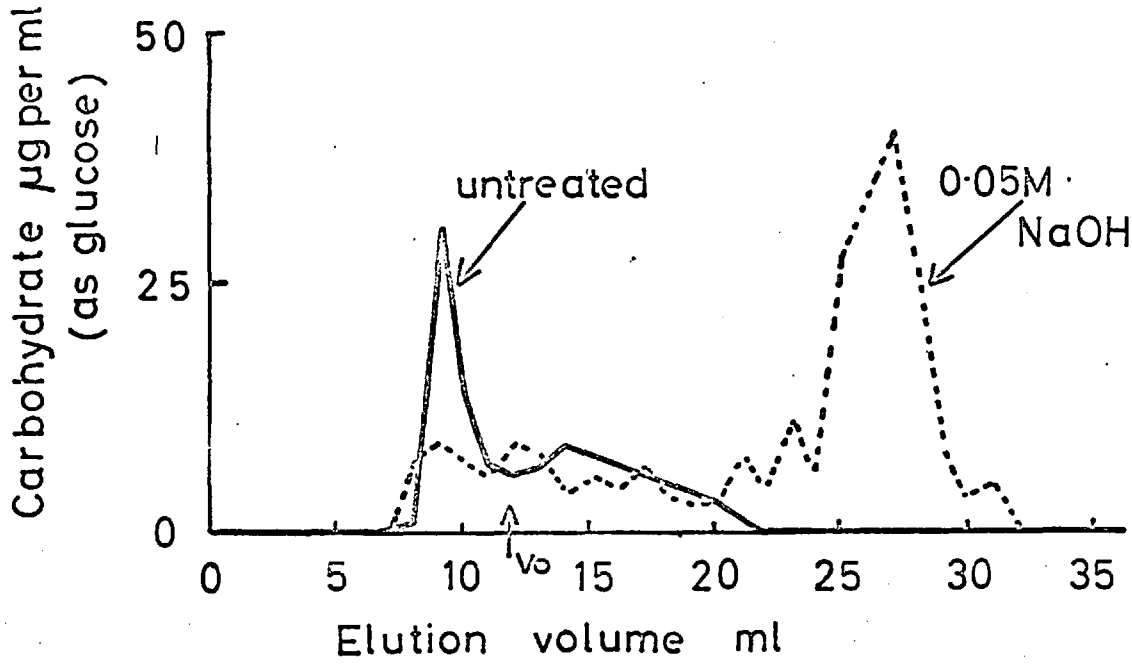
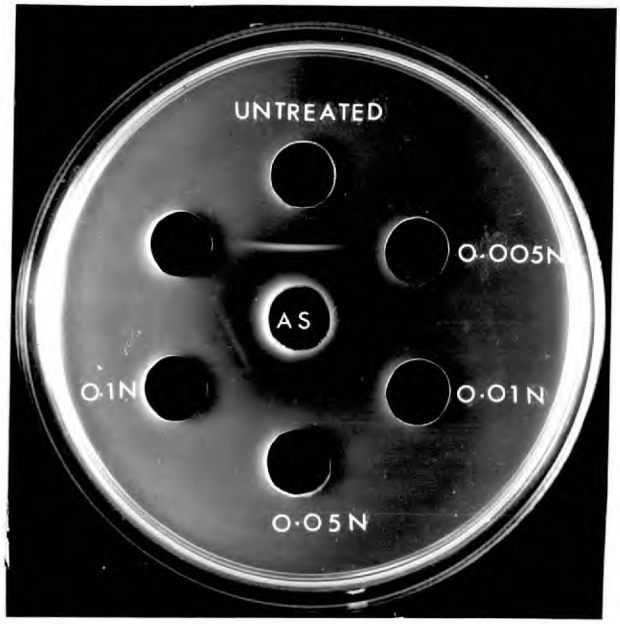

Carbohydrate μg per ml (as glucose)
50
25
0
untreated
0·05M NaOH
Vo
0
5
10
15
20
25
30
35
Elution volume ml

Fig. 48. Effect of sodium hydroxide on the K antigen from *E. coli* WF82.

The wells contained: AS - antiserum raised against live WF82, untreated K antigen and antigen that had been incubated overnight at 37°C with 0.005 N, 0.01 N, 0.05 N and 0.1 N sodium hydroxide.

Fig. 49. Effect of sodium hydroxide on the K antigen from *E. coli* WF26.

The wells contained: AS - antiserum raised against live WF26 and untreated antigen or antigen treated with the sodium hydroxide concentrations indicated.


UNTREATED
0·005N
AS
0·1N
0·01N
0·05N

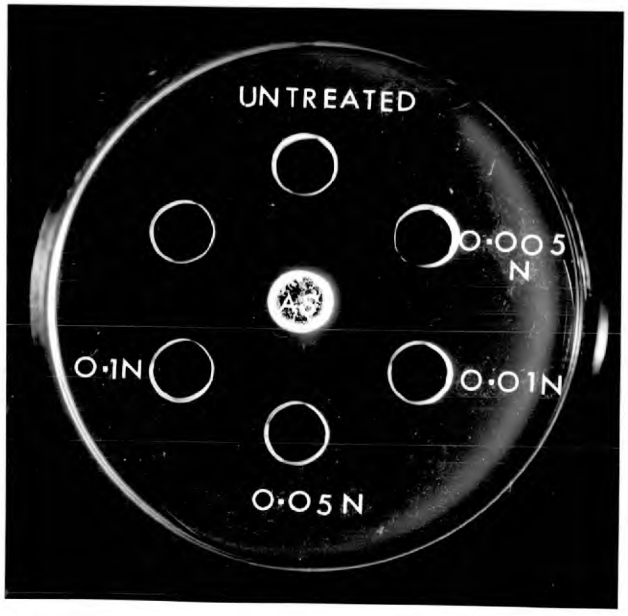
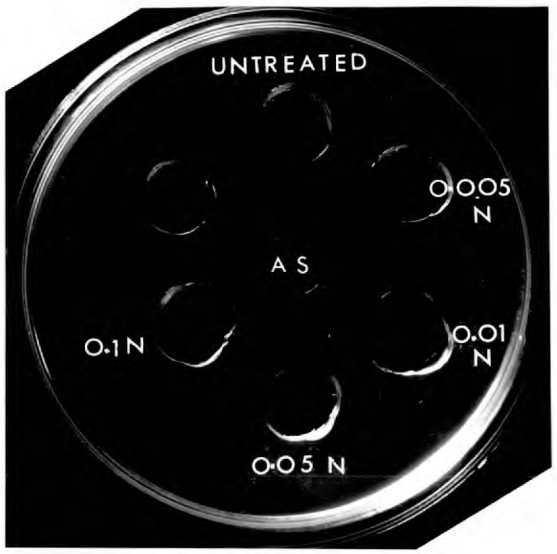

UNTREATED
0·005
N
0·1N
0·01N
0·05N

Fig. 50. Effect of sodium hydroxide on the K antigen from *E. coli* WF96.

The wells contained: AS - antiserum raised against live WF96 and untreated antigen or antigen treated with the sodium hydroxide concentrations indicated.

Fig. 51. Effect of sodium hydroxide on the antibody binding capacity of the K antigen from *E. coli* WF82.

The wells contained: K a/s - anti *E. coli* WF82 OK serum; Anti K serum + Kag - anti WF82 OK serum that had been incubated with untreated K antigen; Anti K serum + K NaOH - anti WF82 OK serum that had been incubated with alkali treated K antigen; Kag - untreated K antigen from WF82; K NaOH - K antigen that had been incubated with sodium hydroxide.


UNTREATED
0·005 N
A S
0·1 N
0·01 N
0·05 N

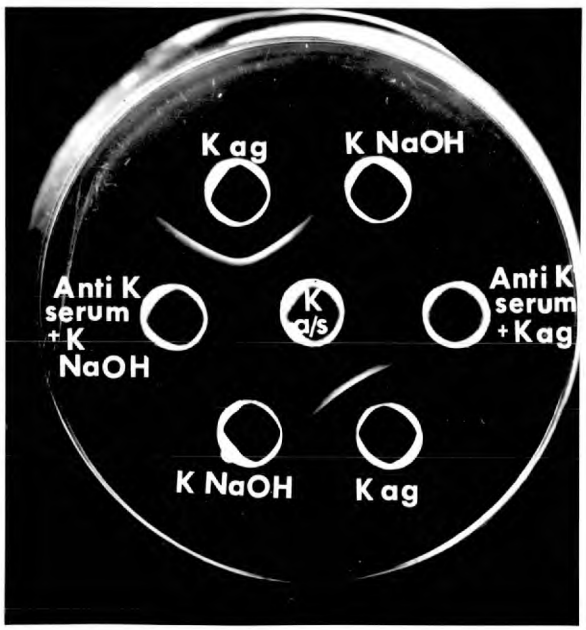

K ag
K NaOH
Anti K serum + K NaOH
K a/s
Anti K serum + K ag
K NaOH
K ag

Fig. 52. Absorption of an anti 82 OK antiserum with a sodium hydroxide treated phenol-water extract of E. coli WF82.

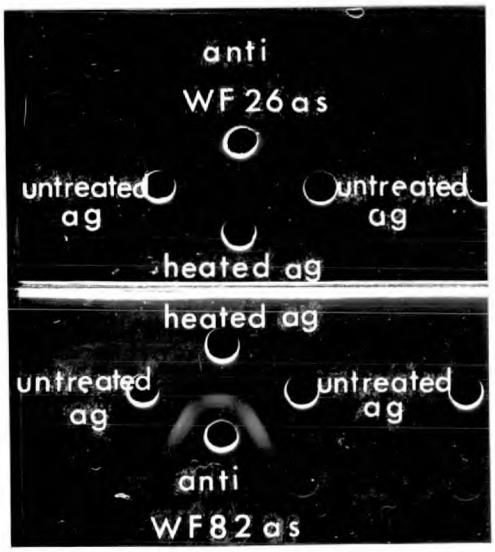
The wells contained: P.E - phenol-water extract of WF82; Kag - K antigen from WF82; Anti OK AS - anti WF82 OK antiserum; Anti OK AS + PE NaOH - anti WF82 OK antiserum that had been incubated with a sodium hydroxide treated phenol-water extract of WF82.

Fig. 53. Effect of heating the K antigens from E. coli strains WF26 and WF82 at 100°C.

In Fig. 53(a) the wells contained untreated K antigen from WF26, heated K antigen from WF26 and anti WF26 OK serum.

In Fig. 53(b) the wells contained untreated K antigen from WF82, heated K antigen from WF82 and anti WF82 OK serum.


PE
Kag
PE
ANTI
OK AS
ANTI OK AS+
PE NaOH


anti
WF 26 as
untreated
ag
untreated
ag
heated ag
heated ag
untreated
ag
untreated
ag
anti
WF 82 as

DISCUSSION

Acidic polysaccharide antigens were isolated from three strains of E. coli. Antibodies directed against these antigens were demonstrable in antisera raised in rabbits against live but not heated bacteria. The isolated antigens were capable of inhibiting an unrelated agglutination system in vitro and were considered to be the K antigens of the strains from which they were isolated.

The "heat labile" K antigens of E. coli have been found to be acidic polysaccharides in the majority of cases by other workers (Luderitz et al., 1968).

The K antigen from E. coli WF82 could be purified by ion exchange chromatography, providing an alternative method of preparation to cetavlon fractionation (Hungerer et al., 1967). Ion exchange chromatography was more time consuming than cetavlon fractionation. The yield was also lower. 500 mg of a phenol extract yielded about 20 mg of K antigen using cetavlon and only 4 mg using ion exchange chromatography. K antigens were therefore prepared using cetavlon.

K antigens isolated from different strains of E. coli were found to differ in their ability to inhibit red cell agglutination. Although the antigen from WF96 was less effective at inhibiting agglutination than the antigen from WF82 it had a higher electrophoretic mobility. This suggested

activity was not directly related to charge.

The binding of a molecule to D.E.A.E. cellulose is dependent on its negative charge. The more negatively charged a molecule is the higher the buffer molarity needed to elute it from the resin. No relation was demonstrable between the inhibitory activities of the antigens and the buffer molarities needed to elute them from D.E.A.E. cellulose.

Although the inhibitory activities of the different antigens was not directly proportional to the negative charge on the molecule a common feature of molecules capable of inhibiting agglutination in antigenically unrelated systems has been shown to be their acidic nature (Ceppellini and Landy, 1963). It seems likely therefore that negative charge was a necessary contributory factor.

When the sizes of the isolated antigens were compared using Sepharose chromatography the least inhibitory antigen from E. coli WF96 was found to be the smallest of the three. The antigens from strains WF26 and WF82 were not homogeneous with respect to molecular weight. They consisted of a fraction containing polysaccharides with a molecular weight of $1\text{-}5 \times 10^6$ and a fraction containing larger molecules.

The fraction of the antigen from strain WF82 which contained the larger molecules was more effective at inhibiting red cell agglutination than the fraction containing the smaller molecules.

The inhibitory activity of K antigens therefore appeared to be related to the size of the isolated polysaccharide molecule whether different antigens or fractions of antigens were compared.

D.E.A.E. and Sepharose chromatography separated the antigen isolated from WF82 into two similar fractions. The 0.25M D.E.A.E. fraction corresponded to the antigen molecules with a molecular weight of 1-5 x 10^6 which were held up by the 2B gel. The 0.35M D.E.A.E. fraction corresponded to the part of the isolated antigen which was excluded from Sepharose 2B.

Although two K antigen fractions were obtained from D.E.A.E. cellulose using 0.25M and 0.35M buffer, when a phenol extract of WF82 was fractionated on D.E.A.E. using a molarity gradient only one peak corresponding to K antigen was obtained (Figs. 32 and 39).

It therefore seems likely that the 0.35M D.E.A.E. fraction was not a homogeneous sample but was a collection of molecules combined into one fraction when a stepwise method of elution was used. Possibly the molecules comprising the 0.35M D.E.A.E. fraction were polymers or aggregates of the antigen molecules with a molecular weight of 1-5 x 10^6 which were eluted from D.E.A.E. by 0.25M buffer.

Alkali reduced the size of the antigen molecule. Associated with the destruction of the structural integrity

of the molecule was a loss of agglutination inhibiting activity. All three antigens lost their inhibitory activity at a similar sodium hydroxide concentration suggesting there was some common feature in the breakdown of the molecules. At least the reduction in size of the isolated polysaccharides was associated with loss of inhibitory activity, again suggesting that activity was dependent on size.

The possibility exists that differences in the inhibitory activity of different antigens was due to different amounts being absorbed onto red cells. This seems unlikely, at least in the case of alkali treated antigen since red cells were still sensitised by this to agglutination with anti K antibody. Although inhibitory activity had been lost alkali treated polysaccharide was still being attached to the red cell.

Hungerer et al. (1967) isolated the K antigen from a strain of E. coli serotype 09:K30(A):H12. It was an acid polysaccharide composed of glucuronic acid, mannose and galactose residues. The isolated polysaccharide was not homogeneous with respect to molecular weight. Hydrolysis with sodium hydroxide digested the isolated antigen to give a polysaccharide with a molecular weight of 1.5×10^5. Alkali had no effect on the sugar composition of the antigen.

The structure proposed for this K antigen was that linear molecules with a molecular weight of 1.5×10^5, composed of

the three sugar residues, were joined between carboxyl groups of the uronic acid residues and hydroxyl groups of the sugars to form larger polymers.

Jann et al. (1968) examined the acid polysaccharide K antigen from a strain of E. coli serotype 08:K27:H⁻. The isolated polysaccharide had a molecular weight of 3×10^6. Hydrolysis with sodium hydroxide produced a molecule with a molecular weight of 1×10^5. This antigen appeared to be structurally similar to the K30 antigen described by Hungerer et al. (1967).

Jarvis et al. (1967) found that alkali reduced the molecular weight of the Vi antigen isolated from a strain of Escherichia from 1.7×10^6 to 1×10^5. Jarvis et al. proposed the same structure for the Vi antigen as Hungerer et al. (1967) proposed for the K30 antigen, i.e. linear subunits with a molecular weight of 1×10^5 were joined by ester bonds to produce the intact molecule found on the bacterial surface.

Hungerer et al. (1967) stated that they have found A types of K antigens to be physically inhomogeneous but not the B or L types. It was also suggested that the reduction in size caused by alkali was peculiar to the A types.

The antigens studied here were all B or L types according to the definition proposed by Kauffmann (1954) since bacteria

became O agglutinable after heating at 100°C for 1 hour. They were physically inhomogeneous and alkali labile. These properties are therefore not confined to K antigens of the A type. The Vi antigen can be considered as an example of the B type of K antigens (Kauffmann, 1954). This antigen has also been shown to be alkali labile (Jarvis et al., 1967).

Besides reducing the size of the isolated molecule sodium hydroxide affected the serological specificity of some antigens. The effect of alkali on serological specificity varied for different antigens and was independent of its effect on agglutination inhibiting activity and the size of the molecule.

When the antigen from E. coli WF82 was incubated with alkali the position of the precipitin line it produced in an Ouchterlony plate changed (Fig. 48).

The possibility that the change in position of the K precipitin line was because alkali reduced the size of the molecule was eliminated since 0.005N sodium hydroxide affected the serological specificity of the molecule but not its size (Fig. 46).

An antiserum raised against live E. coli WF82 gave a precipitin line with both untreated antigen and alkali treated antigen.

When untreated antigen was incubated with the antiserum

neither of the precipitin lines were produced. Incubation with alkali treated antigen removed antibody directed against alkali treated antigen but the antiserum still precipitated with untreated antigen (Fig. 51).

It was therefore concluded that the acid polysaccharide from E. coli WF82 contained two immunodeterminant groups, one of which was alkali labile the other being alkali stable.

Alkali labile O-acetyl groups were shown to comprise part of the immunodeterminant group of the K30 antigen (Hungerer et al., 1967). It seemed likely that loss of O-acetyl or some other alkali labile group resulted in the change in serological specificity of the K antigen from strain WF82 due to sodium hydroxide.

Although Kauffmann (1947) considered K antigens to be thermolabile this has been disputed. It has been suggested that K antigens are not necessarily thermolabile but rather that they are eluted from the bacterial surface on heating (Ørskov and Ørskov, 1968). When studying the antigens by means of bacterial agglutination tests they therefore gave the appearance of being thermolabile.

The findings reported here that steaming for 2½ hours did not affect the serological specificity of the antigens, judged by immunodiffusion, or the ability of the antigens to inhibit red cell agglutination supports the hypothesis that these antigens are not in fact heat labile.

Kauffmann (1947) proposed the term K antigen to simplify the serological scheme used for typing strains of E. coli. He emphasised that the three types of antigens designated L, A and B, which were united by the term K antigen, were distinct groups distinguished by their heat lability and sensitivity to acid and alcohol. He thought that a chemical investigation of their nature would emphasise the differences between these three types.

Examples of K antigens of the L, A and B types have all been shown to be acid polysaccharides (Luderitz et al., 1968). Jann et al. (1968) considered that it was difficult to reconcile the chemical similarities of the K antigens with their subdivision into the different classes which Kauffmann (1947) considered to be quite distinct. Even the physical inhomogeneity and alkali lability which Jann et al. (1968) thought might be a property of the A types of K antigen relating to their ability to form capsules has been shown here to be also characteristic of K antigens which were not A types.

As a group the K antigens are united by their ability to cause live bacteria to be O inagglutinable (Kauffmann, 1954) and by the finding that in the majority of cases they are acidic polysaccharides (Luderitz et al., 1968).

VIII. CHANGES INDUCED IN THE COMPLEMENT SENSITIVITY OF ESCHERICHIA COLI BY DIPHENYLAMINE

INTRODUCTION

When strains of E. coli that were normally resistant to C⁰ were grown in the presence of diphenylamine they became C⁰ sensitive. Associated with this change in serum sensitivity was a reduction in the carbohydrate content of the lipopolysaccharide (Feingold, 1969). It was suggested that this phenotypic conversion from a serum resistant to serum sensitive state was analogous to the smooth-rough mutation and was due to partial loss of the polysaccharide component of the O antigen.

Since K antigens were not considered by Feingold although they have been reported to affect the serum sensitivity of E. coli (Sjostedt, 1946; Muschel, 1960), the effect of growth in the presence of diphenylamine on the K and O antigens and serum sensitivity of E. coli was examined.

MATERIALS AND METHODS

Diphenylamine (B.D.H. analar) was dissolved in alcohol to give a final concentration of 50 mg/ml and serilized by filtration. Diphenylamine in alcohol was then added to nutrient broth to give the desired final concentrations.

Bacteria

E. coli strains WF82 and WF98 were grown overnight at 37°C in nutrient broth and nutrient broth containing various concentrations of diphenylamine. Bacteria that were to be examined for their sensitivity to C⁹ were harvested by centrifugation washed by resuspension in tris buffer, 0.04M pH 8.4, and resuspended in the same buffer to give the required concentration by optical density. Bacteria that were to be examined for the effect of diphenylamine on antigen and carbohydrate content were harvested by centrifugation washed by resuspension in saline and acetone dried.

Sensitivity to C⁹

The effect of diphenylamine on sensitivity to killing and lysis by human serum was examined. Bacteria, N.H.S., Ca^{++}, Mg^{++} and egg white lysozyme were mixed in a cuvette in the proportions recorded below. The % transmission at 542 nm was measured in an absorptiometer (Vitatron) and recorded automatically. % lysis at any given time could be calculated from the % transmission values.

The effect of diphenylamine on sensitivity to killing by C⁹ was examined by taking samples for viable counts from the cuvette at timed intervals. The samples were put immediately into cold nutrient broth to arrest the reaction and suitable dilutions plated.

Mixture used in cuvette

	Concentration	ml.	Final concentration of constituents	Final Ionic Strength
Bacteria	1.5×10^9	0.4	$3 \times 10^8 ml^{-1}$	0.008
Ca^{++}	0.003 M	0.1	0.00015 M	-
Mg^{++}	0.01 M	0.1	0.0005 M	-
Egg white lysozyme	200 $\mu g\ ml^{-1}$	0.2	20 $\mu g\ ml^{-1}$	-
Normal human serum		0.2	1/10	0.018
NaCl	0.17 M	0.4	0.034 M	0.034
Water		0.6	-	-

Final volume 2.0 ml

Final Ionic Strength 0.060

(Glynn and Milne, 1967).

Antigen and carbohydrate estimations

Acetone dried bacteria were suspended in 0.15M saline to give a final concentration of 3.0 mg per ml. and extracted with phenol, final concentration 45% v/v at 65°C. After centrifugation the aqueous phase was collected and dialysed against 0.15M saline to remove residual phenol. All antigen and carbohydrate analyses were performed on this extract.

O and K antigens were measured by radial immunodiffusion

(Mancini et al., 1965 and Section II). K antigen extracted from E. coli WF82 with 45% phenol at 65°C and purified by ion exchange chromatography on D.E.A.E. cellulose (Section VII) was used as the standard for K antigen estimations. Lipopolysaccharide was extracted from the same strain with 45% aqueous phenol at 65°C. The aqueous phase was dialysed against 0.15M saline and lipopolysaccharide sedimented by centrifugation at 100,000g for 2 hours used as the standard for O antigen estimations. The total carbohydrate present in the bacterial extracts was measured by the phenol-sulphuric acid method of Dubois et al. (1956). This reaction measures hexoses, pentoses, methylpentoses and their derivatives and hexuronic acids. Carbohydrate was also estimated by the Molisch reaction (Dische, 1955), which measures hexoses, pentoses and methyl pentoses but not hexuronic acids or hexosamines. Glucose was used as standard for both methods. Heptose was measured by the Osborn modification of the method of Dische (Osborn, 1963) using D-glucoheptose (Koch-light) as standard.

Antisera

An anti O antiserum was raised in a rabbit against E. coli WF82 that had been heated at 100°C for 2½ hours, washed and resuspended in 0.15M NaCl (Edwards and Ewing, 1962).

The γ globulin fraction of the antiserum was prepared by precipitation with sodium sulphate (Kekwick, 1940) and labelled with ^{125}I by the method of McFarlane (1958).

Assay of the amount of anti O antibody bound to the bacterial cells grown in diphenylamine

Bacteria that had been grown in the presence of various concentrations of diphenylamine were harvested by centrifugation, washed and resuspended in 0.15M saline so that the optical density was 0.50 on an absorptiometer (Spekker) using a neutral grey filter. 1.0 ml of ^{125}I labelled γ globulin was mixed with 1.0 ml of bacteria and incubated on ice for 60 minutes. The cells were then spun and washed three times in saline, the radioactivity of 2.0 ml volumes measured in a scintillation counter, and the optical density measured. The amount of antibody bound to bacteria was calculated as counts per 100 sec. per 2 ml of cells of O.D. 0.2.

RESULTS

Reduced bacterial growth due to diphenylamine

When bacteria were grown in nutrient broth containing diphenylamine the yield of bacteria, measured as the number of mg. of acetone dried cells that were harvested from one litre

of culture medium, fell as the diphenylamine concentration increased (Table 16). 75 µg diphenylamine per ml. was the highest concentration that could be dissolved in the culture medium.

Changes in C⁹ sensitivity induced by diphenylamine

Two strains of E. coli WF98 and WF82 were grown in nutrient broth containing 0, 10, 25, 50 and 75 µg of diphenylamine per ml.

As the diphenylamine concentration was increased up to 50 µg per ml. the cells became more sensitive to lysis by C⁹. Bacteria grown in media containing 75 µg of diphenylamine per ml. were more sensitive to lysis by C⁹ than cells grown in media which did not contain diphenylamine but they were less sensitive than bacteria grown in media containing 50 µg diphenylamine per ml. (Figs. 54 and 55). In the same way as for the lytic experiments killing increased as the diphenylamine concentration was raised to 50 µg per ml. Increasing the concentration from 50 µg per ml. to 75 µg per ml. did not make the bacteria more sensitive to killing by C⁹ (Fig. 56).

Decrease in extractable carbohydrate due to growth in the presence of diphenylamine

The amount of carbohydrate extractable from acetone dried

cells is recorded in Table 17. The amount of carbohydrate extracted from bacteria grown in the presence of diphenylamine is given as a percentage of the amount extracted from bacteria grown in media which did not contain diphenylamine in Fig. 57. As the diphenylamine concentration was increased the amount of both total carbohydrate and of heptose extracted from acetone dried bacteria decreased.

Heptose is one of the components of the basal core of the O antigen (Luderitz et al., 1966). It is therefore apparent that basal core polysaccharide synthesis is affected by the diphenylamine.

Reduction in the amount of O and K antigens due to growth in media containing diphenylamine

The amounts of both K and O antigens extracted from acetone dried cells decreased as the diphenylamine concentration in the culture medium increased (Table 17). The amounts of O and K antigens extracted are shown as percentages of the amount of antigen extracted from cells grown in nutrient broth which did not contain diphenylamine in Fig. 57.

Diphenylamine therefore seems to exert some general inhibitory effect on cell wall polysaccharide synthesis.

Effect of diphenylamine on the amount of anti O antibody bound to the bacterial cell

Although the amount of O antigen extracted from bacteria was reduced as the diphenylamine concentration was increased synthesis was not completely inhibited. O antigen could still be detected by radial immunodiffusion in extracts of cells grown in media containing 75 µg diphenylamine per ml. Despite this fall in O antigen content bacteria grown in the presence of diphenylamine bound more anti O antibody than cells grown in media that did not contain diphenylamine (Fig. 58).

Table 16. Reduction in bacterial growth due to diphenylamine

Diphenylamine concentration in the culture medium µg per ml.	Mg. of acetone dried cells harvested per litre of culture medium
0	170.2
10	150.4
25	120.2
50	107.6
75	87.3

Table 17. The effect of increasing diphenylamine concentration on the amount of antigen and carbohydrate extracted from E. coli WF82

Diphenylamine conc. in culture medium µg per ml.	µg K antigen extracted per mg. dried cells	µg O antigen extracted per mg. dried cells	µg carbohydrate extracted per mg. dried cells		
			Dubois	Molisch	Heptose
0	13.5	39.0	33.2	25.6	4.7
10	9.5	35.0	24.0	18.0	3.0
25	4.6	16.1	18.9	16.2	1.3
50	2.4	7.1	17.0	15.6	1.3
75	2.1	4.9	13.8	11.6	0.7

Fig. 54. Complement lysis of *E. coli* WF98 grown in nutrient broth and nutrient broth containing various concentrations of diphenylamine. The figures on the graph indicate the diphenylamine concentration in the culture medium.

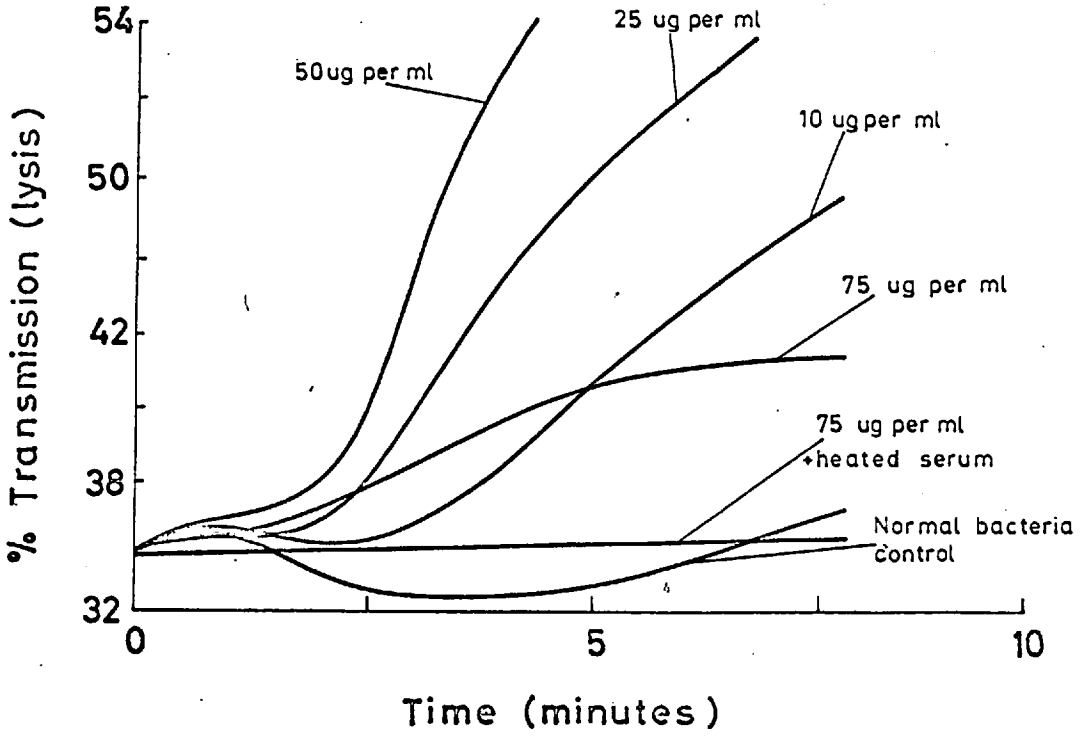

54
50
42
38
32
% Transmission (lysis)
0
5
10
Time (minutes)
50ug per ml
25 ug per ml
10 ug per ml
75 ug per ml
75 ug per ml
+heated serum
Normal bacteria
control

Fig. 55. Complement lysis of *E. coli* WF82 grown in nutrient broth and nutrient broth containing various concentrations of diphenylamine. The figures on the graph indicate the diphenylamine concentration in the culture medium.

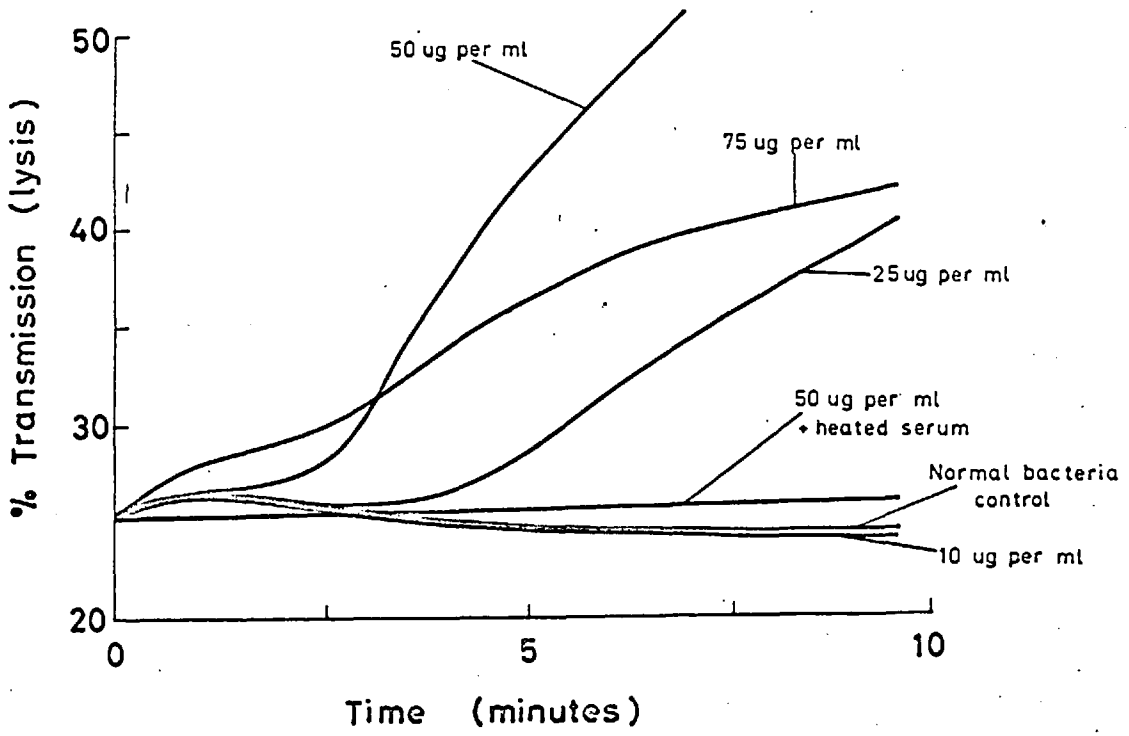

50
40
30
20
% Transmission (lysis)
0
5
10
Time (minutes)
50 ug per ml
75 ug per ml
25 ug per ml
50 ug per ml
+ heated serum
Normal bacteria
control
10 ug per ml

Fig. 56. Increased sensitivity to lysis and killing by complement of *E. coli* WF82 grown in the presence of diphenylamine.

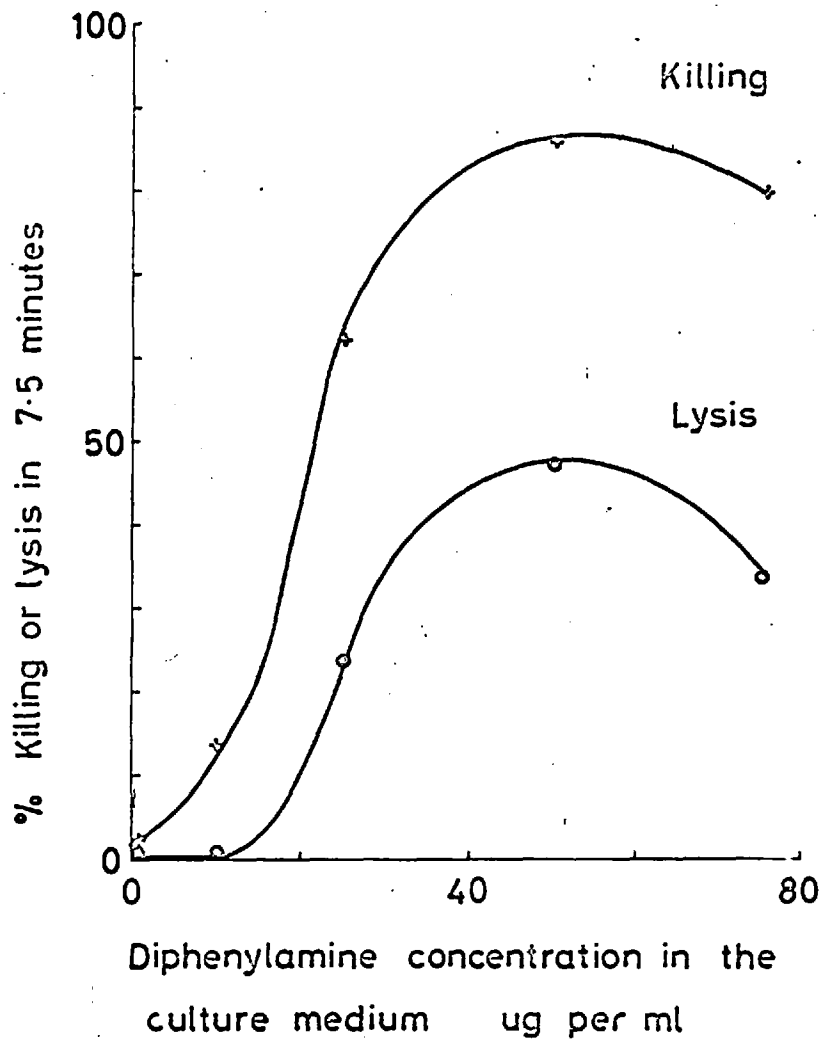

100
50
0
0
40
80
Killing
Lysis
% Killing or lysis in 7·5 minutes
Diphenylamine concentration in the culture medium ug per ml

Fig. 57. Per cent carbohydrate and antigens extracted from *E. coli* WF82 grown in media containing diphenylamine.

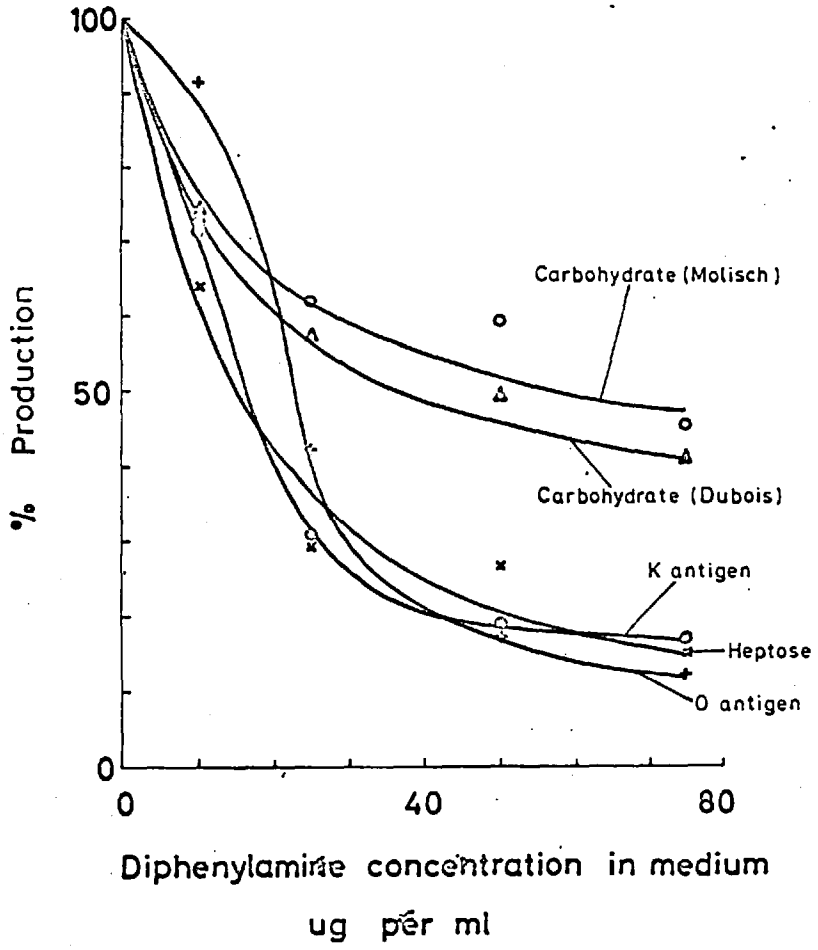
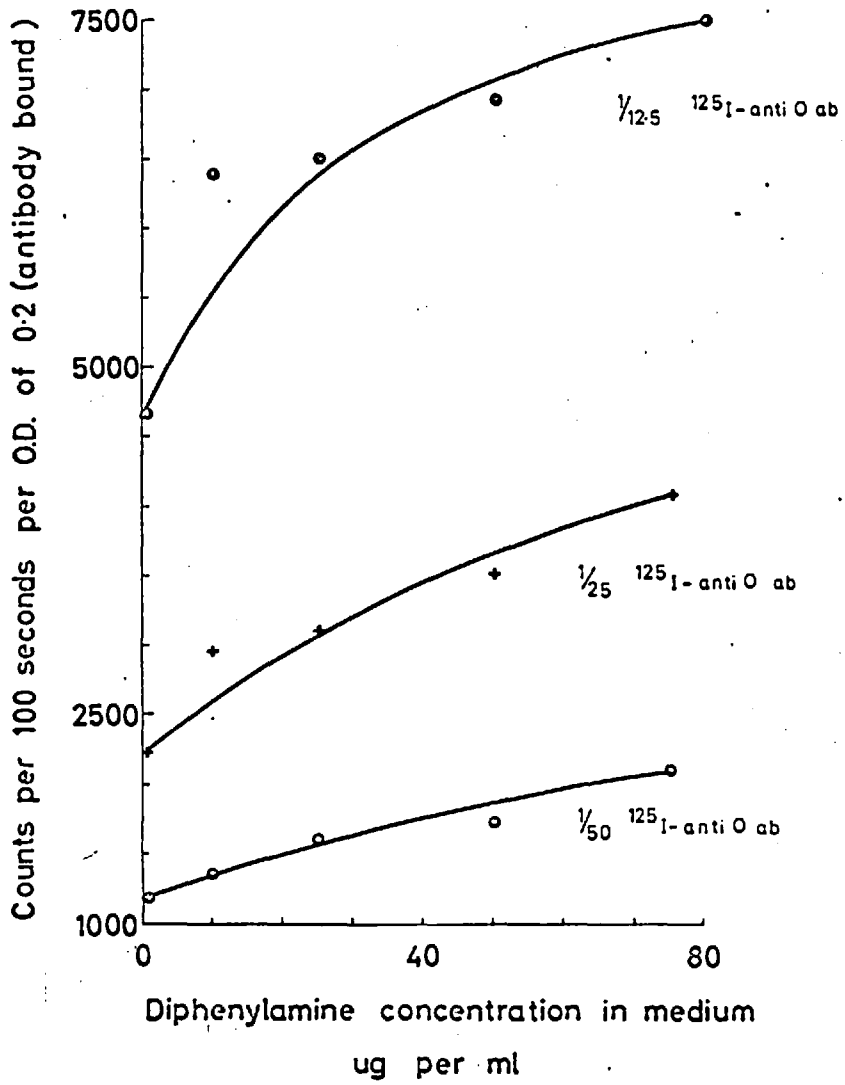

100
50
0
% Production
0
40
80
Diphenylamine concentration in medium
ug per ml
Carbohydrate (Molisch)
Carbohydrate (Dubois)
K antigen
Heptose
O antigen

Fig. 58. The effect of growth in the presence of diphenylamine on the amount of anti O antibody bound to bacteria.
1/12.5 ^{125}I-anti O ab indicates the concentration of ^{125}I labelled anti O antibody which was mixed with bacteria.


7500
5000
2500
1000
0
40
80
Counts per 100 seconds per O.D. of 0·2 (antibody bound)
Diphenylamine concentration in medium
ug per ml
1/12·5 125I-anti O ab
1/25 125I-anti O ab
1/50 125I-anti O ab

DISCUSSION

When C^9 resistant strains of *E. coli* were grown in media containing diphenylamine they became more sensitive to the bactericidal action of N.H.S. These observations confirm those of Feingold (1969). Associated with this increased serum sensitivity was a reduction in the amounts of O and K antigens extracted from the dried cells.

Besides this fall in specific antigen content there was also a reduction in the amounts of total carbohydrate and heptose extracted. Synthesis of the basal core of the O antigen as well as of the specific side chains was therefore affected.

A likely explanation of the mechanism by which diphenylamine affected C^9 sensitivity is that it had some general inhibitory effect on cell wall polysaccharide synthesis. It would therefore cause the amount of both the O and K antigens produced to be reduced. Both the K antigen and the specific side chains of the O antigen, which were being detected by the radial immunodiffusion technique, are polysaccharides. Since both O and K antigens contribute to the C^9 sensitivity of *E. coli* and as both were affected by diphenylamine one cannot be separated from the other as being the main factor causing the change in serum sensitivity. The change in sensitivity to the bactericidal action of N.H.S. is probably the result of several factors dependent on the

changes in cell wall structure caused by the diphenylamine.

Although the amount of specific O antigen extracted from the bacteria was reduced synthesis was not completely inhibited even when a diphenylamine concentration of 75 µg per ml was used. Despite this fall in O antigen content with increasing diphenylamine concentration more anti O antibody was bound to bacteria grown in diphenylamine than to normal bacteria.

The amount of K antigen present in the cell is thought to influence the amount of anti O antibody that binds to the bacterium (see Sections III and IV). The increase in the amount of anti O antibody bound to cells grown in media containing diphenylamine could therefore be explained in terms of the K antigen content of the cell, i.e. as the amount of K antigen present was reduced due to growth in diphenylamine more anti O antibody was able to reach its substrate in the bacterial cell wall. As a result diphenylamine grown bacteria would have been more effectively sensitised to C^9 than normal bacteria.

Loss of K antigen may affect the C^9 sensitivity in another way. The inhibitory effect of K antigen on C^9 per se rather than by inhibiting antibody binding, demonstrated in Section III, may also be reduced as the K antigen content of the cell is reduced.

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When Micrococcus lysodeikticus, Sarcina lutea or Chromobacterium violaceum were grown in the presence of diphenylamine membranes were synthesised that were virtually devoid of unsaturated carotenoids. There was no effect on total lipid production (Cho et al., 1964). Salton and Schmitt (1967) showed that diphenylamine prevented the formation of conjugated carotenoids in M. lysodeikticus membranes. Diphenylamine was also shown to be incorporated into the cell membrane. Although these effects were shown the mechanism by which they occurred remained undiscovered.

Wardlaw (1964) compared rough and smooth strains of E. coli for their lipopolysaccharide content and Michael and Landy (1961) compared paired smooth enterobacteria with the same serotype but different C^9 sensitivities for their endotoxin content. Both publications recorded evidence supporting the view that the amount of lipopolysaccharide or endotoxin present in the cell wall influenced the bacteriums sensitivity to C^9. Strains with less lipopolysaccharide or endotoxin were more sensitive. Bacteria grown in diphenylamine therefore could also be more sensitive to serum because of the loss of O antigen.

Rowley and Turner (1968) showed that if proteins were attached to the surface of a C^9 sensitive strain of S. adelaide C^9 became less effective at killing the bacteria,

in the presence of antibody directed against the attached molecule, as the size of the attached antigen increased. Based on these results it was suggested that the proximity of C^9 activation to its substrate was important in determining C^9 sensitivity. Loss of both O and K antigens may well enable C^9 to be activated nearer its substrate.

When bacteria were grown in media containing diphenylamine synthesis of cell wall polysaccharide including the O and K antigens was inhibited. The changes in sensitivity to human serum observed when diphenylamine grown cells were compared with normal cells is likely to be due to several factors caused by the gross changes in cell wall structure due to the diphenylamine. Since both O and K antigens are affected one cannot be said to be more important than the other and it seems likely that both are involved.

1

IX. SCANNING ELECTRON MICROSCOPE EXAMINATION OF STRAINS OF ESCHERICHIA COLI

INTRODUCTION

The structure of the bacterial cell wall affects the sensitivity of strains of E. coli to the bactericidal action of human serum. Loss of O specific polysaccharide from the lipopolysaccharide component of the gram negative cell wall, smooth-rough mutation, is associated with an increased sensitivity to C^9 and differences in colonial morphology are visible when this mutation occurs (Wilson and Miles, 1964).

Other authors have associated differences in colonial morphology with the K or Vi antigen content of bacteria. Giovanardi (1954) reported that bacteria containing Vi antigen produced opaque colonies whereas bacteria which did not contain the Vi antigen were translucent.

Wolberg and DeWitt (1969) used the method proposed by Landy (1950) to compare the K antigen content of different strains of E. coli. This method involved using incident light and judging the translucency of colonies.

It seemed likely that differences in colonial morphology that were related to composition of the bacterial cell were the result of differences in the morphology of the individual cells comprising the colony.

Murphy and Campbell (1969) were able to demonstrate

characteristic ridges and ribs on Bacillus polymyxa spores using a scanning electron microscope. Changes in the surface structure of bacteria due to antibiotics has been demonstrated by Greenwood and O'Grady (1969).

A scanning electron microscope was used to study the surface structure of different strains of E. coli known to differ in their cell wall composition and C' sensitivity. The purpose of this examination was to see if differences in cellular morphology were demonstrable between different strains of E. coli and to see if these differences could be related to the structure of the cell.

Bacteria that had been killed with human serum and bacteria killed with human serum to which excess lysozyme had been added were examined to see if lysozyme caused any demonstrable differences in the manner in which bacteria were killed. Lysozyme was known to promote the bacteriolytic action of serum (Glynn and Milne, 1967).

Bacteria that had been grown in the presence of diphenylamine were examined as this was known to affect cell wall polysaccharide synthesis (see Section VIII). Bacteria grown in the presence of diphenylamine, which made them C' sensitive, were killed by serum and studied to see if differences were demonstrable in the manner in which they and strains of E. coli which were normally sensitive to C' were killed.

MATERIALS AND METHODS

Bacteria

Various strains of *E. coli* known to differ in their sensitivity to C' and K antigen content were compared. *E. coli* WF96 (02:K1) was sensitive to C', *E. coli* WF82 (0117:K?) and *E. coli* WF26 (06:K13) were resistant to C'. *E. coli* Lilly which was a rough strain was also examined. *E. coli* WF82 that had been grown in broth containing 50 µg of diphenylamine per ml was studied since growth under these conditions had been shown to reduce the O and K antigen content of the cell and to convert it phenotypically to a C' sensitive state (Section VIII). Overnight 37°C nutrient broth cultures were used in each case.

E. coli WF96 was grown in broth, washed and resuspended in tris buffer and treated with N.H.S., final concentration 1/10, with or without added lysozyme, 20 µg per ml, as described in Section VIII.

E. coli WF82 that had been grown in the presence of diphenylamine, 50 µg per ml, was incubated with C' and lysozyme in the same way as above. The action of C' was stopped by the addition of five volumes of cold nutrient broth (Muschel and Treffers, 1956a).

Preparation of slides for examination

Bacteria were fixed by adding an equal volume of 1% aqueous glutaraldehyde to nutrient broth containing 10^6 - 10^7 cells per ml. After incubation overnight at 4°C the bacteria were harvested by centrifugation at 1500 R.P.M. for 10 minutes, washed twice by resuspension in water and resuspended in water to give 10^6 - 10^7 cells per ml.

Two or three drops of the fixed cells were pipetted onto a glass cover slip and allowed to dry in air. The cover slips supporting the specimens were fixed with "Durafix" to a "specimen stub", provided by the Cambridge Instrument Company.

Before being examined the slides were covered with a layer of gold palladium to render their surfaces conducting. Samples were examined using a steroscan microscope belonging to the Cambridge Instrument Company.

RESULTS

In Figs. 59-61 *E. coli* strains WF26, WF82 and WF96 respectively have been compared. Strains WF26 and WF82 were resistant to the bactericidal action of serum and strain WF96 was sensitive. All of the strains had a similar convoluted surface. Differences in the K antigen content of the strains did not cause any demonstrable difference in their appearance.

Fig. 62 is an electronmicrograph of *E. coli* Lilly, a rough C° sensitive strain of *E. coli* (Wardlaw, 1964).

Although differences in the colonial appearance of rough and smooth strains of E. coli are apparent the surface of this bacterium was the same as that of smooth E. coli strains. In Fig. 62 the constriction of the cell prior to division can be seen.

Figs. 63 and 64 show the appearance of E. coli WF82 grown in the presence of diphenylamine. Although diphenylamine reduces the amounts of O and K antigens synthesised no difference in the surface structure was found between these bacteria and bacteria grown in media which did not contain diphenylamine (c.f. Fig. 60). Diphenylamine did affect the shape of some of the cells. Markedly elongated forms are visible in Fig. 64 indicating cell division was impaired.

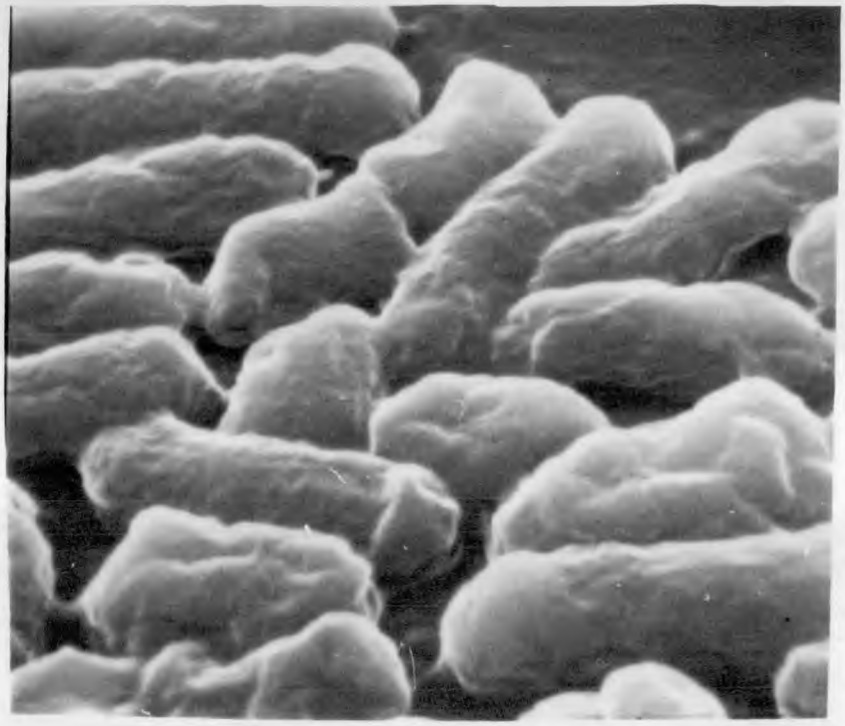
The effect of C' with and without added lysozyme on E. coli WF96 can be seen in Figs. 66 and 67. Heated serum did not destroy the cells (Fig. 65).

The structural integrity of the bacterium was lost after exposure to C' and what appeared to be cell walls remained (Fig. 66). When lysozyme had been added the cell remains after exposure to C' appeared more amorphous (Fig. 67). It is likely that this is due to digestion of the mucopeptide by lysozyme since mucopeptide contributes to the rigidity of the cell.

When E. coli WF82 that had been grown in the presence of

diphenylamine was incubated with C^{9} the resultant debris appeared much the same as the remains of the strain WF96, which is normally sensitive to C^{9} (Fig. 68). Control cells incubated with heated serum were not destroyed (Fig. 69).

Fig. 59. *E. coli* WF26 (a) x 21,000 (b) x 54,000.



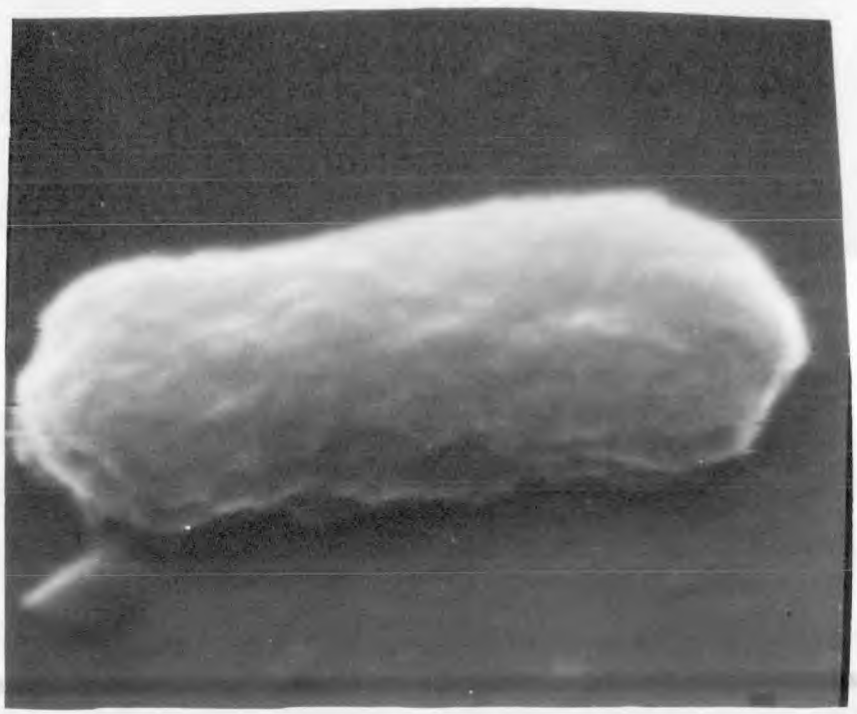
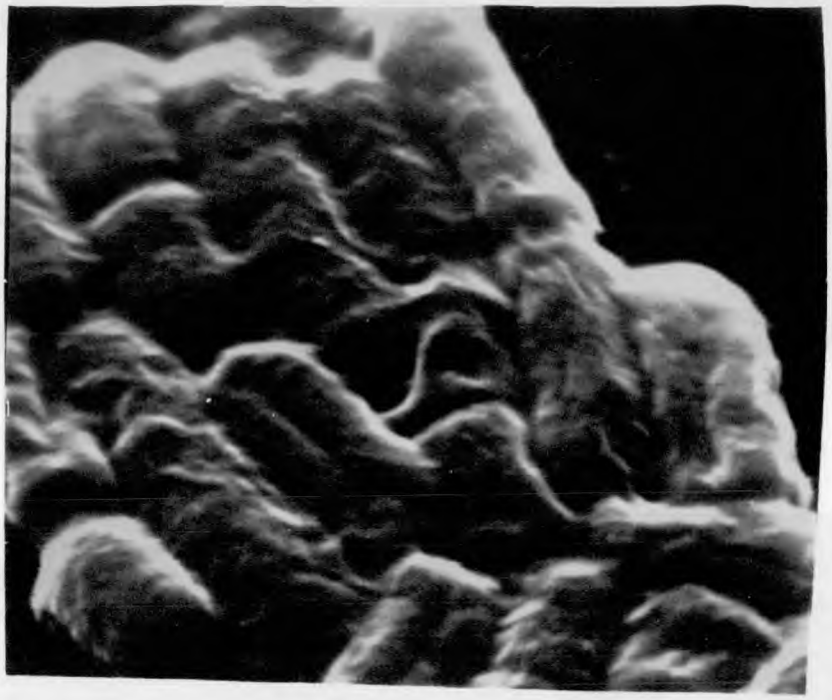


Fig. 60. *E. coli* WF82 x 21,000.

Fig. 61. *E. coli* WF96 x 21,000.



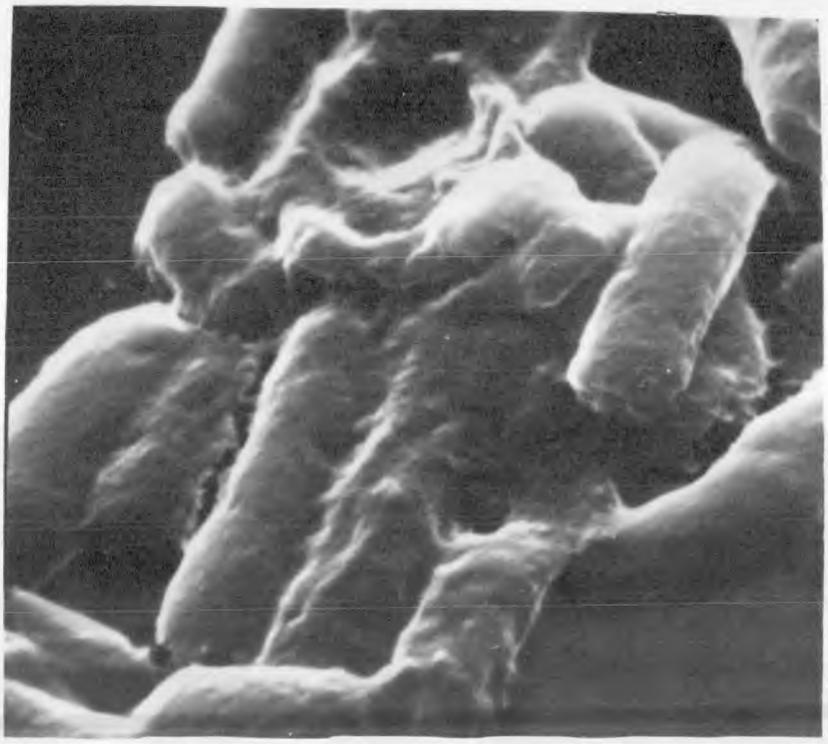


Fig. 62. *E. coli* Lilly x 24,000.

Fig. 63. *E. coli* WF82 grown in the presence of diphenylamine x 53,000.

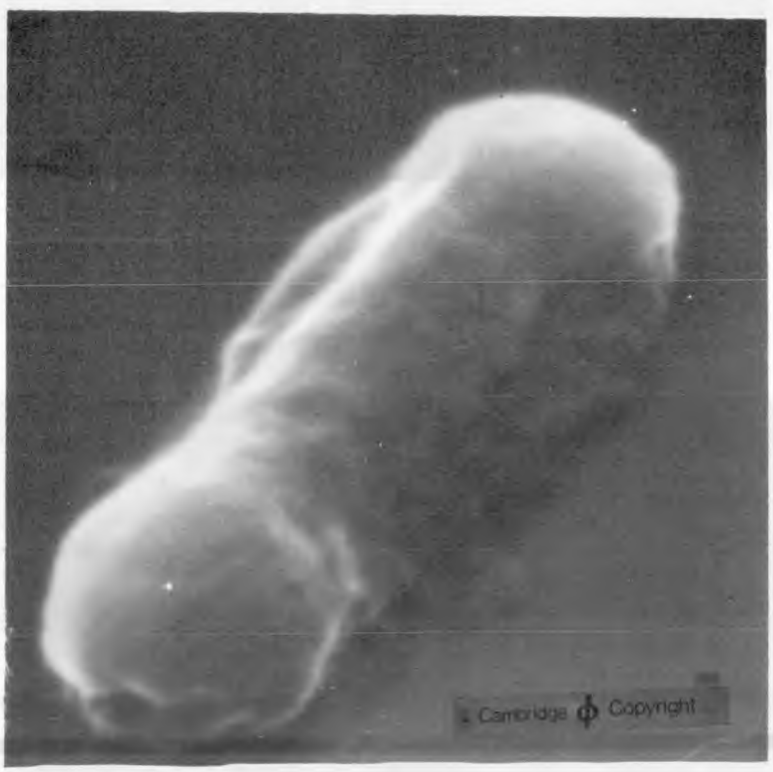

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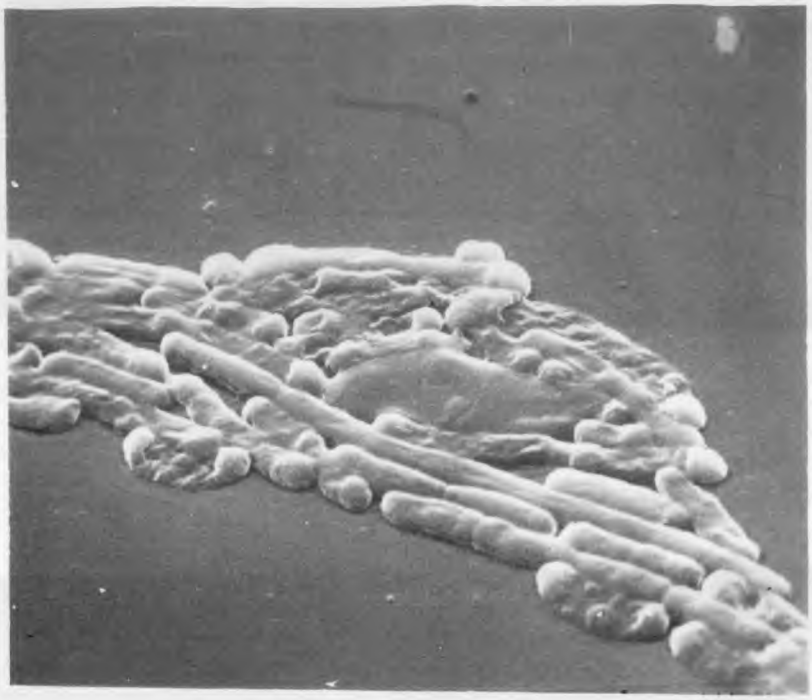
Fig. 64. *E. coli* WF82 grown in the presence of diphenylamine x 5,300.

Fig. 65. *E. coli* WF96 after exposure to heated serum and added lysozyme x 11,000.



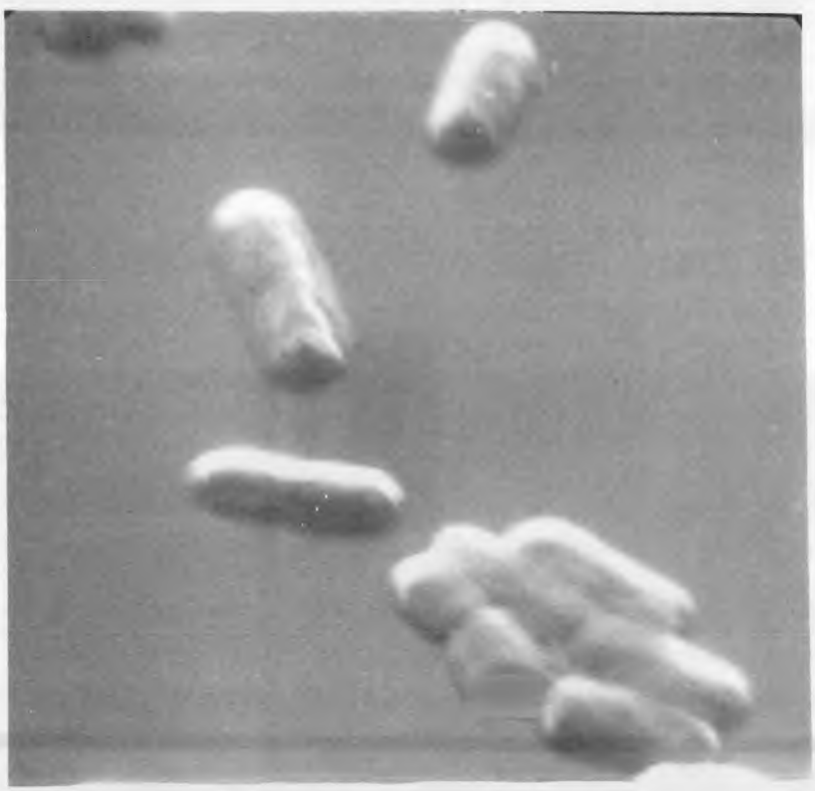
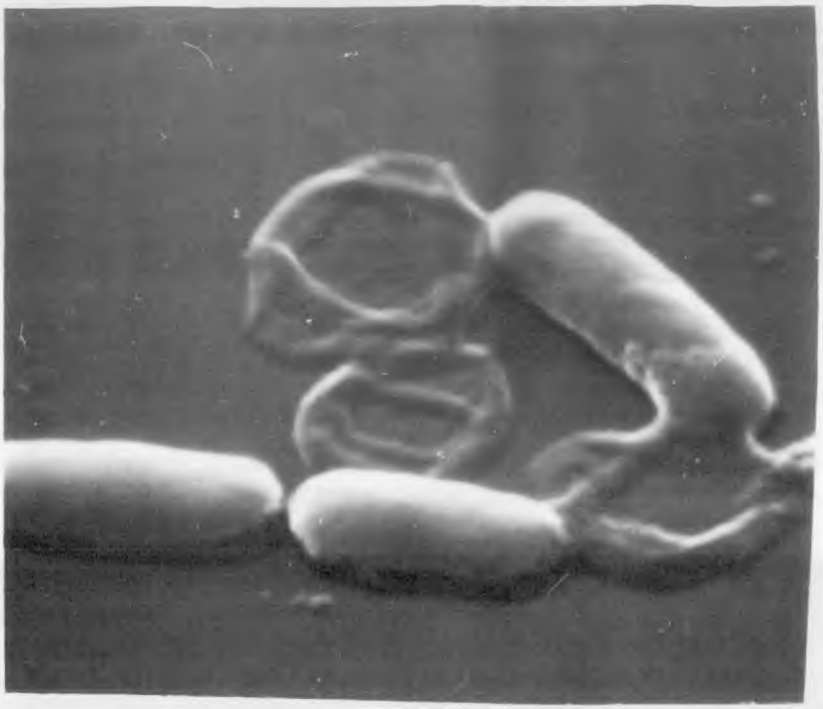


Fig. 66. *E. coli* WF96 after exposure to normal human serum x 22,000.

Fig. 67. *E. coli* WF96 after exposure to normal human serum and added lysozyme x 11,500.



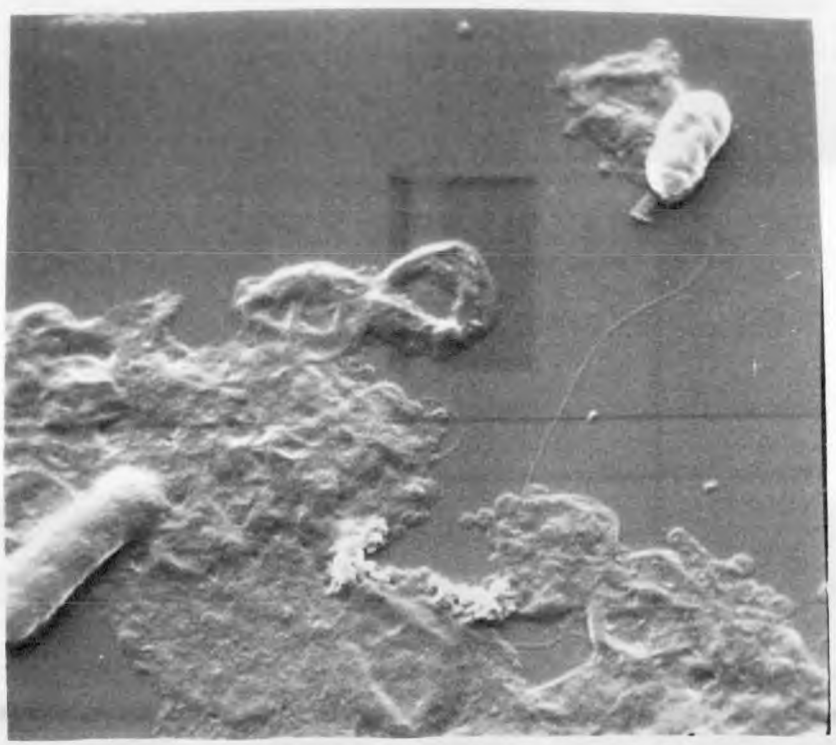
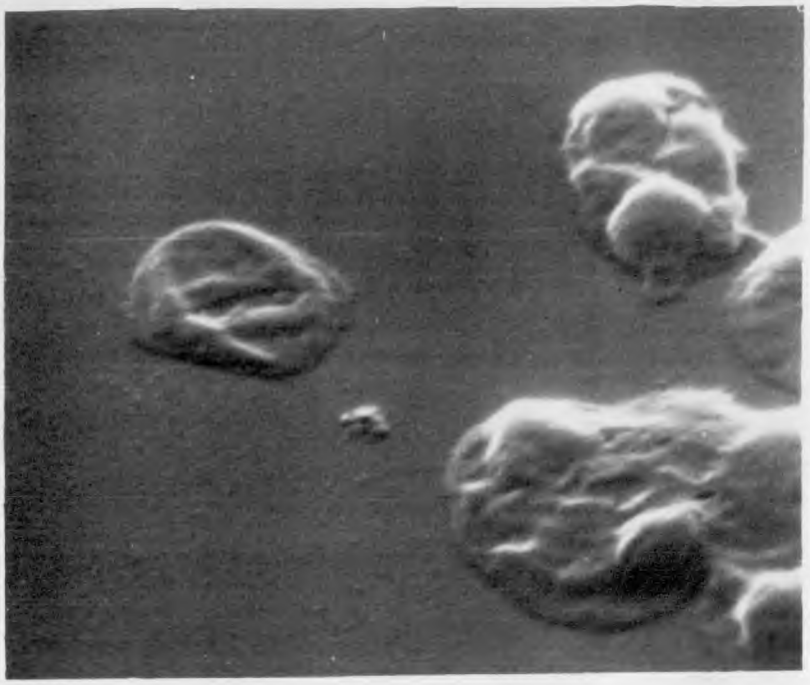
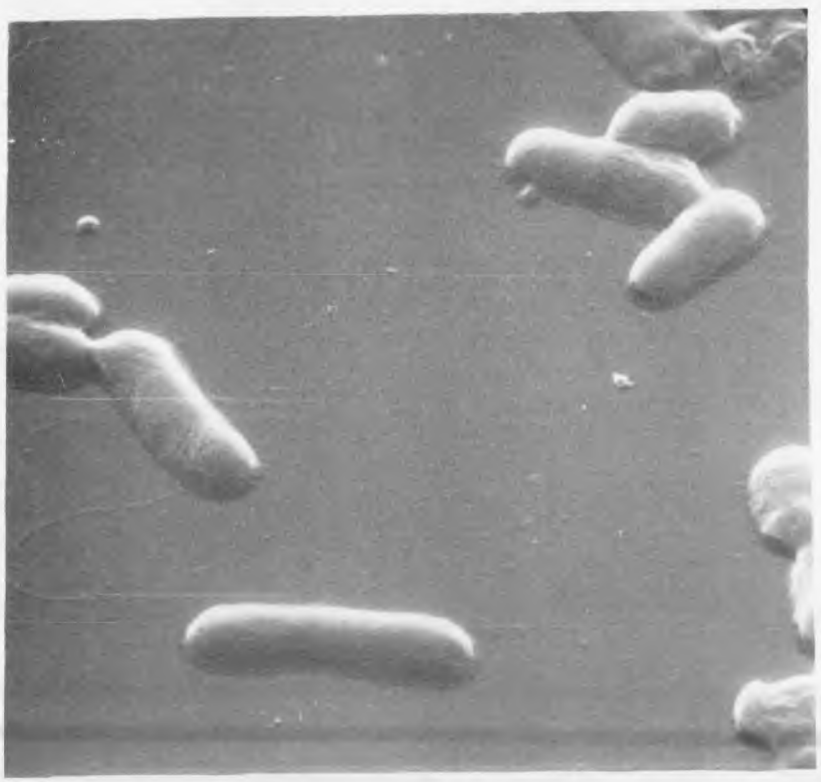


Fig. 68. *E. coli* WF82 grown in media containing diphenylamine and incubated with normal human serum and added lysozyme x 22,000.

Fig. 69. *E. coli* WF82 grown in media containing diphenylamine and incubated with heated serum and added lysozyme x 11,500.





DISCUSSION

Strains of E. coli were examined using a scanning electron microscope in an attempt to relate differences in the surface structure of the bacteria to known differences in the amounts of O and K antigens present.

No differences in the surface structure of smooth strains that differed in their sensitivity to C°, or of a rough strain or of a strain grown in the presence of diphenylamine were observed.

Feingold (1969) using a transmission electron microscope was able to demonstrate a loss of the convoluted appearance of the bacterial surface due to growth in the presence of diphenylamine. The lack of success achieved using a scanning electron microscope may reflect the lower resolution of this type of machine compared with the transmission electron microscope.

The cidal effect of C° was clearly demonstrated. C° treated cells lost their shape possibly as a result of the lesions in the bacteria that have been demonstrated to be caused by C° (Dourmashkin and Humphrey, 1967). Leakage of cytoplasmic contents may occur resulting in a loss of structural integrity. Addition of lysozyme caused the resulting debris to become more amorphous. Since lysozyme digests the mucopeptide layer which is responsible for the

rigidity of the bacterial cell wall this appearance is not surprising.

When bacteria that were normally C⁹ resistant were grown in the presence of diphenylamine and then exposed to C⁹ the appearance of the dead cells was much the same as that of bacteria that were sensitive to C⁹ without it being necessary to grow them in media containing diphenylamine. This suggests there was no peculiarity in the mode of action of C⁹ on cells induced to be C⁹ sensitive with diphenylamine.

Although no correlation between surface structure and cell wall structure was obtained from this study other authors have successfully used the scanning electron microscope to study the bacterial surface.

Greenwood and O'Grady (1969) were able to detect changes in the surfaces of bacteria that had been treated with antibiotics and Murphy and Campbell (1969) were able to show the characteristic ridges and ribs present on the spores of Bacillus polymyxa.

Using a transmission microscope Shands (1965) demonstrated that the somatic O antigen of E. coli extended as fibrils up to 150 mμ beyond the confines of the bacterial cell wall. The use of embedding and sectioning techniques coupled with an electron microscopic study of strains of E. coli might therefore provide evidence on the way in which K antigens

are distributed on the cell surface which this study, using a scanning electron microscope was unable to provide.

CONCLUSIONS

The correlation between sensitivity to the bactericidal action of serum, susceptibility to phagocytosis and mouse virulence of strains of *E. coli* can be explained in terms of the K antigens.

It was found that the ability of K antigens to inhibit haemolysis was due in part to the prevention of the union of anti red cell antibody with the red cell and in part to some inhibitory effect of K antigen on C' at some stage subsequent to the binding of antibody to antigen.

Based on these results it was suggested that K antigens might prevent normal anti O antibody from combining with the bacterial cell and sensitising it to C' or opsonising it.

As the K antigen content of strains increased bacteria became more virulent possibly as they were able to evade two of the hosts defence mechanisms, killing by C' and phagocytosis. Bacteria containing sufficient K antigen are likely to persist in host tissues when strains which only contained small amounts of K antigen or none would be killed. It has been shown that it was not the possession of K antigens that determined resistance to C' and phagocytosis but the amount present on the organism.

The size of the K antigen molecule was found to be related to its ability to inhibit agglutination in vitro. Agglutination was prevented in the main because of the effect of K antigens on lattice formation although its inhibitory action on antibody binding may have affected agglutination to a lesser extent.

Anti K antibody was able to opsonise bacteria which were resistant to killing by C'. It would therefore be expected to protect against infection by C' resistant strains of E. coli.

The relative ineffectiveness at sensitising bacteria to C' of anti K antibody compared with anti O antibody could be explained in terms of the size of the K antigen and its site on the cell. The union of anti K antibody with K antigen probably occurred too far from the lipopolysaccharide for the C' activated by the antibody-antigen complex to be effective.

Besides their importance in animal infections K antigens affected the human pathogenicity of E. coli. When strains isolated from urinary tract infections were examined bacteria which were able to cause tissue invasion were shown to contain more K antigen than strains confined to the bladder. It seems likely that this is for the same reasons as for increased mouse virulence due to K antigen and is because killing by C' and phagocytosis of the bacteria in the host tissues are prevented.

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The sensitivity to complement of strains of *Escherichia coli* related to their K antigens

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The Sensitivity to Complement of Strains of *Escherichia coli* Related to their K Antigens

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Summary. We have confirmed that K antigens influence the sensitivity to complement of strains of *Escherichia coli*. Resistant strains bound more polycation and by inference therefore had a higher surface negative charge than sensitive strains.

Extracts containing K antigen non-specifically inhibited red cell agglutination and this inhibitory activity was roughly proportional to complement resistance. All of five resistant strains became more sensitive to complement when grown at unusual temperatures and extracts from them then had less inhibitory activity. In four strains of serotype O6 K13 complement resistance was proportional to K antigen content measured by immunodiffusion. However, purified K antigen from a resistant strain (WF82) had much greater agglutination inhibiting activity weight for weight than purified K antigen from a sensitive strain (WF96).

In experiments with ^{125}I-labelled haemolysin K antigens decreased the binding of both IgG and IgM antibodies and also directly reduced complement activity.

The mechanisms of action of K antigens and their relation to virulence are discussed.

INTRODUCTION

A correlation between sensitivity to complement and virulence for animals has been shown for strains of *Salmonella* by Maaloe (1948) and of *Escherichia coli* by Rowley (1954). Although Rosenberg (1965), in a critical review, suggested that there was little evidence for a significant protective role of complement in infections the position is still open and factors affecting the complement sensitivity of bacteria remain of interest. Wardlaw (1963) suggested that rough strains of *E. coli* were more sensitive than smooth because the former contain less lipopolysaccharide. It now seems likely from the work of Lüderitz, Staub and Westphal (1966) that the terminal sugars of the lipopolysaccharide are more important than the overall amount. However, salmonellae may have chemically complete lipopolysaccharides and still be sensitive to complement (Nelson and Roantree, 1967).

Within a given species or even serotype of enterobacteria there may be both sensitive and resistant strains and Muschel (1960) suggested that this was due to differences in their Vi or K antigens. Muschel, Chamberlin and Osawa (1958) showed that complement resistant strains of *Salmonella typhi* are not agglutinated by anti-O sera and contain large amounts of Vi antigen. The Vi antigen was measured by its ability to specifically inhibit the agglutination of Vi coated red cells by anti-Vi. Jude and Nicolle (1952) had

found that *S. typhi* does not produce Vi antigen when grown at 14° or at 45°. When Muschel and his colleagues (1958) grew resistant strains of *S. typhi* at these temperatures, they became complement sensitive. Muschel (1960) found similar relationships between O inagglutinability, temperature and complement sensitivity in *E. coli* but did not measure the Vi or K antigen content directly.

Previous work therefore indicates a close relationship between K antigens and sensitivity to complement in *E. coli* and in the salmonellae. However, in a preliminary study of 100 smooth strains of *E. coli* Milne (1966) found no correlation between complement sensitivity and any particular K (or O) specificity. For example, four strains, all of serotype O55 B5 varied in complement sensitivity from 14 per cent killing to 20 per cent growth in a 1 : 5 dilution of serum (Milne, 1966). Although such differences could be due simply to differences in the amount of K antigen present, the possibility of qualititative differences between K antigens unrelated to their antigenic specificity cannot be excluded.

The results of an investigation into the properties of K antigens present in smooth strains of *E. coli* of known complement sensitivity are reported below. In some strains the effect on complement sensitivity and K antigens of growth at unusual temperatures, 18° and 45° instead of 37°, was also studied.

K antigens were examined *in situ* by measuring the capacity of strains of *E. coli* to bind lysozyme labelled with ^{125}I.

With the exception of K88 (L), which is a protein, the K antigens of *E. coli* so far studied are polysaccharides, usually acidic (Hungerer, Jann, Jann, Ørskov and Ørskov, 1967) chemically distinct from those described by Goebel (1963) and Anderson and Rogers (1963). It seemed likely that K antigens are responsible for a large though undetermined and perhaps variable part of the negative charge on the bacterial surface. The capacity of a strain of *E. coli* to bind a cationic protein was taken as a measure of this charge. Since under the conditions used lysozyme had no detectable effect on the bacteria and since its substrate the mucopeptide is deep in the cell wall, it was thought that lysozyme was acting as a convenient polycation rather than as a specific enzyme (Glynn, 1969).

A general property of K or Vi antigens described by Ceppellini and Landy (1963) is their ability to inhibit agglutination in serologically unrelated systems. Extracts and purified preparations of K antigens from strains of *E. coli* were, therefore, tested quantitatively for their ability to inhibit agglutination of sheep red cells by a rabbit antiserum. Because of its obvious relevance to complement sensitivity this inhibitory effect was investigated further. IgM and IgG type antibodies to sheep red cells were labelled with ^{125}I and the amounts bound to cells measured in the presence and absence of K extracts. The effect of such extracts on the lysis by complement of red cells sensitized by known amounts of antibody was then examined.

The results have been analysed to try to answer the questions: are K antigens important determinants of complement resistance in *E. coli* and if so, how do they act?

METHODS

Bacteria

Twenty-five smooth strains of *E. coli* were studied. Of these twenty were from the Wright-Fleming Institute or the Central Public Health Laboratory, Colindale, and have been described previously (Milne, 1966). Strains *E. coli* K235 L+T, L−O and L+O (mucoid) were kindly sent by W. F. Goebel (Rockefeller University). Strains *E. coli* 5396/38 and

E. coli 136 were from A. Abrams (Walter Reed Institute) and E. E. Baker (Boston University School of Medicine). Two other strains of *E. coli* (M.R.E. 162 and M.R.E. 600) were supplied by R. Elsworth, Microbiological Research Establishment, Porton.

Bacteria used for estimation of complement sensitivity or lysozyme binding were always in the logarithmic growth phase. From an overnight culture in nutrient broth, 0·5 ml were taken to inoculate 5 ml of fresh broth and grown for 1 hour at 37° in rocking L tubes (Gorrill and Needs, 1958). The organisms were then harvested by centrifugation, washed in water and adjusted to the concentration required by optical density.

Bacteria used to prepare extracts were grown overnight on nutrient agar plates at 37° harvested in saline, precipitated by the addition of 3 volumes of acetone in the cold, spun, resuspended in acetone and dried under vacuum. In addition strains WF82 and WF96 were grown in bulk at the M.R.E. Porton and supplied as wet frozen bacteria. These were stored at −60° and acetone dried as required.

Antibody and complement for bactericidal experiments

Normal human serum (NHS) from one subject (C.J.H.) was stored at −60° and used as a source of both complement and antibody. The haemolytic titre measured by Mayer's method (Kabat and Mayer, 1961a) was 36 $C'H_{50}$/ml. The concentration of complement was varied and the antibody concentration kept constant by diluting the NHS with NHS previously heated to 56° for 30 minutes. This treatment inactivated complement but presumably did not affect normal IgM antibody (Michael, Whitby and Landy, 1962).

Determination of the complement sensitivity of strains of E. coli

Complement sensitivity is expressed as the amount of complement in $C'H_{50}$ units required to kill 50 per cent of a standard inoculum in 15 minutes and was measured by the method described by Muschel and Treffers (1956). For some screening experiments a plate method was devised. In this suspensions of *E. coli* were spread on nutrient agar in small (7·5 cm diameter) Petri dishes to give an expected 50–100 colonies/plate. The plates were then immediately flooded with 0·3 ml of neat or diluted (1 : 2 or 1 : 4) fresh serum and incubated. Controls were treated with heated serum. Sensitive strains showed a marked fall in colony count after serum treatment. The method was rapid and easy to perform but was less sensitive and less precise than tube methods. It was also relatively extravagant with serum.

The effect of growth at different temperatures

Five strains of *E. coli* were grown into the logarithmic phase on the rocking water bath at 18°, 37° and 45°. They were then washed and their concentration adjusted. Complement sensitivity was measured at 37° and extracts were tested for agglutination inhibiting activity.

In this particular group of experiments complement sensitivity was determined by the percentage growth or killing of *E. coli* after 30 minutes exposure to 7 $C'H_{50}$ units/ml (equivalent to a 1 : 5 dilution of fresh serum).

Agglutination inhibiting activity was also measured of extracts from bacteria grown on nutrient agar at different temperatures. The results were no different from those with extracts of the log phase fluid cultures.

Estimation of [^{125}I]*lysozyme uptake by* E. coli

Crystalline egg white lysozyme (EWL) (Armour) was labelled with ^{125}I by the method

of Macfarlane (1958), adjusted to a concentration of 300 μg/ml by optical density (OD_{280} of EWL 100 mg/ml = 0·24) and the radioactivity measured in a scintillation counter.

Two millilitres of [^{125}I]lysozyme (300 μg/ml) were added to 2 ml samples of *E. coli* (5×10^8/ml), mixed and kept at 0° for 5 minutes. The bacteria were then centrifuged, washed three times with water and made up in 2 ml water. The concentration of lysozyme used was sufficient to produce maximum uptake by the *E. coli*. The number of bacteria in the final solution was checked by optical density and their radioactivity measured. From this and the specific activity of the lysozyme the quantity of lysozyme bound per bacterium was calculated and expressed as femtogram per cell (fg/cell).

Preparation of extracts of E. coli

Acetone dried cells were suspended in 0·15 M saline at a concentration of 10 mg/ml and homogenized on ice with an 'Ultra Turrax' homogenizer using 30-second bursts for a total of 3 minutes. In this way overheating was avoided. Bacteria and debris were removed by centrifugation at 1200 ***g*** for 30 minutes. To the clear crude extract so obtained 3 volumes of alcohol was added in the cold, the precipitate centrifuged and redissolved in 0·15 M saline to give a crude concentrated extract. Traces of alcohol were removed by dialysis against saline. In some experiments further purification was carried out by cetavlon precipitation as described for K antigens by Hungerer *et al.* (1964). The cetavlon precipitated material was redissolved in saline and precipitated with 3 volumes of ethanol. This sequence was repeated three times (Bolanos and De Witt, 1966) and the final precipitate dried in acetone and ether.

Crude and purified extracts of various strains were tested for agglutination inhibiting activity, K and O antigens and carbohydrate content.

Carbohydrate estimations

The following methods were used:

Total sugar given as glucose equivalents was estimated by the Molisch reaction (Dische, 1955) which detects hexoses, pentoses, and methyl pentoses but not hexuronic acids or amino sugars.

Total hexoses also as glucose equivalents were measured by the anthrone reaction (Dische, 1955).

Hexuronic acids were measured by the carbazole reaction (Dische, 1955).

Hexosamines were measured by the method of Reissig, Strominger and LeLoir (1955).

To compare the concentration of extracts made on different occasions from *E. coli* WF82, and used to inhibit haemolysis and antibody binding the carbohydrate content was measured by the phenol sulphuric acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956).

Antisera

All antisera were raised in rabbits according to the method of Edwards and Ewing (1962).

Anti-OK sera were prepared against live bacteria and anti-O sera against bacteria heated at 100° for 2½ hours.

Estimation of agglutination inhibiting activity

The ability of extracts to inhibit the agglutination of sheep red cells by rabbit antibody was measured as follows. A series of doubling dilutions of 0·5 ml of crude or purified

extract corresponding to a known weight of bacteria was made in 0·15 M phosphate buffered saline, pH 7·2 (PBS). A volume of 0·5 ml of 5 per cent sheep red cells in PBS were added to each dilution and to a PBS control and incubated at 37° for 30 minutes. Then 0·04-ml amounts of such coated or of control uncoated red cells were added to 0·2-ml amounts of doubling dilutions of rabbit anti-sheep serum in haemagglutination trays (WHO pattern) in a chequer board titration such that antibody dilutions were read horizontally and extract dilutions vertically. The agglutination inhibiting activity of an extract was taken as the reciprocal of the dilution required to double the minimal agglutinating dose of the anti-red cell serum. Although this was only a one-step difference the activity of any individual extract was very constant in repeated titrations. The anti-sheep red cell antibody used was prepared against boiled red cell stroma as described in Kabat and Mayer (1961b) or obtained from Staynes Laboratories Ltd. Both gave identical results.

Inhibition of haemolysis by bacterial extracts

Sheep red cells were coated as before with various concentrations of the extract under study, washed in PBS and resuspended in the veronal calcium magnesium buffer used for complement estimations. Coated cells and uncoated controls were then sensitized with haemolysin in the standard way for complement titrations (Kabat and Mayer, 1961c). A volume of 0·5 ml of coated sensitized cells together with controls (coated unsensitized, uncoated sensitized and uncoated unsensitized cells) were added to 7·0-ml amounts of a suitable dilution of human complement and incubated for 90 minutes at 37°. The cells were then spun down, the haemoglobin in the supernatant read at 541 mμ and the per cent haemolysis calculated. The amount of complement used was that found by preliminary titration to give 80 per cent lysis in sensitized cells not coated with extract.

Labelled antibody experiments

The γ-globulin was precipitated from the anti-sheep red cell serum with 12 per cent sodium sulphate. From this IgG and IgM fractions were prepared on DEAE-cellulose (Adinolfi, Glynn, Lindsay and Milne, 1966). IgM was further purified on Sephadex G-200 (Flodin and Killander, 1962). The crude precipitated γ-globulin and the IgG and IgM fractions were labelled with ^{125}I by Macfarlane's method, and used in agglutination and haemolysis experiments as above. In this way the effect of extracts on cells sensitized with measured quantities of antibody was determined.

RESULTS

The maximum amount of lysozyme which could be bound by different strains of *E. coli* was directly related to their resistance to complement (Fig. 1). An exception was strain WF70 (O22K?) which although resistant to complement (LD_{50} $C'H_{50}$ units), bound relatively little lysozyme. Extracts of WF70 did not inhibit red cell agglutination.

THE POLYSACCHARIDE CONTENT OF CRUDE EXTRACTS OF STRAINS OF *E. coli*

Crude extracts of eight smooth strains of *E. coli* were analysed (Table 1). In general the total carbohydrate content (Molisch reaction) and glucose content (Anthrone reaction) rose with resistance to complement although WF20 was more resistant than the polysaccharide analysis would have suggested. The extracts used contained both K and O

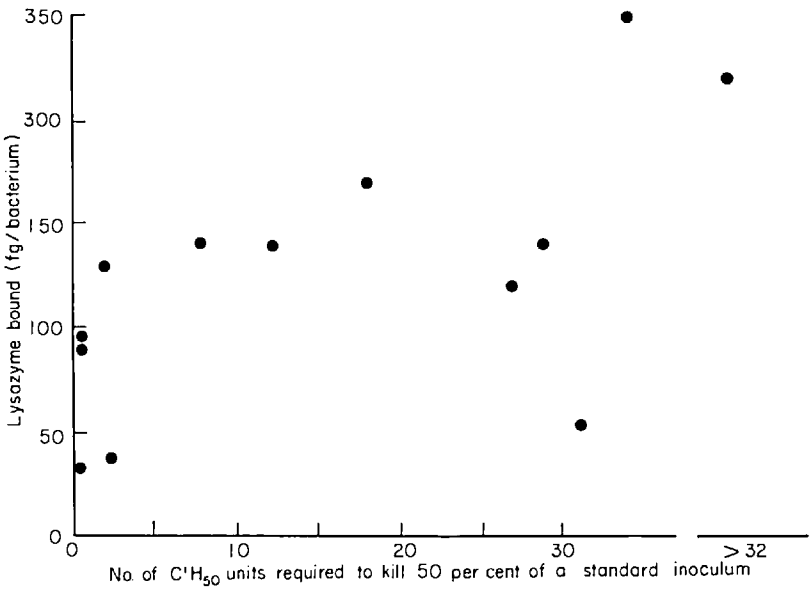

FIG. 1. Uptake of egg white lysozyme by strains of *E. coli* related to their sensitivity to human complement. Neat serum contained 36 $C'H_{50}$ units/ml. 1 femtogram (fg) = 10^{-15} g.

TABLE 1

CARBOHYDRATE ANALYSIS OF CRUDE EXTRACTS OF *Escherichia coli* RELATED TO COMPLEMENT SENSITIVITY

Strain of *E. coli*	Complement sensitivity (No. of $C'H_{50}$ units to give an LD_{50})	Total carbohydrate (glucose equivalent) (μg/ml)		Hexuronic acid (μg/ml)
		Molisch	Anthrone	
WF41	<0·72	287	376	–
WF96	0·72	312	480	–
WF98	7·9	450	400	–
WF86	27	425	525	–
WF82	28·8	588	ND	65
WF20	32·4	400	325	–
WF8	>32·4	625	663	–
WF26	>32·4	1000	1210	60

ND, not done.

–, No detectable hexuronic acid. Higher concentrations of extracts could not be used as the concentrated sulphuric acid reacts with hexoses present producing a brown colour that masks the reaction for uronic acids.

antigens so that no conclusions as to their relative importance can be drawn. Somewhat surprisingly hexuronic acid was only detected in two strains. Hexosamine could not be measured by the method used because of interference by the hexoses present.

AGGLUTINATION INHIBITING ACTIVITY OF K EXTRACTS

In general the more complement resistant a strain, the more agglutination inhibiting activity could be extracted from it (Fig. 2). The exceptions were again WF70 which produced little inhibitory activity and WF6 which produced a lot but was only moderately resistant, the LD_{50} being 12 $C'H_{50}$ units.

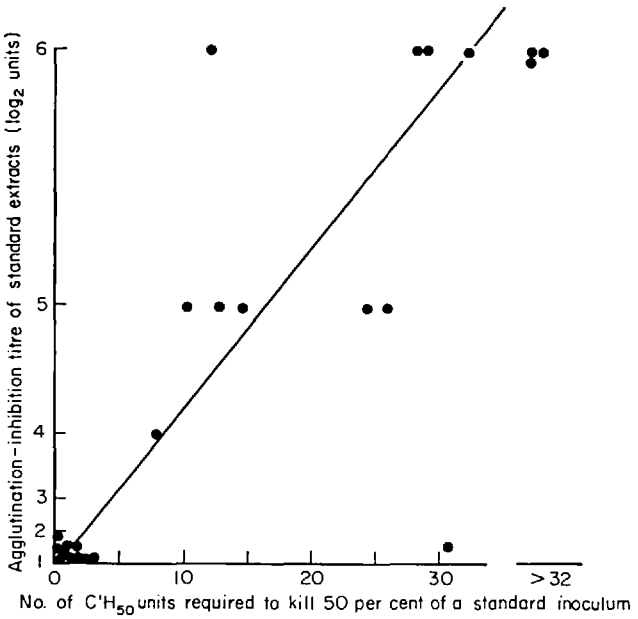

FIG. 2. Sheep red cell agglutination inhibition titre of standard extracts of strains of *E. coli* related to their complement sensitivity.

THE EFFECT OF GROWTH AT DIFFERENT TEMPERATURES

Strains of *E. coli* which were resistant to complement when grown at 37° usually became more sensitive when grown at 18° or 45°. The increased sensitivity (Table 2) was accompanied by a marked fall in the agglutination inhibiting activity of crude extracts. Strain WF82 showed only slight increase in sensitivity after growth at 18° and extracts from the 18° culture still inhibited agglutination although the titre had fallen. Strain WF20 was more sensitive after growth at 45° than at 18° but no agglutination inhibiting activity was obtained after culture at either of these temperatures.

TABLE 2

RELATION OF COMPLEMENT SENSITIVITY AND AGGLUTINATION INHIBITING ACTIVITY OF EXTRACTS OF STRAINS GROWN AT DIFFERENT TEMPERATURES

Strain of *E. coli*	Growth temperature	Reciprocal of titre of agglutination inhibiting activity	Per cent growth (+) or killing (−) after 30 minutes in $7C'H_{50}$ units of complement
WF60	37°	32	+74
	18°	0	−41
	45°	4	−40
WF26	37°	64	+170
	18°	0	+20
	45°	0	−50
WF38	37°	64	+120
	18°	1	−12·5
WF20	37°	64	+5
	18°	0	−30
WF82	37°	64	+20
	18°	8	−10

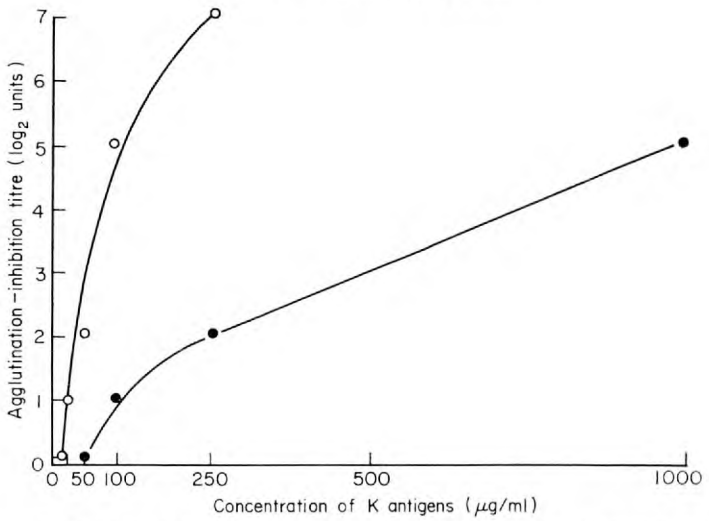

FIG. 3. Sheep and cell agglutination inhibition titre of purified K antigens from two strains of *E. coli*. ○, K ag from *E. coli* WF82; ●, K ag from *E. coli* WF96.

COMPARISON OF PURE K ANTIGENS FROM WF96 (K1) AND WF82 (K UNKNOWN)

Purified K antigen was prepared by cetavlon precipitation from the complement sensitive strain WF96 and the resistant strain WF82. Solutions of known polysaccharide content were tested for agglutination inhibiting activity. It is clear (Fig. 3) that weight for weight K antigen from the resistant strain WF82 was much more active than K1 from WF96.

Fig. 4 shows the immunoelectrophoretic behaviour of the two antigens. K1 from WF96 moves a little further than the K from WF82.

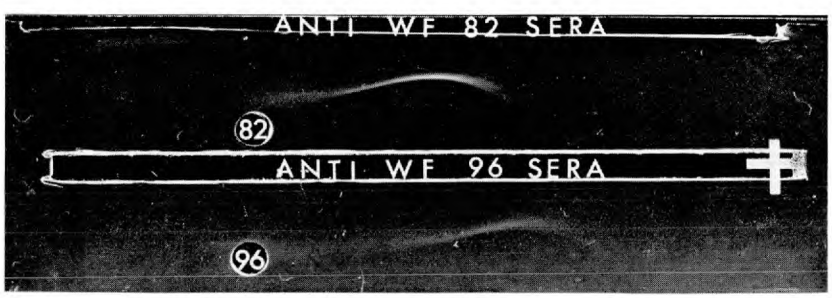

FIG. 4. Electrophoretic mobility of K antigens from *E. coli* WF96 and WF82. In the wells: 82,K ag from WF82, 96,K ag from WF96. In the troughs antiserum to live bacteria.

COMPARISON OF K13 FROM STRAINS WF26, WF8, WF86 AND WF98

These four strains grown under identical conditions differ markedly in their sensitivity to complement. Since all the strains are of the antigenic type O6, K13, the difference in sensitivity is unlikely to be due to variations in the specificities of the antibodies involved.

Comparable extracts were made from all four strains and their K content measured by the technique of Mancini, Carbonara and Heremans (1965) using a pure anti-K13 serum

against live WF26 and absorbed with WF60 (O,K?) which had been steamed for 2 hours.

Table 3 shows that the more resistant strains produced more K antigen.

TABLE 3

THE K ANTIGEN CONTENT MEASURED BY IMMUNODIFFUSION, OF STANDARD EXTRACTS FROM FOUR STRAINS OF *Escherichia coli* SEROTYPE O6K13 RELATED TO RESISTANCE TO COMPLEMENT

Strain	K antigen (μg/ml)	Complement sensitivity (No. of $C'H_{50}$ units to give an LD_{50})
WF98	160	8·0
WF86	295	27·0
WF8	550	>32·4*
WF26	1020	>32·4*

*32·4 $C'H_{50}$ units killed 20 per cent of the inoculum of *E. coli* WF8. There was no demonstrable killing of *E. coli* WF26.

THE EFFECT OF EXTRACTS OF *E. coli* ON ANTIBODY BINDING AND COMPLEMENT DEPENDENT HAEMOLYSIS

The addition of extracts with agglutination inhibiting activity to the sheep red cell system reduced the degree of lysis obtained (Fig. 5), e.g. an extract containing 7 mg/ml glucose equivalents reduced lysis nearly four-fold. The extracts reduced lysis mediated by

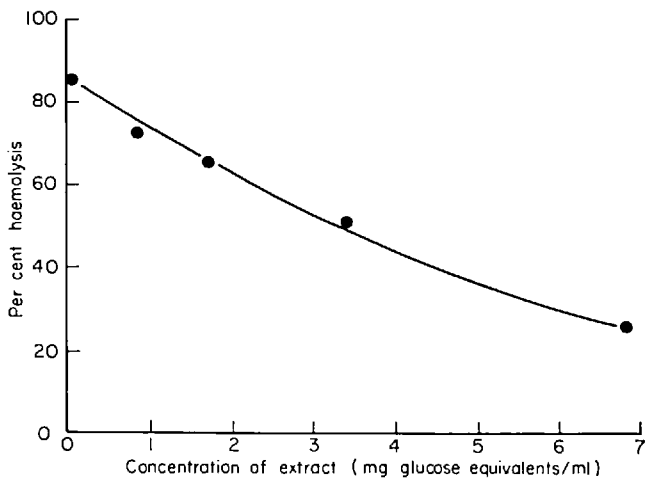

FIG. 5. Effect of saline extract of *E. coli* on immune haemolysis of sheep red blood cells.

either IgG or IgM antibody (Fig. 6). This effect was due partly to a decrease in the amount of antibody which became attached to the red cells and partly to interference with the action of complement.

When red cells were sensitized with a constant amount of labelled antibody in the presence of increasing concentrations of extract, the amount of antibody actually bound to the cells fell progressively. This was seen whether the antibody used was a crude preparation of γ-globulin precipitated with 12 per cent sodium sulphate (Fig. 7) or had been separated into pure IgG and IgM fractions (Fig. 8). Red cells were then sensitized with increasing amounts of antibody both in the presence and absence of extract, washed, and lysed with complement. The results are shown for crude γ-globulin in Fig. 9.

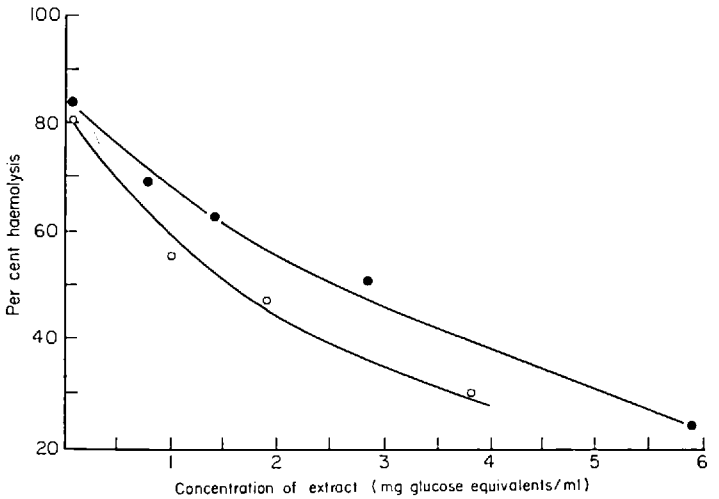

FIG. 6. Inhibition by *E. coli* extract of complement lysis of red cells sensitized with IgG or IgM antibody. ●, RBC treated with IgM; ○, RBC treated with IgG.

Whether extract was used or not haemolysis increased as the quantity of sensitizing antibody per red cell increased up to a limiting value. However, at any given level of sensitization there was always less lysis in the tubes where extract had been used.

A similar result was obtained when red cells were sensitized initially with antibody, washed, treated with increasing concentrations of extract, washed again and then lysed with complement (Fig. 10). Here all the tubes contained red cells sensitized as one batch but lysis fell as the amount of extract used rose.

Finally (Fig. 11) a similar result was given by a highly purified extract thought to contain only K antigen. Fig. 12(a) and (b) show an immunoelectrophoretic analysis of the purified extract to show that O antigen had been removed. The patterns given by anti-K serum with crude and purified K differ and suggest that a slow moving fraction of K had been removed during purification.

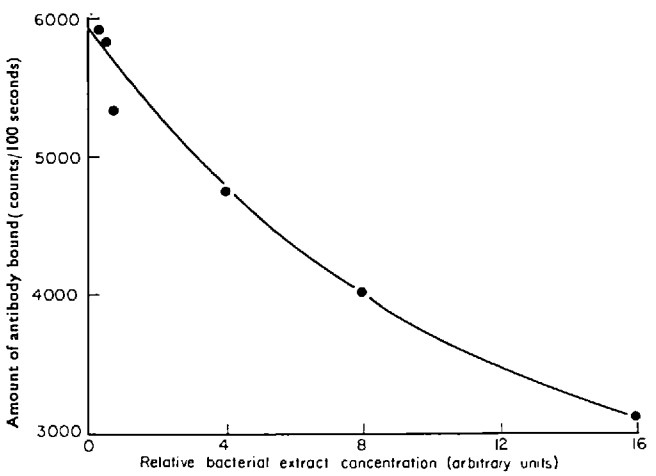

FIG. 7. Effect of saline extract of *E. coli* on the binding of rabbit antibody to sheep red blood cells.

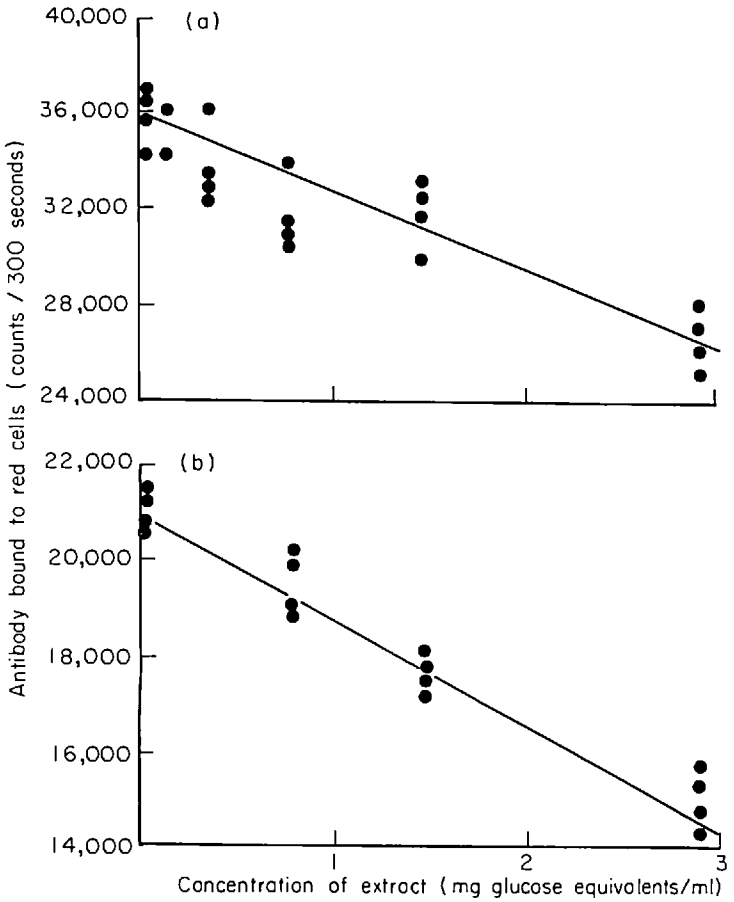

FIG. 8. Inhibition of: (a) IgM, and (b) IgG antibody binding by extracts of *E. coli*.

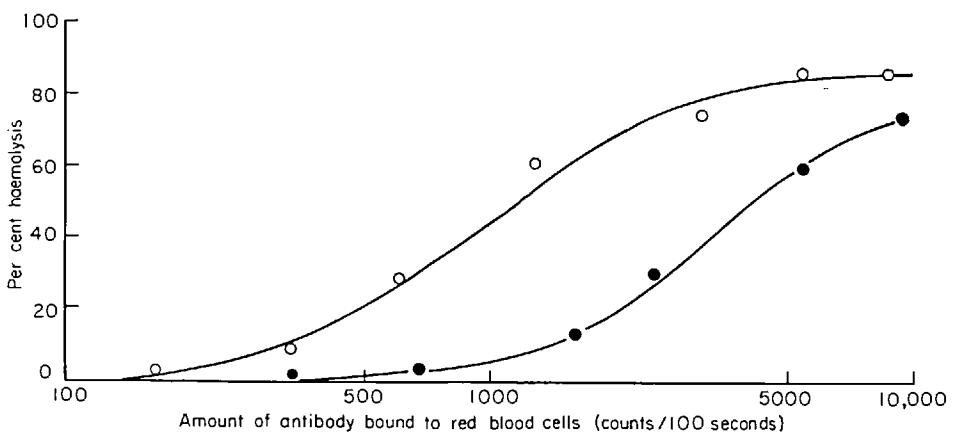

FIG. 9. Degree of haemolysis as a function of the amount of haemolysin bound to red cells in the presence and absence of *E. coli* extract. ●, Sheep RBC treated with bacterial extract; ○, sheep RBC treated with saline.

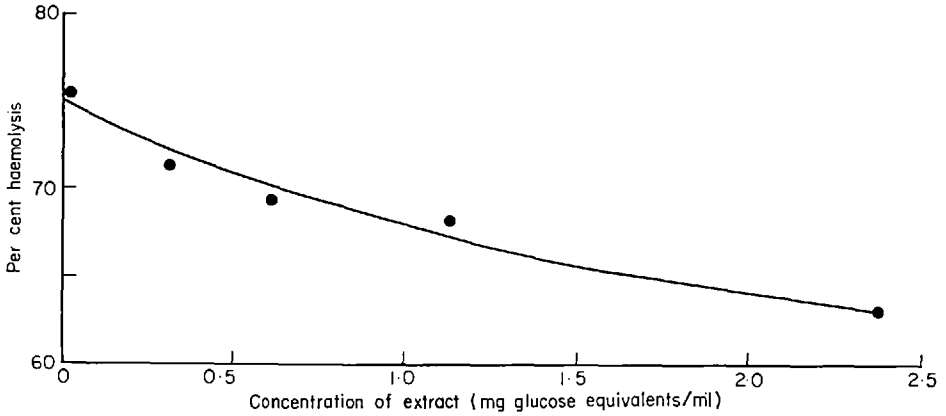

FIG. 10. Inhibition by *E. coli* extract of immune haemolysis of sensitized red cells.

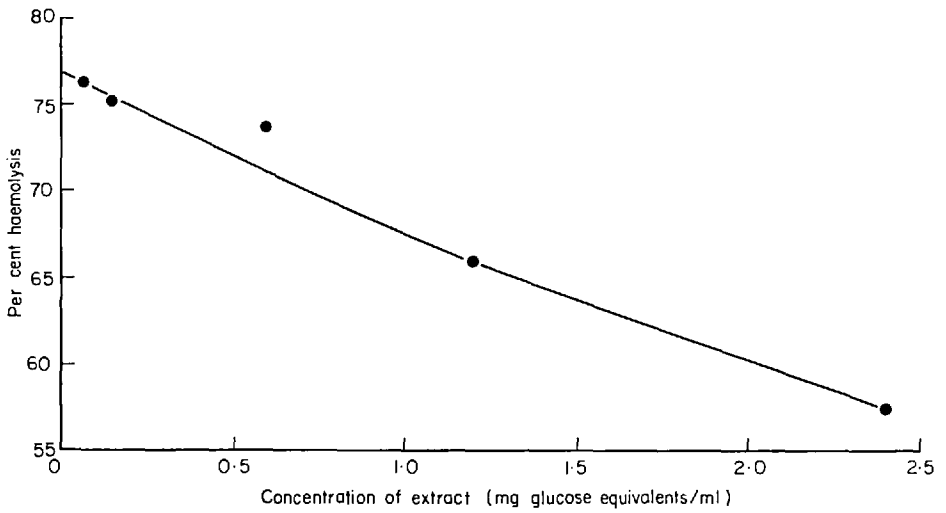

FIG. 11. Effect of purified *E. coli* extract (K antigen) on immune haemolysis.

DISCUSSION

The results confirm previous work showing that the K antigen of *E. coli* is an important determinant of a strain's resistance to complement. This is so whether one compares strains with different K antigens, different strains with the same K antigen or growths of the same strain at different temperatures with different amounts of K antigen.

The increased binding of lysozyme by the more resistant strains is probably a more direct method of measuring the surface charges which are responsible for the acriflavine effects discussed previously (Glynn and Priest, 1970).

The carbohydrate was estimated in crude extracts containing both O and K antigens so that it is difficult to make precise deductions from the results. However, it is of interest that White (1929) observed that smooth strains of *Salmonella* reacted more vigorously than rough with Molisch's reagent in a crude qualitative test. The quantitative results reported here

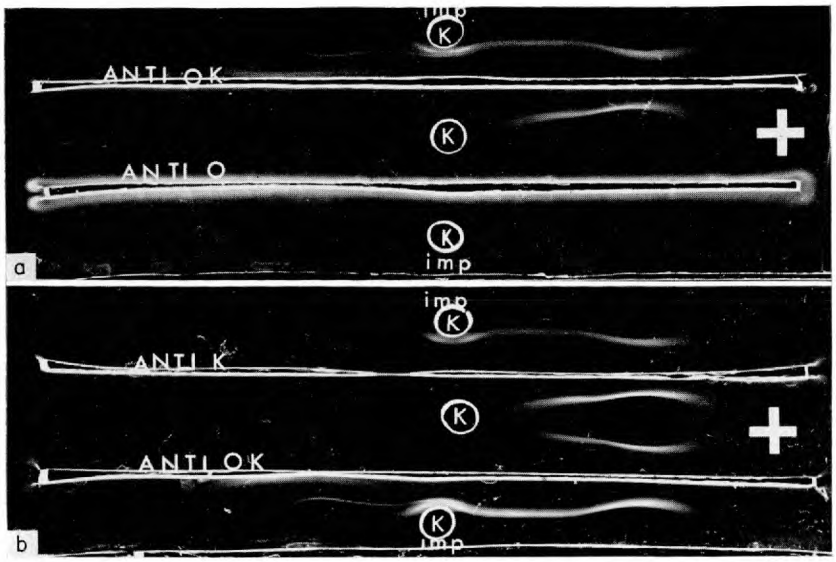

FIG. 12. (a) Immunoelectrophoresis of crude and purified extracts to demonstrate presence of K antigen using anti-O and anti-OK serum. In the wells: 'Imp. K' is the original saline extract of *E. coli* WF82. 'K' is the purified K antigen. (b) Similar using anti-K and anti-OK serum.

are more likely to be detecting extra K antigens than extra lipopolysaccharide since the comparison is between smooth strains only. The extracts tested precipitated specifically with the appropriate O and K antisera.

The effect of temperature and still more the findings with the K13 strains show that the amount of K produced is important. Nevertheless, comparison of K1 from WF96 with the K antigen (type unknown) from WF82 demonstrates that weight for weight some K antigens are much more effective than others.

The possibility that the K13 antigens from the four strains studied differ physically from each other in any way has not been excluded.

Milne (1966) found that the resistance to complement of smooth strains of *E. coli* was not related to any particular K specificity.

Ceppellini and Landy (1963) have suggested that the ability of K antigens to suppress agglutination was related to viscosity. In a different context Taylor and Walton (1957) demonstrated that the anti-complementary effect of dextran sulphate increased with both molecular size and the number of sulphate groups added. They suggested that the relevant factor was the charged surface area available.

The finding (Fig. 4) that K antigen from WF96 moves faster in gel electrophoresis than does the more active K from WF82 is at first sight contrary to expectations based on charge. However, the two kinds of molecule may differ enormously in size and other physical properties so that interpretation at this stage should be cautious. These and other properties of K antigens are being investigated further.

Whatever the molecular properties of K involved, it seems clear from the experiments with labelled antibody that they act first by impeding antibody binding to cells and then on complement itself. Possibly, although so far there is no evidence either way, one or

more complement components are also prevented from attaching themselves to the antibody or to the cell. If this were so, K antigen could be regarded as having a general action on protein binding.

These results may at first sight appear to disagree with those of Cepellini and Landy (1963) who showed that although Vi antigen prevented agglutination, antibodies to the red cell could still be detected by building up globulin–anti-globulin layers. From this and from the results of absorption and elution experiments they concluded that Vi did not reduce or impair the binding of antibody by red cells, but prevented subsequent lattice formation and agglutination. They did not find any effect of Vi on haemolysis by complement.

The experiments we report used a somewhat higher polysaccharide concentration and if Figs. 3, 5 and 8 are compared it can be seen that the K antigen had a much more pronounced effect on the agglutinating ability of anti-sheep sera than on haemolysis or the binding of IgG and IgM.

Thus although 25 μg of the polysaccharide WF82 reduced the agglutinating ability of anti-sheep red cell sera by 50 per cent, more than 2·5 mg would have been needed to reduce 50 per cent of its binding. In other words inhibition of agglutination is mainly due to the prevention of lattice formation and to a smaller part due to prevention of antibody binding.

However, if K antigen had no effect on antibody binding, or complement dependent lysis then the results of Muschel (1960) and Muschel *et al.* (1958) relating O inagglutinability to complement resistance in Enterobacteriaceae would be difficult to understand.

It is possible that with fewer antibody molecules per unit area of cell surface it is more difficult to build up a lattice. In addition the binding forces involved in the primary cell antibody union and in the secondary lattice formation are very similar. Both may be affected by K antigens. Moreover, bactericidal and haemolytic reactions do not need a secondary lattice formation but would be affected by anything interfering with the primary union with antibody or with any component of complement.

According to Rowley and Turner (1968) only thirty molecules of IgM antibody per bacterial cell are sufficient for complement killing of a Gram-negative bacterium. In the haemolytic reaction only two to three molecules of IgM or about 2000–3000 molecules of IgG (Humphrey and Dourmashkin, 1965) are required per red cell.

The effect of K antigen on antibody binding described above clearly does not diminish the number of antibody molecules per red cell to anything like this extent. Yet in some strains of *E. coli* the K antigen may completely abolish killing by normal human serum. It is likely that the K antigen normally *in situ* on the surface of a bacterium is present in greater quantity and is more efficiently arranged sterically than the K antigen artificially coated on a red cell.

The specificity of K antigens is useful taxonomically and their ability to suppress agglutination has been exploited for technical serological purposes by Coombs, Jonas and Lachman (1966). Their pathological significance arises from their presumed relation to virulence for man. Such a relationship might well depend on the ability of K antigens to produce resistance to complement. The endotoxic qualities of O antigens, the mass of genetic and chemical data about them acquired in recent years (Lüderitz *et al.*, 1966) and the comparative failure of alcohol-killed alcohol-preserved vaccine to protect against typhoid in the clinical trials in Yugoslavia (Yugoslav Typhoid Commission, 1962) has concentrated attention on the role of O antigens in virulence (Roantree, 1967). However,

virulence is more likely to have a multifactorial basis and it would seem unwise to dismiss K at present.

Recent reports (Nelson and Roantree, 1967; Medearis, Camitta and Heath, 1968) have related loss of virulence and increased serum sensitivity to genetically induced deficiencies in O antigens. Unfortunately it is not clear whether K antigens were present in the original strains used or had been lost or altered in the mutants. The strain of *E. coli* from which Medearis *et al.* (1968) derived their mutants was descended by periodic subculture from a strain of serotype O111 B4. The serotypes of the strains actually used are not given. It would be useful in future work of this sort to know not only whether specific K antigens were detectable but also whether their nature and quantity were such as to affect bactericidal and haemolytic reactions as described above.

Medearis *et al.* (1968) suggest that Vi and K antigens may be regarded as extensions in quantity and complexity of the saccharide components of O. Semantically this seems confusing and unnecessary. Chemically, there may well be loose links between K and O just as there are between O antigens and the mucopeptide layer. However, the distinctive chemical and peculiar immunological properties of K antigens warrant their separate consideration.

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