Ferric iron and the antibacterial effects of horse 7S antibodies to *Escherichia coli* O111

H. J. ROGERS *National Institute for Medical Research, Mill Hill, London*

Summary. Previous work showed that the virulence of *Escherichia coli* O141 was related to its ability to secrete catechols capable of transporting iron from serum transferrin to the bacterial cell. *E. coli* O111 also produced similar compounds in synthetic medium but was unable to do so in serum. It was postulated that antibody interfered with the production of these substances by this strain. The present experiments show that horse antiserum to *E. coli* O111 can induce bacterial killing in foetal calf serum, which, in contrast to the bactericidal effect of normal rabbit serum, cannot be reversed by Fe^{3+}. Five IgG subfractions of increasing electrophoretic mobility were isolated from the 7S fraction of the antiserum, all of which exerted a bactericidal effect on *E. coli* O111 in calf serum which could not be prevented by Fe^{3+}. The bacteriostatic effect induced by the fractions in the presence of transferrin could, however, be reversed by Fe^{3+}. IgG and IgT were isolated from normal serum but neither of these activated calf serum complement or induced bacteriostasis in the presence of transferrin.

These results show that specific antibody is responsible for these antibacterial effects and point to new problems concerning the role of iron compounds in the antibacterial effects of normal and specific immune serum.

Correspondence: Dr Henry J. Rogers, National Institute for Medical Research, The Ridgeways, Mill Hill, London NW7 1AA.

INTRODUCTION

There is good evidence to show that iron compounds can enhance the growth of relatively avirulent strains of *E. coli* both *in vitro* (Bullen and Rogers, 1969) and *in vivo* (Bullen, Leigh and Rogers, 1968; Fletcher and Goldstein, 1970). These, and related studies led to the conclusion that the extremely high association constant of serum transferrin for ferric iron (Bullen, Rogers and Lewin, 1971) restricted the availability of this element to the bacterial cell. Both the bacteriostatic and bactericidal effects exerted by serum on these organisms depend upon the presence of unsaturated transferrin. A second class of organism, which appeared to be more virulent in experimental animals, was distinguished by its ability to multiply in serum *in vitro*. Work with *Pasteurella septica* showed that this class of organism must possess an active mechanism for removing iron from its transferrin complex (Bullen *et al.*, 1971; Bullen, Wilson, Cushnie and Rogers, 1968). In the case of *E. coli* O141 which can also multiply in serum relatively easily, this process is accomplished by endogenously produced conjugates of 2,3-dihydroxybenzoyl serine which transport iron from transferrin to the bacterial cell. It was concluded that this ability to utilize transferrin-bound iron constitutes a virulence factor of the organism (Rogers, 1973). *E. coli* O111 which is less virulent than strain O141 and also fails to multiply in serum could nevertheless be induced to secrete iron-binding catechols in a synthetic medium.

The failure of this organism to multiply in serum was therefore attributed to the presence of natural antibodies which interfere with either the synthesis or secretion of the catechols. This idea was supported by the observation that specific immune serum was 100 times more active than normal serum in inducing bacteriostasis of strain O111 in the presence of transferrin. It was suggested that these antibodies formed an important part of host defences (Rogers, 1973). Further work has now been carried out to examine both the nature and antibacterial effects of these antibodies and also compare the properties of antibody fractions from normal and immune sera.

It is well known that immunization of horses gives rise to sera which can be highly protective against virulent bacteria and bacterial toxins (Kabat and Mayer, 1961). It became clear, however, that both the molecular weight and electrophoretic mobility of the antibodies varied both with the antigen employed and the course of immunization (van der Scheer, Lagsdin and Wychoff, 1940; van der Scheer, Wychoff and Clarke, 1941). Recently much effort has been directed towards unravelling the complexity of horse antibodies. A number of authors have discussed this complexity and the problems of isolation (McGuire, Crawford and Henson, 1973; Montgomery, 1973; Raynaud and Iscaki, 1970). It appears that there are six distinct classes of antibody, three of which can be divided immunologically into a total of eight subclasses. All these classes and subclasses can be shown to display antibody activity (Helm and Allen, 1970; Raynaud and Iscaki, 1970; Rockey, Klinman and Karush, 1964). In the present work it has not been possible to characterize completely the 7S antibody fractions present in immune horse serum but an attempt has been made to give some indication of the antibody classes employed in the antibacterial tests.

MATERIALS AND METHODS

Bacteria

Organisms from a 3-h broth culture of *E. coli* O111/B4/H2 were collected by centrifugation and suspended in 10 per cent (vol/vol) papain digest broth in saline. The bacterial population was then estimated by nephelometry with the aid of a standard curve. Viable counts were made by spreading 0·10-ml

volumes of appropriate dilutions on blood agar plates.

Human transferrin

Since some commercial samples of iron-free human transferrin failed to induce bacteriostasis of *E. coli* in the presence of 0·5 per cent specific antiserum, the following method was employed to remove Fe^{3+} from iron-saturated human transferrin (Kabi Pharmaceuticals). All operations were carried out at 4°. The pH of 10 ml of a 10 per cent aqueous solution of transferrin was lowered to 3·8 by careful addition of a 10 per cent solution of citric acid. In order to remove ferric citrate the mixture was immediately passed over a 4·0-ml column of Deacidite FF ion exchange resin (200–400 mesh) equilibrated with 0·1 M sodium citrate, pH 3·8. The pH of the eluate was raised to 7·5 with 1 N NaOH and the solution was finally dialysed overnight against 1 l of 0·1 M NaCl containing 0·02 M $NaHCO_3$, final volume, 21 ml.

Sera

E. coli O111 antiserum was prepared in a horse and had an O agglutinating titre of 8000, the bacteriostatic titre in the presence of transferrin was 200. The 'normal' serum was obtained 4 years previously from the same horse after immunization with *Clostridium welchii* type A; it was stored at −20°. Previous experience showed that the specific activities of these sera remained unchanged over several years. The serum had an O agglutinating titre of 16 and a bacteriostatic titre of 2 (Rogers, 1973). Foetal calf serum was obtained in the frozen state from Wellcome Reagents and stored at −70°. It was considered to be a satisfactory source of complement if >99 per cent of an inoculum of 10^4 *E. coli* O111 was killed in 2 h in the presence of 1:200 O111 antiserum.

Serum iron-binding capacity

Unsaturated iron-binding capacity was determined by titration with 1·0 mM ferric nitrilotriacetate (Bullen, Rogers and Leigh, 1972c). Saturated iron-binding capacity was determined after denaturation with urea (Bullen *et al.*, 1972c) by extraction with 2·5 mM bathophenanthroline in amyl alcohol (Peterson, 1951).

Immunoelectrophoresis and immunodiffusion

Microimmunoelectrophoresis and immunodiffusion

were carried out in 1 per cent agarose gel containing 0·03 ionic strength barbital–acetate buffer, pH 8·6. Precipitin lines were developed with rabbit anti-horse serum prepared by immunizing rabbits with alum-precipitated O111 antiserum (Weir, 1973). Anti-horse IgG, anti-human IgM and IgA were obtained from Miles Laboratories Inc.

Bacterial growth in serum and serum fractions

Samples were tested for their bactericidal effects in 95 per cent foetal calf serum; controls contained saline in 95 per cent calf serum. Three-millilitre amounts were stirred by means of magnetic followers in jacketed culture vessels at 37° under a gas mixture containing 10 per cent O_2–5 per cent CO_2–85 per cent N_2 flowing at 50 ml/min (Rogers, 1973). Tests for the bacteriostatic effects of the fractions in the presence of human transferrin which had a total iron-binding capacity of 38 μM, and was 10 per cent saturated with Fe^{3+} were carried out in normal saline containing one-quarter strength medium 199, 0·2 per cent $NaHCO_3$ and 0·1 per cent bovine serum albumin (fraction V) under the same conditions. Samples were diluted 1:10 and homogenized (MSE microhomogenizer) for 1 min prior to viable counting (Rogers, 1973).

Gel filtration

Gel filtration was carried out on a 2·2 × 95 cm column of Sephadex G-100 (fine) coupled in series to a similar column of Sephadex G-200 (superfine). The columns were pumped upwards at 12 ml/h with phosphate-buffered saline, ionic strength 0·15, pH 7·4. Extremely fine particles were removed from the Sephadex G-200 prior to swelling, by repeated sedimentation in ether (Kawata and Chase, 1968). Serum samples, 3·0 ml, were pumped into the columns sandwiched between 2 × 3·0 ml volumes of 5 per cent sucrose in PBS.

Ion-exchange chromatography on diethylaminoethyl-cellulose

A 2·0 × 50 cm column of DEAE–cellulose (Whatman DE52, microgranular) was equilibrated with 0·015 M Tris (hydroxymethyl) aminomethane (Tris–hydrochloride) pH 8·3 at 4°. Whole serum, 3·0 ml, was diluted with 27 ml water and applied to the column. After washing with 50 ml of starting buffer, linear gradient elution to a limiting buffer of 0·05 M Tris–hydrochloride + 0·15 M NaCl, pH 8·3 (400 ml each) was employed. The flow rate was 35 ml/h and

7·5-ml fractions were collected. The 7S fraction isolated from whole serum by gel filtration was concentrated to 10 ml and then dialysed overnight against 500 ml 0·012 M Tris–hydrochloride, pH 8·3. Chromatography of this material was carried out as described above except that only 350 ml of buffer was used in each chamber and the fraction size was 3·5 ml.

Concentration of fractions

Fractions were concentrated in a Sartorius high pressure filter no. 16208 fitted with membrane no. 12136. The sample volume was finally adjusted to 3·0 ml with PBS pH 7·8. Protein concentrations were determined by optical density measurements at 280 nm using $E^{1\ per\ cent}_{1\ cm} = 13\cdot8$ for the 7S globulin fractions (Kim and Karush, 1973).

RESULTS

Properties of normal and immune horse serum

Previous work has shown that immunization enhanced both the agglutinating and bacteriostatic effects against *E. coli* O111 (Rogers, 1973). Immunoelectrophoresis developed with rabbit anti-horse serum failed to reveal any significant difference between the two sera but the complexity of the β_2-γ region was similar to that found by other workers (Raynaud and Iscaki, 1970).

The bacteriostatic effect of a 1:200 dilution of the antiserum plus transferrin was abolished by adding iron at a concentration of twice the total iron-binding capacity of the transferrin (Rogers, 1973).

Raising the saturation level of the transferrin from 10 to 90 per cent however, did not abolish bacteriostasis (Fig. 1). Addition of the iron-transporting catechol (1 μm) isolated from *E. coli* O141 (Rogers, 1973) to 1:200 antiserum plus 10 per cent saturated transferrin also abolished bacteriostasis (Fig. 1).

Bactericidal effect of specific antiserum in foetal calf serum

Since foetal calf serum normally contains little or no γ-globulin (Barta, Barta and Ingram, 1972a; Barta, Barta, Ingram and Hubbert, 1972b), it was decided to use this as a source of complement (C) in order to characterize further the antibacterial
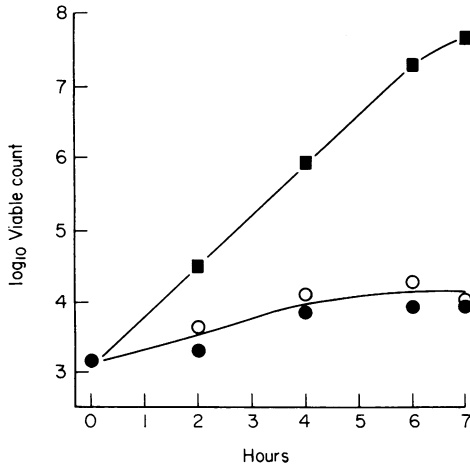
Figure 1. Bacteriostatic effect of 1:200 *E. coli* O111 antiserum in one-quarter strength medium 199 containing human transferrin (TIBC) (38 μM). (●) Transferrin 10 per cent saturated; (○) transferrin 90 per cent saturated; (■) transferrin 10 per cent saturated but 1 μM iron-transporting catechol (Rogers, 1973) added.

antibodies. *E. coli* O111 multiplied with a generation time of 18 min at pH 7·4 in foetal calf serum which had a total iron-binding capacity of 72 μm of which 82 per cent was saturated. Immune serum at a dilution of either 1:200 or 1:2000 produced a rapid bactericidal effect which displayed a prozone effect at 1:20 (Fig. 2) (Muschel, Gustafson and Larsen,
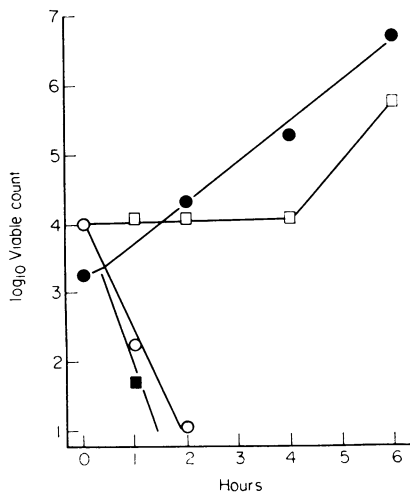

Figure 2. Effect of antiserum concentration on growth of *E. coli* O111 in foetal calf serum: (●) no addition; (○) antiserum, 1:2000; (■) antiserum, 1:200; (□) antiserum, 1:20.

1969). Heating the calf serum to 56° for 30 min abolished its ability to kill the bacteria in the presence of 1:200 antiserum, in fact the bacteria multiplied with a generation time of 20 min. In contrast, bacteriostasis was observed in heated rabbit serum (Bullen and Rogers, 1969). A further difference in the two systems was noted in their response to iron compounds, the bactericidal effect of normal rabbit serum was immediately reversed by Fe^{3+} (Bullen and Rogers, 1969). Neither saturating the transferrin with Fe^{3+}, nor adding iron-binding catechol from *E. coli* O141, immediately reversed the bactericidal effect of 1:200 antiserum in calf serum; in both cases the initial rate of killing was reduced and was followed by multiplication of the surviving bacteria (Fig. 3). Ammonium citrate added at the same concentration as ferric ammonium citrate (0·13 mM) had no effect on the bactericidal action of the antiserum (data not shown).
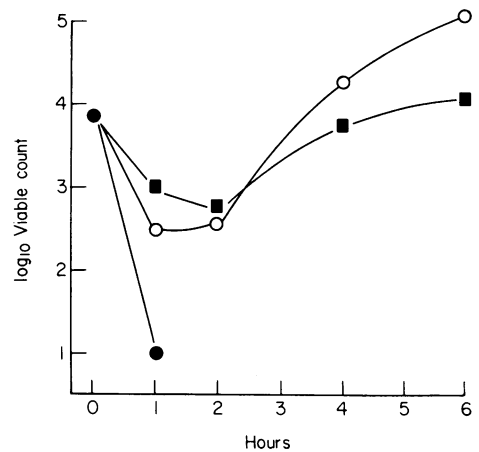

Figure 3. Effect of iron compounds on the bactericidal action of antiserum (1:200) on *E. coli* O111 in foetal calf serum: (●) control, no addition; (○) ferric ammonium citrate equivalent to twice the total serum iron-binding capacity; (■) 1 μM iron-transporting catechol from *E. coli* O141.

Fractionation of *E. coli* O111 antiserum on diethyl-aminoethyl–cellulose

The procedure is based upon that of Vaerman, Querinjean and Heremans (1971) except that linear gradient elution was used instead of stepwise elution. Six major peaks were obtained from whole serum, the first two of which appeared to be IgG_{ab}, and IgG_c as judged by immunoelectrophoresis (Vaerman
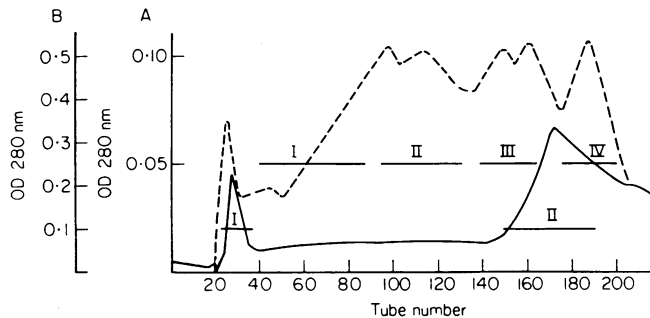
Figure 4. Chromatography of 7S fractions from horse sera on DEAE–cellulose, pH 8·3. (A) (– – –) *E. coli* O111 anti-serum; (B) (——) normal serum. Horizontal lines indicate pooling of fractions.

et al., 1971). Gel filtration showed that both fractions were eluted in the same volume as human IgG.

Isolation of the 7S globulin fraction by combined gel filtration and ion-exchange chromatography

Antiserum
Because of the high IgG content of these horse antisera it was necessary to use Sephadex G-200 and G-100 columns in series in order to obtain a clear separation between the 7S and albumin peaks. The anti-O titre of the 7S fraction obtained in this

Figure 5. Immunoelectrophoresis of fractions from DEAE–cellulose chromatography of the 7S fractions of *E. coli* O111 antiserum (Fig. 4) developed with anti-horse IgG: (a) fraction I; (b) fraction II; (c) fraction III; (d) fraction IV. The anode is to the left.

way was found to be 20,000. When subjected to ion-exchange chromatography on DEAE–cellulose, the 7S peak could be separated into four major fractions (Fig. 4). The pattern of lines given by the four fractions after immunoelectrophoresis is shown in Fig. 5.

It is quite clear that the electrophoretic mobility of the fractions increase with the NaCl concentration of the gradient. The anti-horse IgG also showed that antiserum fraction IV gave a line having the same mobility as the most anionic component present in whole serum. The recovery of protein from the column was apparently only 25 per cent, some of this loss can be accounted for by the appearance of a precipitate on storage of the 7S fraction at − 20°. The main component lost appeared to be IgG$_{ab}$ since the recovery was only 13 per cent of that obtained by chromatography of the whole serum on DEAE–cellulose. Ion-exchange chromatography of the third fraction obtained by gel filtration showed that transferrin and albumin were present but little or no γ-globulin could be detected by immunoelectrophoresis. Repeated tests on both whole serum and the fractions by both immuno-diffusion and immunoelectrophoresis using anti-human IgA, failed to give a positive reaction although Vaerman *et al.* (1971) detected horse IgA by this method. The fractions from DEAE–cellulose (Fig. 4) also failed to react with anti-human IgM although horse IgM could easily be detected in whole serum with this reagent.
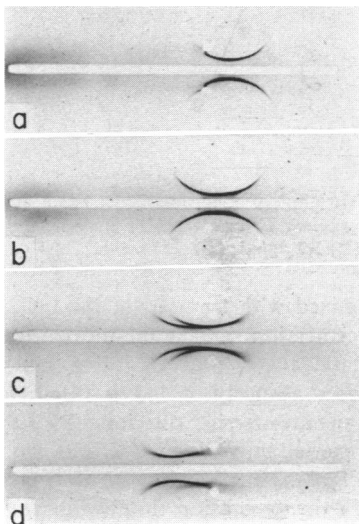
Normal serum
The fraction containing the 7S globulins was isolated initially by gel filtration as described above, when subjected to further fractionation on DEAE–cellulose, two major peaks were obtained

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(Fig. 4). The overall recovery of protein was 42 per cent and specifically in the case of IgG_{ab} it was 45 per cent. Immunoelectrophoresis confirmed the fact that normal serum fraction I consisted of IgG_{ab}. The high concentration and β_2 mobility of fraction II suggested that this was the IgT (anti-toxic) fraction.

Antibacterial activity of the 7S fraction

IgG_{ab} and IgT from normal serum

Both the IgG_{ab} (50 μg/ml) and the IgT (560 μg/ml) fractions failed to activate calf serum complement and the bacteria multiplied rapidly. When tested at the same concentration with transferrin in one-
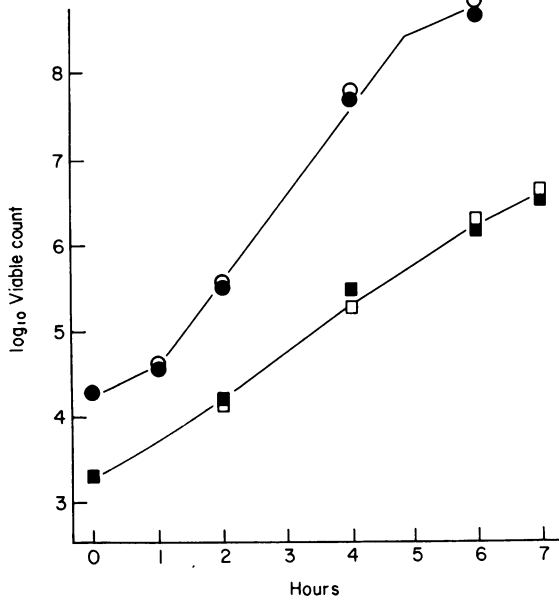

Figure 6. Absence of antibacterial activity against *E. coli* O111 from normal serum fractions (Fig. 4): (●) IgG_{ab} (50 μg/ml); (○) IgT (560 μg/ml) in foetal calf serum; (■) IgG_{ab}; (□) IgT in one-quarter strength medium 199 containing human transferrin, TIBC, 38 μM, 10 per cent saturated with Fe^{3+}.

quarter strength medium 199, however, the generation time of the bacteria was increased from 25 min (Rogers, 1973) to 35 min but there was no indication of a bacteriostatic effect (Fig. 6).

Fractions from antiserum

IgG_{ab} (140 μg/ml) and IgG_c (37 μg/ml) and the four 7S subfractions (60–100 μg/ml) were all bactericidal in calf serum in that >99 per cent of the inoculum of 10^4 *E. coli* O111/ml were killed in 2 h. Titration of the bactericidal effect of IgG_c showed that 90 per cent of the inoculum was killed during the first hour using an antibody concentration of 3·7 μg/ml, after which the survivors multiplied. When diluted to 0·37 μg/ml no bactericidal effect was observed (Fig. 7). Titration of the immune IgG_{ab} fraction gave a similar pattern of killing and regrowth at 2·1 μg/ml, suggesting that the specific activities of the two fractions were similar.
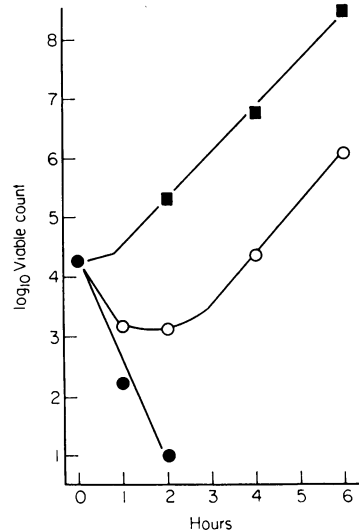

Figure 7. Titration of the bactericidal effect of IgG_c from antiserum, against *E. coli* O111 in foetal calf serum; (●) 37 μg/ml; (○) 3·7 μg/ml; (■) 0·37 μg/ml.

When mixed with transferrin, the IgG_{ab} fraction at a concentration 140 μg/ml produced a typical bacteriostatic effect, that is, initial multiplication which ceases abruptly at 2–3 h (Fig. 10). In the presence of transferrin, the four 7S subfractions (Fig. 4) exerted a variety of effects on bacterial growth (Fig. 8). Thus fractions I and II produced an increase in the generation time whilst fractions III and IV appeared to initiate bacteriostasis after which the viable count did in fact decrease. The importance of the concerted action of the antibody and transferrin is emphasized by the observation that the bacteria multiplied with a generation time of 22
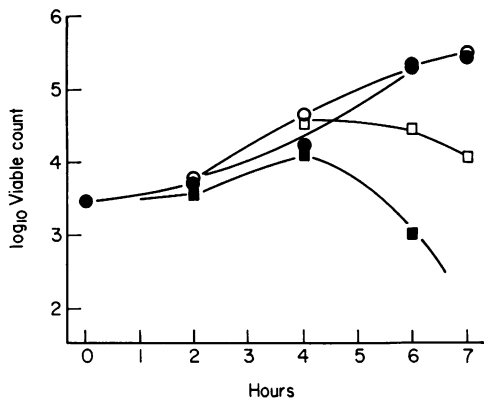
Figure 8. Antibacterial effects of 7S antiserum fractions (Fig. 4) in one-quarter strength medium 199 containing human transferrin, TIBC 38 μM, 10 per cent saturated with Fe^{3+}: ($\bullet$) fraction I (67 μg/ml); ($\bigcirc$) fraction II (100 μg/ml); ($\blacksquare$) fraction III (80 μg/ml); ($\square$) fraction IV (60 μg/ml).

min in samples of one-quarter strength medium 199 containing the same 7S subfraction in the absence of transferrin.

Effect of iron compounds on the antibacterial action of the fractions

Since iron compounds can abolish the bactericidal and bacteriostatic effects of whole serum on bacteria such as *E. coli* O111 and O141 (Bullen and Rogers,
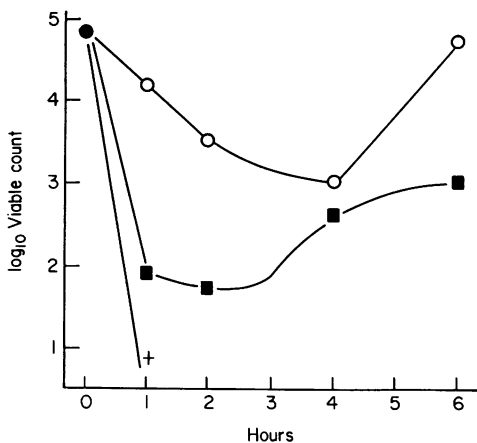
1969), *Pasteurella septica* (Bullen et al., 1971) and *Clostridium welchii* (Rogers et al., 1970), it was of some importance to compare the effect of iron on the isolated antibody fractions, with the results obtained using whole serum under the same conditions (Figs 1 and 3). Although comparison with whole serum (Fig. 3) is difficult, it appears that after some delay Fe^{3+} can abolish the bactericidal effect of immune IgG_{ab} in calf serum (Fig. 8). Addition of 1 μM iron-transporting catechol (Rogers, 1973) prevented the complete killing of the inoculum by IgG_{ab} in calf serum but then failed to support significant multiplication of the survivors (Fig. 9). Similar results were obtained with the subfractions of the 7S globulins (Fig. 4) in calf serum. Fe^{3+} abolished the inhibitory effects of the immune IgG_{ab} fraction in the presence of transferrin only when present in excess of the total iron-binding capacity of the transferrin (Fig. 10), an effect also found
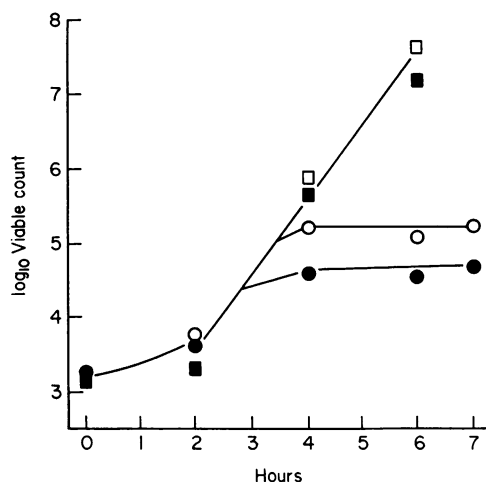

Figure 10. Effect of iron compounds on the bacteriostatic action of IgG_{ab} from antiserum (140 μg/ml) on *E. coli* O111 in one-quarter strength medium 199 containing human transferrin. ($\bullet$) Transferrin, 10 per cent saturated; ($\bigcirc$) transferrin, 90 per cent saturated; ($\blacksquare$) transferrin, 200 per cent saturated; ($\square$) transferrin, 10 per cent saturated but 1 μM iron-transporting catechol added.

Figure 9. Effect of iron compounds on the bactericidal action of IgG_{ab} (140 μg/ml) from antiserum on *E. coli* O111 in foetal calf serum; ($\bullet$) control, no addition; ($\bigcirc$) ferric ammonium citrate equivalent to twice the total serum iron-binding capacity; ($\blacksquare$) 1 μM iron-transporting catechol from *E. coli* O141 (Rogers, 1973) +less than ten bacteria per millilitre.

with 1:200 antiserum and transferrin. Similarly, the iron-transporting catechol (1 μM) abolished bacteriostasis (Fig. 10). The bacteriostatic effects exerted by the individual 7S subfractions as shown in Fig. 8 were also abolished either by saturating the transferrin with Fe^{3+} or by adding iron-transporting catechol (data not shown).

DISCUSSION

Previous work with *E. coli* O141 showed that the ability of large inocula of this organism to multiply in serum arose from its capacity to secrete derivatives of 2,3-dihydroxybenzoyl serine which transport iron from transferrin to the bacterial cell. Strain O111 produces similar compounds and grows in synthetic medium containing transferrin but is unable to do so in whole serum. It was concluded that horse and rabbit serum and human and bovine milk contained antibody which interfered with either the synthesis or release of these iron-transporting compounds. Immunization of a horse with *E. coli* O111 resulted in a 100-fold increase in the bacteriostatic titre of the serum against that organism. Thus there is good circumstantial evidence that specific antibody, acting in the presence of transferrin, is responsible for the bacteriostatic effect which can be abolished by iron compounds (Rogers, 1973).

Both normal serum and antiserum to *E. coli* O111 were fractionated either by means of ion-exchange chromatography or a combination of gel filtration and ion-exchange chromatography (Fig. 4). Antibacterial antibody, as defined by the bactericidal effect in foetal calf serum could be detected in antiserum fractions ranging in electrophoretic mobility from slow γ to α_2. It appears that the immune 7S subfraction IV (Fig. 4) contains mainly IgT but it may be that the small amount of IgG also present in this fraction is responsible for the bactericidal effect since it appears that IgT does not activate heterologous complement (McGuire *et al.*, 1973). Immunization with strain O111 leads to a large increase in the anti-O titre of the 7S fraction. It did not prove possible to detect horse serum IgA in the present experiments (Vaerman *et al.*, 1971). The normal serum to *E. coli* O111, which had in fact been obtained 4 years earlier as an antiserum to *Clostridium welchii* contained two major 7S fractions which appeared to be IgG$_{ab}$ and IgT containing some fast-running IgG (Fig. 4). Both these fractions were devoid of antibacterial activity against *E. coli* O111 (Fig. 6). Thus the antibodies of normal serum (Rogers, 1973) do not appear to reside in either of these fractions and this is supported by preliminary experiments which show that the IgM fraction exerts a bactericidal effect on the organisms in calf serum (unpublished observations).

The inhibitory effects exerted by the fractions from antiserum on bacterial growth in the presence of transferrin were somewhat variable, thus some produced the typical sigmoidal growth curve (Figs 8 and 10) as found previously with whole serum (Rogers, 1973). In other cases the fractions appeared to increase the generation time of the bacteria (Fig. 8). The fact that the bacteria multiplied rapidly in the presence of the IgG fractions in one-quarter strength medium 199 again serves to emphasize the importance of the concerted action of antibody plus transferrin in producing these antibacterial effects. The antibacterial effects of the transferrin and the specific antibody fractions could, however, be reversed by saturating the transferrin with iron (Fig. 10). Increasing the saturation of the transferrin from 10 to 90 per cent failed to abolish the bacteriostatic effect of either 1:200 antiserum or the specific antibody fractions. Iron-transporting catechol from *E. coli* O141 abolished bacteriostasis in both cases (Figs 1 and 10).

Foetal calf serum in which the transferrin was 82 per cent saturated with iron has been used as a source of complement in order to examine the bactericidal effect of the antiserum and its fractions. The organisms multiplied rapidly in calf serum but addition of antiserum, 1:200 or 1:2000 produced a bactericidal action which displayed a prozone effect at 1:20 (Fig. 2) (Muschel *et al.*, 1969). The bactericidal effect was abolished by heating calf serum. The results also suggested that the specific activities of the immune IgG fractions were comparable. These observations are similar to those of Barta *et al.* (1972a, b) who showed that although two rough strains of *E. coli* were killed by precolostral calf serum, strain O127 was killed only after the addition of specific antiserum.

These results suggest therefore that upon immunization with *E. coli* O111, the horse produces 7S antibody molecules having a wide range of electrophoretic mobility and high O agglutinating titre which are responsible for both the bactericidal and bacteriostatic effects *in vitro*. Furthermore, the fact that the bacteriostatic effect of normal serum (Griffiths, 1972), immune serum (Fig. 1) (Rogers, 1973) and immune IgG plus transferrin (Fig. 10) can be reversed by iron compounds or iron-transporting catechol, suggests that in each case production of endogenous bacterial iron-transporting catechols is inhibited by antibody. On the other hand, some of the results of this investigation pose certain problems. Thus the complex patterns of viable counts which result from the addition of

either Fe^{3+} or iron-transporting catechols when each is added to either antiserum or its fractions in calf serum is quite different from the rapid and complete abolition of the bactericidal effect of normal rabbit serum by Fe^{3+} (Bullen and Rogers, 1969). It remains to be determined whether this is due to differences in the antibody molecules or complement systems involved. It is interesting that Griffiths (1971) found that Fe^{3+} did not immediately reverse the bactericidal effect of specific antiserum against *P. septica.* Differences were also observed between heated rabbit serum which still induced bacteriostasis of *E. coli* O111 (Bullen and Rogers, 1969) and heated calf serum containing 1:200 antiserum where rapid bacterial multiplication occurred. This type of observation only serves to emphasize the complex nature of the interaction of the organism, transferrin, antibody and complement. Finally, since iron compounds abolished both the antibacterial effects of normal serum *in vitro* (Griffiths, 1972; Rogers, 1973) and natural resistance in experimental animals (Bullen, Leigh and Rogers, 1968; Fletcher and Goldstein, 1970), it was concluded that in both situations transferrin and natural antibody acted together to prevent bacterial multiplication (Rogers, 1973). The present work suggests that Fe^{3+} is less effective in abolishing the bactericidal action of specific antibody and complement; this suggests that it may be worth while investigating the effect of Fe^{3+} on passive immunity to *E. coli.*

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