Antibacterial Effect of the Scandium and Indium Complexes of Enterochelin on *Escherichia coli*

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Enterochelin, the iron chelator produced by a number of pathogenic enterobacteria, appears to be an essential metabolite for multiplication within the host, where it transports iron from the host iron-binding proteins to the bacteria. Previous work showed that complexes of enterochelin containing either scandium (Sc^{3+}) or indium (In^{3+}) exerted a bacteriostatic effect on *Klebsiella pneumoniae* in serum, whilst the Sc^{3+} complex exerted a significant therapeutic effect on mice infected with *K. pneumoniae*. These observations have now been extended to a number of pathogenic serotypes of *Escherichia coli* including those carrying either the K1 antigen or the ColV plasmid. The Sc^{3+} and In^{3+} complexes each exert a bacteriostatic effect on these organisms growing in either whole serum or media containing an iron-binding protein. Evidence is presented that the Sc^{3+} complex may act as a competitive inhibitor of the Fe^{3+} complex. In contrast to their effects on *K. pneumoniae*, sideramines other than enterochelin fail to reverse the bacteriostatic effect of the Sc^{3+} complex of enterochelin in *E. coli*, suggesting that the complex produces a more profound derangement of metabolism in this organism. The Sc^{3+} complex exerts a significant therapeutic effect on *E. coli* infections in mice although the In^{3+} complex is less active.

INTRODUCTION

The physiochemical state of iron in body fluids is now considered to be of fundamental importance in host resistance to many bacterial infections (for general reviews of iron and infection see Bullen *et al.*, 1978; and Weinberg, 1978). Bacteria entering the body become exposed to the iron-binding proteins, lactoferrin on mucosal surfaces and transferrin in the plasma. It has been postulated that in order to multiply in the presence of these iron-binding proteins, with which the ferric ion is very strongly associated (Aasa *et al.*, 1963; Aisen & Liebman, 1972), the bacteria must possess a specific mechanism for assimilating this protein-bound iron (Bullen *et al.*, 1971).

Under iron-deficient conditions, a number of enterobacterial pathogens including *Escherichia coli* (Rogers *et al.*, 1977), *Salmonella typhimurium* (Pollack & Neilands, 1970) and *Shigella sonnei* (Perry & San Clemente, 1979) secrete enterochelin, the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl serine. This compound appears to be a virulence factor for both *E. coli* (Rogers, 1973) and *S. typhimurium* (Yancey *et al.*, 1979). This idea is supported by the observations that enterochelin can remove Fe^{3+} from its complex with transferrin (Carrano & Raymond, 1979) and that it can abolish the bacteriostatic effect of serum on *E. coli* (Rogers *et al.*, 1977). Since enterochelin appears to be an essential growth factor for this important group of pathogens under the iron-restricted conditions imposed by the host, it was reasoned that complexes of enterochelin with ions other than Fe^{3+} might act as antimetabolites to ferric enterochelin. Of 19 complexes tested, only those containing either scandium or indium exerted a bacteriostatic effect on *Klebsiella pneumoniae*. The scandium complex exerted a therapeutic effect similar to that of kanamycin sulphate on *K. pneumoniae* infections in mice (Rogers *et al.*, 1980). The present paper describes the antibacterial activity of these complexes on certain pathogenic serotypes of *E. coli*. A preliminary account of this work has been published (United Kingdom Patent 2022076, 1979).

METHODS

Bacteria. *Escherichia coli* O111/K58/H2 was isolated from a case of neonatal gastro-enteritis whilst strain O141/K85/H4 was isolated from a scouring piglet. Serotypes O1/K1/H- (Sarff *et al.*, 1975; provided by I. Ørskov, Statens Serum Institute, DK 2300, Copenhagen 5, Denmark) and O18/K1/H7 ColV+ (provided by H. Williams Smith, Houghton Poultry Research Station, Huntingdon, Cambs., U.K.) were isolated from cases of neonatal meningitis. Inocula were prepared and viable counts obtained as previously described (Rogers *et al.*, 1980).

Sera. Normal rabbit serum was stored at -20°C . Heparinized rabbit plasma was heated for 30 min at 56°C and then stored at -20°C . Foetal calf serum and normal horse serum no. 2 were obtained in the frozen state (Wellcome Reagents) and stored at -70°C and -20°C respectively. Specific antiserum to *E. coli* O111 was prepared from a horse. The antiserum had an O agglutinating titre of 1:8000. Serum taken from the horse prior to immunization had a titre of 1:16 (Rogers, 1973).

Bacterial growth in serum and trypticase soy broth. Bacteria were grown in serum at pH 7.4, 37°C , and viable counts were obtained on homogenized samples (Rogers, 1973). Trypticase soy broth (TSB; BBL Microbiology Systems) supplemented with $10\text{ }\mu\text{M}$ -ferrous ammonium sulphate and $1\text{ }\mu\text{M}$ - MnCl_2 , was also used in some experiments. The addition of 0.071 M - NaHCO_3 under 5% (v/v) CO_2 in air at 37°C gave a pH of 7.5.

Conalbumin. Conalbumin (Sigma, type I; 10 mg ml^{-1} in 0.15 M - NaCl containing 0.02 M - NaHCO_3) was dialysed for 24 h at 4°C against the same solution to remove low molecular weight chelators. The conalbumin solution was sterilized by membrane filtration and stored at -20°C .

Enterochelin complexes. A 20 mM solution of enterochelin in *n*-butanone appeared to be stable at -20°C for at least two months. Metal chlorides, final concentration 10 mM , were dissolved in 0.1 M - HCl /ethanol mixture (1 vol. 10 M - HCl to 99 vol. ethanol). For experiments *in vitro*, 0.2 mM solutions of the complexes were prepared daily by mixing $20\text{ }\mu\text{l}$ of the enterochelin solution with $40\text{ }\mu\text{l}$ metal chloride solution and then adding 1.94 ml water followed by a few mg solid NaHCO_3 . Samples for the mouse protection tests were prepared by mixing 1.0 ml 20 mM -enterochelin solution with 2.0 ml 10 mM -metal chloride solution. In the case of the Sc^{3+} complex, one drop of sterile glycerol was also added. After removing the solvent under vacuum, the residue was suspended by sonication in 20 ml sterile 1% NaHCO_3 containing 0.85% NaCl . The In^{3+} complex formed a clear solution whilst the Sc^{3+} complex formed a fine suspension which settled out on standing but could be dispersed again by vigorous shaking. Without the addition of glycerol, the Sc^{3+} complex proved extremely difficult to wet and disperse. These solutions appeared to be stable for at least one week at 4°C . Solutions of the Fe^{3+} complex were prepared in the same way as the In^{3+} complex but appeared to undergo some decomposition within 24 h.

Microbial iron chelators. Enterochelin, terregens factor, rhodotorulic acid and ferrioxamine B were all obtained as previously described by Rogers *et al.* (1980).

Haematin solution. A freshly prepared solution of 1 mM -haematin hydrochloride in 0.01 M - NaOH was added to heat-inactivated rabbit plasma to give a final concentration of $10\text{ }\mu\text{M}$ immediately before inoculation with *E. coli* O111.

Mice. Female TO mice weighing 23–25 g were used.

RESULTS

Antibacterial effects in serum

The addition of either the Sc^{3+} or In^{3+} complexes of enterochelin ($2\text{ }\mu\text{M}$; 1.4 and $1.6\text{ }\mu\text{g ml}^{-1}$ respectively) to normal rabbit serum in which *E. coli* O141 had been growing for 2 h inhibited further growth. In the case of the Sc^{3+} complex, this was followed by a bactericidal effect (Fig. 1). When heat-inactivated serum or plasma was used as a growth medium, addition of the complexes resulted in bacteriostasis. The Sc^{3+} complex of enterochelin also exerted a bacteriostatic effect on *E. coli* O1/K1 growing in foetal calf serum. This experiment may be considered as a very simple model of neonatal septicaemia. The observation by Williams (1979) that a ColV-linked locus may enhance iron acquisition in invasive strains of *E. coli* prompted the testing of the Sc^{3+} complex ($2\text{ }\mu\text{M}$) against one such strain (*E. coli* O18/K1/H7 ColV+), growing in normal rabbit serum. The pattern of growth in the control and test samples was almost identical to that of *E. coli* O1/K1.

Role of the iron-binding protein

It was postulated that the ferric enterochelin uptake system would be induced by exposing the bacteria to the iron-binding proteins of the host. Only then would the bacteria become sensitive to the inhibitory effects of the Sc^{3+} complex. The data in Fig. 2 suggest that this is the case, since only a combination of iron-binding protein, in this case conalbumin, and the Sc^{3+} complex of

Fig. 1

Fig. 2

Fig. 1. Antibacterial effect of complexes of enterochelin on *E. coli* O141 growing in normal rabbit serum. At 2 h, the additions made were: none (control, ●); 2 μ M-indium complex (○); 2 μ M-scandium complex (■).

Fig. 2. Effect of conalbumin (1 mg ml⁻¹) and the scandium complex of enterochelin (2 μ M) on the growth of *E. coli* O111 in trypticase soy broth supplemented with 10 μ M-Fe²⁺ and 1 μ M-Mn²⁺. Growth: in TSB containing conalbumin (●); in TSB to which the scandium complex of enterochelin was added at 2 h (○); in TSB containing conalbumin to which the scandium complex of enterochelin was added at 2 h (■).

Table 1. Growth of *E. coli* O111 in the presence of various enterochelin complexes

Normal horse serum containing 0.4% (v/v) O111 antiserum was inoculated with *E. coli* O111 to give an initial population of 3.5×10^2 organisms ml⁻¹. Portions (1.0 ml) were added to 25 ml bottles containing the test sample (final concentration 2 μ M). The bottles were incubated for 6 h on a rotary shaker at 37 °C under a stream of moist 5% (v/v) CO₂ in air.

Complex	Viable count (no. of bacteria ml ⁻¹)
Control (no addition)	< 10 ³
Fe ³⁺	2.3×10^7
Al ³⁺	2.0×10^5
Ga ³⁺	2.0×10^5
In ³⁺	< 10 ³
Sc ³⁺	< 10 ³
Y ³⁺	2.1×10^6
VO ²⁺	1.7×10^6
Ti ⁴⁺	5.0×10^5
Zr ⁴⁺	4.3×10^6

enterochelin were capable of inhibiting the growth of *E. coli* O111 in TSB supplemented with 10 μ M-Fe²⁺ and 1 μ M-Mn²⁺.

Stability of enterochelin complexes in serum

Rogers *et al.* (1980) suggested that some complexes of enterochelin might decompose by exchanging their metal ion with serum transferrin. Since it is known that the bacteriostatic effect of specific antiserum on *E. coli* O111 can be reversed by enterochelin (Rogers *et al.*, 1977), this property has been used in an attempt to differentiate between those complexes which are unstable and those which lack intrinsic antibacterial activity. The results presented in Table 1 show that the VO²⁺, Y³⁺ and Zr⁴⁺ complexes are quite active in stimulating bacterial growth.

Effect of enterochelin on bacteriostasis

Initial experiments showed that low concentrations of enterochelin (0.2 μ M) failed to reverse the inhibitory effect of the Sc³⁺ complex (2 μ M) on *E. coli* O141 in rabbit plasma. Experiments in

Fig. 3

Fig. 4

Fig. 3. Effect of adding the scandium complex of enterochelin (2 μ M) at 2 h on the growth of *E. coli* O141 in heat-inactivated rabbit plasma containing enterochelin at 0.2 μ M (●), 2 μ M (○) or 20 μ M (■).

Fig. 4. Effect of adding ferric enterochelin at 2 h on the growth of *E. coli* O141 in heat-inactivated rabbit plasma containing the scandium complex of enterochelin (2 μ M): none (control, ●), 2 μ M-ferric enterochelin (○) and 20 μ M-ferric enterochelin (■).

Table 2. *Effect of enterochelin complexes on the survival times of mice infected with E. coli O141*

Groups of 10 mice received 10^7 *E. coli* O141 intraperitoneally and 1 h later were given 0.2 ml 67 μ M solution of the complex by the same route. Deaths were recorded at 20 min intervals.

Complex	Time to 50% death (h)
Control	4.75
Al ³⁺	4.75
Ga ³⁺	3.25
In ³⁺	8.5
Sc ³⁺	7.0
Y ³⁺	4.75
La ³⁺	4.25
Co ²⁺	3.5

which the bacteria were grown in the presence of different concentrations of enterochelin before adding the Sc³⁺ complex (2 μ M) showed that bacteriostasis still occurred after a 2 h delay even in the presence of a 10-fold excess of enterochelin (Fig. 3). An attempt was made to determine whether the Fe³⁺ and Sc³⁺ complexes of enterochelin act on the bacteria in a competitive manner. The inhibitory effect of the Sc³⁺ complex (at 0.2 or 2 μ M) on *E. coli* O141 in rabbit plasma was rapidly reversed by the addition, 2 h later, of either 2 μ M or 20 μ M ferric enterochelin (Fig. 4).

Effect of other ferric iron complexes

Microbial iron-transporting compounds, including ferrioxamine B, ferric rhodotorulate, and particularly terregens factor, are all capable of reversing the inhibitory effect of the Sc³⁺ complex on *K. pneumoniae* (Rogers *et al.*, 1980). In the case of *E. coli* O141 however, none of these compounds (at 10 μ M) proved capable of reversing the bacteriostatic effect of 2 μ M-Sc³⁺ complex. When added to the rabbit plasma at a concentration of 100 μ M at the beginning of the experiment, ferric ammonium citrate was able to prevent the bacteriostatic effect of both the In³⁺ and Sc³⁺ complexes of enterochelin (2 μ M) added 2 h later. Haematin (10 μ M) however, did not prevent these bacteriostatic effects (data not shown).

Mouse protection tests

Mice were infected by the intraperitoneal route with 1×10^7 *E. coli* O141 (approximately 1 LD₁₀₀ dose). They then received, 1 h later, 0.2 ml of a 67 μ M solution [approximately 0.4 mg

(kg body weight)⁻¹] of various enterochelin complexes by the same route. Since mice given a lethal dose of the organism die within a few hours, deaths were recorded at 20 min intervals for the first 8 h after infection. The results in Table 2 show that of the complexes tested, only those of Sc³⁺ and In³⁺ were able to delay death.

In a further test of the protective effect of the Sc³⁺ complex, 40 mice were each infected with 8×10^6 *E. coli* O141, and 20 animals received 0.2 ml of 1 mM [5.8 mg (kg body weight)⁻¹] Sc³⁺ complex, 1 and 6 h after infection. Of the untreated mice which served as controls, 90% had died by 32 h, whilst of the treated mice, 80% survived without obvious ill-effects and were killed one month later. In a similar test of the In³⁺ complex in mice receiving 1.2×10^7 *E. coli* O141, the lives of only a few animals were prolonged during the first 6 h of the experiment but there were no survivors from either group at 24 h.

DISCUSSION

These observations provide further support for the idea that complexes of enterochelin with certain ions other than Fe³⁺ act as antimetabolites to ferric enterochelin and so inhibit bacterial multiplication by interfering with iron assimilation in the iron-restricted conditions imposed by the serum iron-binding protein, transferrin (Bullen *et al.*, 1971). Thus, the growth of the four pathogenic serotypes of *E. coli* used in this work was inhibited by the Sc³⁺ complex of enterochelin (at 2 µM).

Although the In³⁺ complex is also active *in vitro* (Fig. 1), its properties have not been examined in great detail since it was less active in the mouse protection test. A bactericidal effect followed the addition of the Sc³⁺ complex to *E. coli* O141 in normal rabbit serum (Fig. 1). Heat-inactivated serum or plasma in which only bacteriostasis occurs were used subsequently in order to avoid complications which might be imposed by this bactericidal effect. It would be of considerable importance, however, if inhibition by the complex were to render the bacteria more susceptible to the natural defences of the host.

Escherichia coli O1/K1 growing in foetal calf serum was employed as a model of neonatal sepsis and meningitis (Sarff *et al.*, 1975). The Sc³⁺ complex caused rapid inhibition of bacterial growth. Recent work by Williams (1979) suggests that certain invasive strains of *E. coli* carry a ColV plasmid which codes for an enhanced ability to sequester protein-bound Fe³⁺. This system appears to operate in addition to the enterochelin system of iron transport. It was found, however, that one such ColV⁺ strain is just as susceptible to inhibition by the Sc³⁺ complex of enterochelin as serotypes O141 and O1.

An attempt has been made in this work to clarify some of the conditions required to render these compounds effective antibacterial agents. Thus, apart from the nature of the complexes themselves (Rogers *et al.*, 1980), it was also postulated that an iron-restricted environment was required. A suitable iron-restricted environment was produced by means of conalbumin, the iron-binding protein from hen eggs. The ferric enterochelin transport system, which is induced by exposing the bacteria to suitable iron chelators (Rogers, 1973), is susceptible to inhibition by the Sc³⁺ complex of enterochelin (Fig. 2). This experiment, together with that carried out in foetal calf serum, also shows that antibody is not involved in the inhibitory process. Previous work showed that complexes of enterochelin containing either Ru³⁺, VO²⁺ or Cr³⁺ stimulated bacterial growth in serum, either as a result of hydrolysis of the complex or exchange reactions with transferrin resulting in free enterochelin (Rogers *et al.*, 1980). Using *E. coli* O111 in which enterochelin release is blocked by specific antibody (Fitzgerald & Rogers 1980), it has been possible to obtain some indication of the stability of the complexes in serum (Table 1). Thus, the growth in the presence of Y³⁺ complex is in accord with its chemical instability. Although the VO²⁺, Ti⁴⁺ and Zr⁴⁺ complexes are moderately stable to decomposition by Fe³⁺ (Rogers *et al.*, 1980), they appear to release sufficient enterochelin in serum to support considerable bacterial growth.

The inhibitory effect of Sc³⁺ enterochelin on bacterial growth can be reversed by relatively high concentrations of the Fe³⁺ complex in a manner that suggests competition between the complexes. The failure of metal-free enterochelin (20 µM) to reverse the inhibitory effect could

then be explained by its inability to remove sufficient iron from transferrin to compete with the Sc^{3+} complex. This, in turn, suggests that in order to overcome the inhibitory effect of the complex, the organism would have to produce very high levels of enterochelin. In the case of *E. coli* O141 growing in serum, the maximum concentration of enterochelin attained was found to be only $0.48 \mu\text{M}$ (Rogers, 1973).

Previous work has shown that a number of microbial iron transporting compounds could reverse the bacteriostatic effect of the Sc^{3+} complex on *K. pneumoniae* (Rogers *et al.*, 1980). In contrast, these compounds failed to reverse the inhibitory effect of the complex on *E. coli* O141. This suggests that exposing the organism to the Sc^{3+} complex may induce biochemical changes beyond an interruption of the iron supply. The In^{3+} complex appears to exert similar effects. Both ferric ammonium citrate and haematin abolish the bacteriostatic effect of serum on *E. coli* O141 (Bullen & Rogers, 1969). In contrast, only the former proved capable of preventing the bacteriostatic effect of the complexes on the organism. It is probable that the initial exposure of the bacteria to ferric ammonium citrate served to repress the ferric enterochelin system thus making the bacteria insensitive to the complexes.

The results of preliminary mouse protection tests (Table 2) and experiments *in vitro* show that the antibacterial properties are confined to the In^{3+} and Sc^{3+} complexes of enterochelin. Definitive tests have shown that, as in the case of *K. pneumoniae* (Rogers *et al.*, 1980), the In^{3+} complex has only a slight therapeutic effect on *E. coli* O141 infections in mice whilst the Sc^{3+} complex exerts a significant therapeutic effect.

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