STUDIES OF THE ANTIBACTERIAL EFFECT OF THE SCANDIUM COMPLEX OF ENTEROCHELIN

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The scandium complex of enterochelin is known to be assimilated and degraded by enterobacterial pathogens, liberating intracellular scandium. Macromolecular synthesis was inhibited in the order RNA, protein, DNA and phospholipid. Some component of heat-inactivated serum acts in concert with the complex inducing RNA degradation and killing. Both processes are inhibited by dinitrophenol. These biochemical changes resemble those found during complement mediated killing. An examination of the effect on synchronously growing cells suggests that the complex may exert its primary effect during the initiation of chromosome replication.

The importance of the interaction of the iron-transporting systems of the host and pathogen in determining the outcome of bacterial infection is now clearly understood[1,2]. A number of enterobacterial pathogens are known to secrete the iron chelator, enterochelin, which consists of three molecules of 2,3-dihydroxy-*N*-benzoyl-L-serine combined in the form of a cyclic trilactone. This molecule, which has a *Ka* for Fe^{3+} of 10^{52} [3] is capable of removing Fe^{3+} from the host iron-binding proteins, transferrin and lactoferrin[4,5,6]. Ferric enterochelin is assimilated by means of a specific receptor located in the bacterial outer membrane[7]. The complex is then transported to the cytoplasmic membrane by the action of the *tonB* function[8] where it is degraded by a specific esterase[9]. Previous work showed that the scandium (Sc^{3+}) and indium (In^{3+}) complexes of enterochelin acted as antimetabolites to the ferric complex in both *Klebsiella pneumoniae*[10] and *Escherichia coli*[11]. The Sc^{3+}-complex was particularly active as a bacteriostatic agent on organisms which would otherwise multiply rapidly in serum and also exerted a significant therapeutic effect on experimental infections with these same organisms[10,11].

It was found that the Sc^{3+}-complex was assimilated by *E. coli* at 20% of the rate of the Fe^{3+}-complex. The Fe^{3+}-enterochelin transport system displayed a marked specificity in that the aluminium complex failed to interfere with Fe^{3+} uptake whilst the gallium complex was assimilated at only 3% of the rate of the Fe^{3+}-complex, although the ionic radius of Ga^{3+} is closer to that of Fe^{3+} than is the radius of Sc^{3+}. The Sc^{3+}-complex was assimilated by the same mechanism as the Fe^{3+}-complex, the ligand being ultimately degraded giving rise to free Sc^{3+} within the cell. Unfortunately, the results did not allow a distinction to be made between an acute deprivation of Fe^{3+} and a specific toxic effect of Sc^{3+} [12]. This paper presents the results of studies of the effect of the Sc^{3+}-complex of enterochelin on bacterial growth, cell division and macromolecular synthesis.

Materials and Methods

Bacteria

Bacteria were maintained and cultured as previously described[12].

Chemicals

[*U*-^{14}C]Protein hydrolysate (57 mCi/milliatom carbon) and [^{32}P]orthophosphate were obtained from Amersham International, UK.

Preparation of the Scandium Complex of Enterochelin

Sc^{3+}-Enterochelin (0.2 mM) was prepared by mixing 50 μl of 20 mM $ScCl_3$ dissolved in 0.1 M HCl with 50 μl of 20 mM enterochelin dissolved in *n*-butanone, followed by the addition of 4.9 ml of distilled water and a little solid $NaHCO_3$ to bring the pH to 7.0. Less concentrated Sc^{3+}-enterochelin was made by diluting the 0.2 mM solution in distilled water[10].

Macromolecular Synthesis

E. coli O18 (*colV*$^+$)[11] and *K. pneumoniae* were grown in 10 ml Trypticase soy broth (TSB) at 37°C overnight after which 5 ml was subcultured in 20 ml TSB in a 500-ml conical flask. The culture was stirred at 37°C for 2 hours, the cells were harvested by centrifugation in an MSE bench centrifuge and resuspended in 0.85% saline at a density of about 10^8/ml as estimated nephelometrically. A 0.1 ml sample from this suspension was added to 10 ml heat-inactivated rabbit plasma (HRP) (30 minutes, 56°C) containing either 0.5 μCi ^{14}C-labelled amino acids per ml for protein synthesis studies or 2 μCi [^{32}P]orthophosphate per ml for nucleic acid and lipid synthesis studies. The culture was incubated at 37°C in a jacketed reaction vessel with stirring under 5% CO_2 in air (100 ml/minute). At 2-hour, 0.2 mM Sc^{3+}-enterochelin was added to give a final concentration of 10 μM and incubation continued for a further 2 hours. Duplicate samples (0.1 ml) were removed at intervals and the bacteria collected on 0.45 μm millipore filters (soaked for 30 minutes in either 5% (w/v) Casamino acids or 0.2 M Na_2HPO_4 - NaH_2PO_4 buffer (pH 7), as appropriate). The filters were washed immediately with 2 ml of ice-cold 5% (w/v) trichloroacetic acid and numbered in pencil for identification. Incorporation of label into protein, DNA or RNA was then determined as described by GRIFFITHS[18]. For incorporation of label into phospholipids, 0.1 ml samples were removed from the culture vessel at intervals and added to 4 ml of cold 2:1 mixture of chloroform - methanol in stoppered vials. The samples were vigorously mixed and stored at 4°C for later analysis. Each extract was washed by the method of JACOBSON and YATVIN[14] with 3 × 0.8 ml of a solution containing chloroform - methanol - aqueous 0.02% calcium chloride in proportions, by volume, of 3 : 48 : 47. The wash solution was added to the extracts and the mixture shaken vigorously. After separation of the phases, the upper aqueous phase was removed and discarded. From each washed extract, two 1 ml samples were dispensed into glass scintillation vials. The samples were dried on a hot plate and counted in 10 ml of scintillation fluid (4 g PPO/liter of toluene) in a Beckman LS-133 liquid scintillation counter.

Microcolonies on Cellophane Films

The methods of ALPER[15] and BROWN and GILLIES[16] were used, except that the cellophane films were 1 cm^2 and stained on slides with 1% methylene blue.

Synchronous Growth of *K. pneumoniae*

From a stock culture of *K. pneumoniae* stored at −70°C, an inoculum was added to 3 ml of HRP and the cells grown at 37°C overnight. A subculture was then made in 7 ml HRP in a 250-ml flask and incubated at 37°C for 2 hours, with stirring under 5% CO_2 in air (100 ml/minute). The culture (10 ml) was centrifuged for 10 minutes at 1,500 × *g* in a warm 15-ml centrifuge tube. Careful control of the temperature at 37°C appeared to be essential throughout this procedure in order to maintain maximum viability subsequently. The centrifuge was decelerated very slowly. From the top of the tube, 0.1 ml of the suspension was removed using a warm syringe and needle and then added to 0.9 ml HRP under CO_2/air in a 37°C water bath. After the cell density had been determined nephelometrically using the top 2 ml of the centrifuged culture, an inoculum from the diluted suspension was added to give 10^4 cells per ml in 3 ml HRP in a jacketed vessel at 37°C. The culture was stirred and gassed with 5% CO_2 in air (100 ml/minute). Sc^{3+}-Enterochelin (40 μM) was added to give a final concentration of 0.4 μM. Samples (0.1 ml) were removed at 5-minute intervals, added to 0.9 ml broth saline and homogenized for 30 seconds in an MSE microhomogenizer. From these, 0.1 ml volumes were plated on nutrient agar and the viable count determined after incubation at 37°C overnight.

Results

Effect of Sc^{3+}-Enterochelin on Macromolecular Synthesis

Macromolecular synthesis in bacteria was studied by measuring the incorporation of ^{14}C-amino acids into protein and [^{32}P]orthophosphate into DNA, RNA and phospholipid. Initially, synthesis was studied in a medium consisting of conalbumin and 2% TSB, with amino acids, phosphate and other essential nutrients at levels similar to those in serum, since there is evidence of good incorporation of

Fig. 1. Effect of 10 μM Sc^{3+}-enterochelin on the incorporation of [^{32}P]orthophosphate into phospholipid, DNA and RNA during growth at 37°C of *K. pneumoniae* in HRP.

○ Control; △ in presence of Sc^{3+}-enterochelin from 2 hours (arrow). Effect on growth is as shown in Fig. 2.

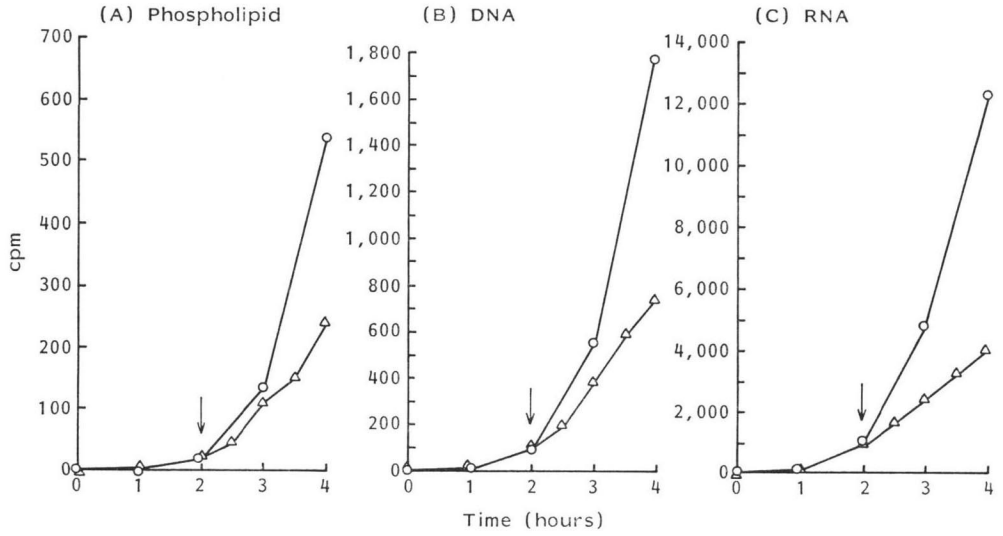
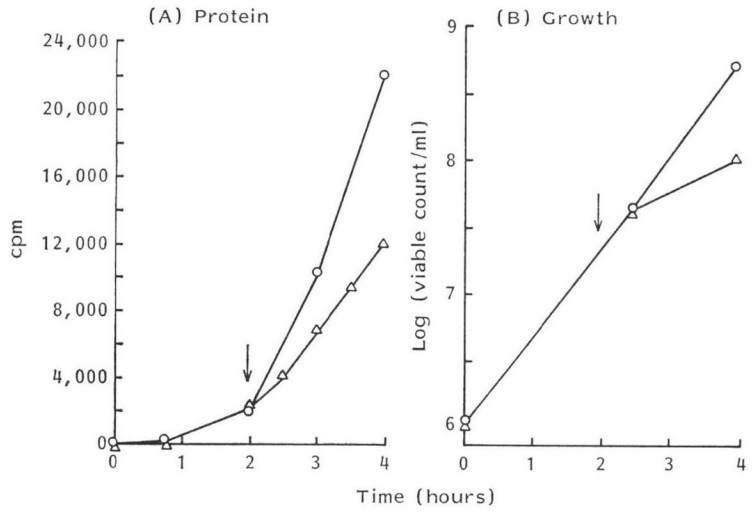


Fig. 2. Effect of 10 μM Sc^{3+}-enterochelin on the incorporation of ^{14}C-amino acids into protein (A) during growth (B) at 37°C of *K. pneumoniae* in HRP.

○ Control; △ in presence of Sc^{3+}-enterochelin from 2 hours (arrow).



the label and also good growth in serum[13]. Sc^{3+}-Enterochelin (10 μM) inhibited the growth of iron-deficient *E. coli* K12 within about one hour in this medium. However, the effects on macromolecular synthesis were neither clear nor reproducible.

As a comparison, therefore, the effect of Sc^{3+}-enterochelin on macromolecular synthesis was studied in a system in which the complex was known to have a clearly defined antibacterial effect[10]. Thus, on addition of Sc^{3+}-enterochelin (10 μM) during growth of *K. pneumoniae* in HRP, RNA synthesis was inhibited immediately, followed by inhibition of protein synthesis and growth after about 30-minute incubation (Figs. 1 and 2). DNA synthesis was inhibited following incubation for about 45 minutes and phospholipid synthesis after about one hour. In no case was synthesis completely stopped by the complex, but the rate of synthesis was reduced.

On addition of 10 μM Sc^{3+}-enterochelin during growth of *E. coli* O18 (*colV*$^+$) in HRP, the order of inhibition was RNA synthesis, protein synthesis and growth after about 30 minutes, followed very closely by DNA and then phospholipid synthesis within the next 30 minutes (Figs. 3 and 4). RNA synthesis may be affected first since further incubation resulted in loss of label from this fraction. Growth was also completely inhibited within about one hour and was then followed by cell death. Although the rate of synthesis of the other components was continually reduced by Sc^{3+}-enterochelin, no label was lost from any of these fractions.

Since Sc^{3+}-enterochelin is less effective in synthetic medium than in rabbit plasma, there may be some component in plasma, such as antibody, which acts synergistically with the scandium complex on the cells. However, the addition of specific antiserum[4] (0.2%) did not affect inhibition by Sc^{3+}-enterochelin (10 μM) of either growth or incorporation of [^{3}H]uracil into RNA in *E. coli* O111 in TSB (data not shown).

2,4-Dinitrophenol (DNP) (4 mM) protected *E. coli* O18 (*colV*$^+$) from killing by Sc^{3+}-enterochelin

Fig. 3. Effect of 10 μM Sc^{3+}-enterochelin on the incorporation of [^{32}P]orthophosphate into phospholipid, DNA and RNA during growth at 37°C of *E. coli* O18 (*colV*$^+$) in HRP.
○ Control; △ in presence of Sc^{3+}-enterochelin from 2 hours (arrow). Effect on growth is as shown in Fig. 4.

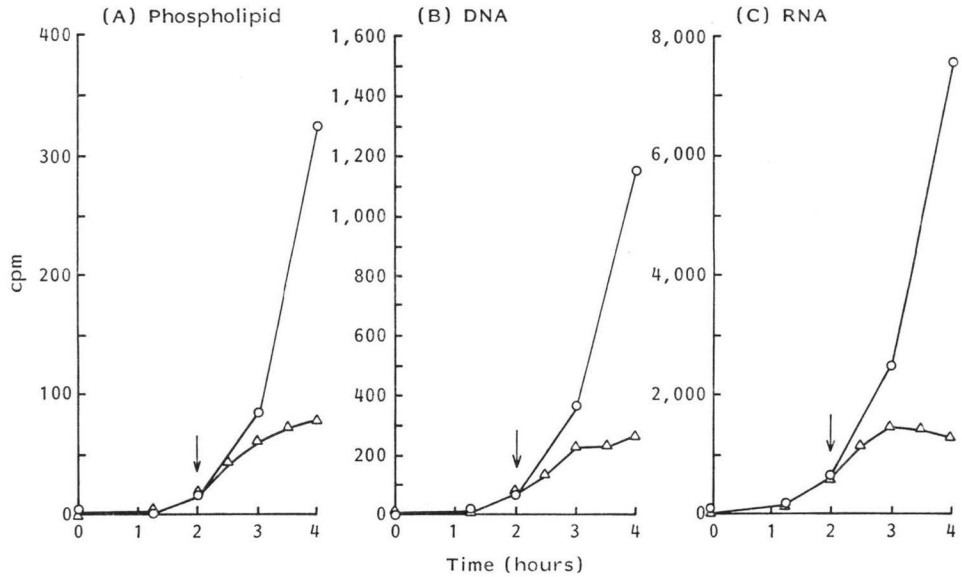


Fig. 4. Effect of 10 μM Sc^{3+}-enterochelin on the incorporation of ^{14}C-amino acids into protein (A) during growth (B) at 37°C of *E. coli* O18 (*colV*$^+$) in HRP.

○ Control; △ in presence of Sc^{3+}-enterochelin from 2 hours (arrow).

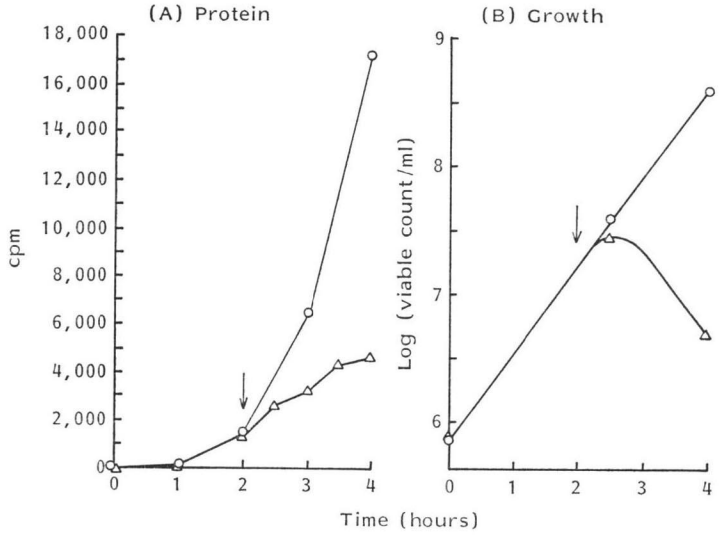
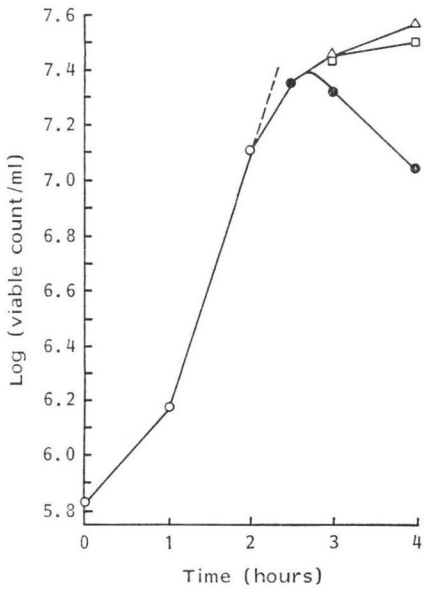


Fig. 5. Growth of *E. coli* O18 (*colV*$^+$) in heat-inactivated rabbit serum in presence of 10 μM Sc^{3+}-enterochelin from 2 hours (●) and DNP (4 mM) from 2 hours 5 minutes (△) or 2 hours 30 minutes (□).



(10 μM) in heat-inactivated rabbit serum, when the uncoupler was added 5 minutes after the scandium complex or at 30-minute, just before the onset of cell death (Fig. 5). DNP also reduced

Table 1. Formation of microcolonies following treatment of *E. coli* K12 in TSB and *K. pneumoniae* in HRP with 10 μM Sc^{3+}-enterochelin for 2 hours.

Numbers in brackets represent percentage of total counts.

	Single cells	Micro-colonies	Total
E. coli K12			
Control	220 (2.2)	9,780 (97.8)	10,000
Treated with Sc^{3+}-entero-chelin	399 (8.0)	4,601 (92.0)	5,000
K. pneumoniae			
Control	4 (2.0)	194 (98.0)	198
Treated with Sc^{3+}-entero-chelin	9 (12.0)	66 (88.0)	75

Following overnight growth at 37°C in TSB from cultures stored at −70°C, *E. coli* K12 was cultured in TSB (containing 1 mg conalbumin per ml and 0.6% $NaHCO_3$), *K. pneumoniae* was grown in HRP giving 10^7 and 2×10^4 cells/ml respectively. The cultures were incubated at 37°C, with stirring under 5% CO_2 in air (100 ml/minute). At 2-hour, 0.2 mM Sc^{3+}-enterochelin was added to give a final concentration of 10 μM Sc^{3+}-enterochelin in the culture, and incubation continued for a further 2 hours. Samples from the *E. coli* K12 culture were diluted 1/10 and those from *K. pneumoniae* diluted 1/100, and 5 μl spread on cellophane films on nutrient agar plates. Following incubation at 37°C for 4 hours, the cellophane films were mounted and stained and microcolonies observed microscopically (see Methods section).

degradation of RNA by Sc^{3+}-enterochelin in *E. coli* O18 in HRP (data not shown).

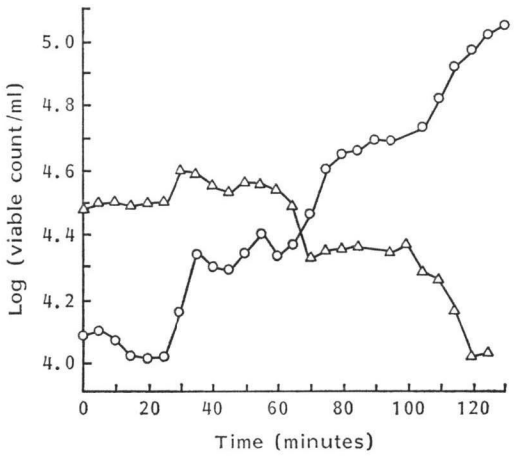
Sc^{3+}-Enterochelin and Cell Viability

Sc^{3+}-Enterochelin (2 μM) induces complete bacteriostasis of *K. pneumoniae* growing in HRP[10]. When added at a concentration of 10 μM to *E. coli* K12 growing in TSB, a 50% reduction of colony forming units ensued. However, microscopic examination of Gram stained films revealed no obvious morphological changes in these treated cells. In order to investigate whether non-viable cells were being produced, treated or untreated cells were grown to the microcolony stage on cellophane films on agar and examined microscopically[15,16] (Table 1). Both in the case of *E. coli* K12 and *K. pneumoniae*, there was an approximately five-fold increase in the percentage of single cells which failed to form microcolonies. Single cells remained as such and all microcolonies gave rise to visible macrocolonies when the plates were incubated at 37°C overnight (data not shown). Hence, treatment with Sc^{3+}-enterochelin results in the production of some non-viable cells.

However, in the case of *K. pneumoniae* when all the microcolonies and single cells present on the cellophane film were counted, there were approximately 60% less microcolonies on the treated as compared to the control films and the difference was not accounted for by a corresponding increase in the number of single cells on the treated films (Table 1). Hence, it appears that when treated with Sc^{3+}-enterochelin, *K. pneumoniae* grows or divides more slowly than untreated cells.

In order to establish whether the complex affected cell growth or division, the effect of Sc^{3+}-enterochelin on synchronously growing *K. pneumoniae* was examined[17]. Initial experiments using Percoll gradients to fractionate the cells were unsatisfactory since many cells were killed on subsequent exposure to heated rabbit plasma. It was found, however, that centrifugation in HRP itself results in a distribution of cells which qualitatively resembled that described by DWEK *et al.*[17]. Thus, 52% of the cells in the fractionated sample had lengths of 0.6 to 1.0 μm whilst only 33% of the starting material fell in this range. It seems quite clear that the effects of the scandium complex could be detected only at the time of division (Fig. 6). There was a slight increase in the number of cells at the onset of the first division cycle, and then killing during the second and third cycles of division. When Sc^{3+}-enterochelin was added just before the onset or during the first division cycle, there was no detectable change in cell numbers at the next expected time of cell division but cells were killed at the time of the following (third) cycle of cell division (data not shown).

Fig. 6. Effect of 0.4 μM Sc^{3+}-enterochelin on synchronously growing *K. pneumoniae* in HRP.
○ Control; △ treated with Sc^{3+}-enterochelin from time zero.



Discussion

During earlier work[12], it was found that Sc^{3+}-enterochelin inhibited Fe^{3+}-enterochelin uptake by bacteria in a competitive manner. It was also shown that the Sc^{3+}-complex enters the bacterial cell where it is degraded by enterochelin esterase. The Sc^{3+} ion so liberated could then exert a toxic effect. The present results demonstrate that Sc^{3+}-enterochelin inhibits the synthesis of RNA, protein, DNA and phospholipid, in that order in both *K. pneumoniae* and *E. coli* O18 (*colV*$^+$) growing in HRP. In the latter organism, both a loss of label from the RNA fraction and cell death occurred approximately one hour after the addition of the complex. Both the nature and order of these events are similar to those which have been observed by other workers whilst studying the biochemical effects of antiserum and complement on *Pasteurella septica*[18] and *E. coli* O111[19]. In the case of *P. septica*, it was shown that not only did the degradation of RNA occur before it left the cell but even before it was released from the ribosomal particles. The extensive loss of RNA which follows the addition of Sc^{3+}-enterochelin to *E. coli* O18 (Fig. 3) may also arise from ribosomal degradation. Since antibody to the O-antigen of *E. coli* O111 initiates complement mediated killing of the organism[20] and also interferes with the release of enterochelin from the cell[21], it was considered possible that this same antibody could also be involved in the events outlined above. In fact, no degradation of RNA occurred in *E. coli* O111 exposed to conalbumin and Sc^{3+}-enterochelin in TSB in either the presence or absence of 0.2% antiserum. It was concluded therefore that some serum component other than antibody is required for the induction of RNA degradation in the presence of Sc^{3+}-enterochelin.

Another striking feature which appears to be common to the action of both antibody plus complement[22] and the Sc^{3+}-complex of enterochelin is the fact that both RNA degradation and killing are prevented by the early addition of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. In the case of the antibody — complement system however, addition of DNP just before the onset of RNA degradation was no longer effective, suggesting that only early events in the process depend upon oxidative metabolism. DNP protected *E. coli* O18 from killing by Sc^{3+}-enterochelin even when the uncoupler was added 30 minutes after the complex, just before the onset of cell death. Since the Sc^{3+}-complex is taken up rapidly and continuously, by 30 minutes there should have been a considerable intracellular concentration of Sc^{3+} [12]. These observations suggest that Sc^{3+} may undergo turnover during its action on the cell thus necessitating a continuous supply of both the ion and the energy for its processing.

With these observations in mind, the work on the effect of Sc^{3+}-enterochelin on bacterial growth and cell division can now be considered. The cellophane film experiments suggest that treatment of either *E. coli* or *K. pneumoniae* with Sc^{3+}-enterochelin results in the formation of non-viable cells. When the complex was added to synchronously growing *K. pneumoniae* in heated rabbit serum, both bactericidal and bacteriostatic effects can be detected, depending on the time of addition in relation to the next cell division (Fig. 6). The occurrence of a bactericidal event at the expected time of cell division was particularly striking. This, together with the lack of any obvious changes in morphology, suggest that the intervening period of cell growth may be relatively undisturbed. The duplication of *E. coli* can be described in terms of the I, C, D concept[23], representing initiation, chromosome replication and cell division respectively. In the present case based on this model, chromosome replication time is assumed to be approximately equal to the cell doubling time[24] of 35 minutes. In turn, this implies that the initiation process extends well into the previous division cycle. It could be, therefore, that when added at the begining of the experiment (0 minute, Fig. 6) the complex partially inhibits initiation of chromosome replication and hence cell division at the end of the first cycle. However, when it is added just before (25 minutes) or during cell division (35 minutes) *i.e.* earlier in the initiation process, the Sc^{3+}-complex allows division to progress through the first cycle but completely inhibits initiation leading to the second cycle. The possible relationship between these temporal events and the degradation of RNA remains to be determined.

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