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Stages in Colicin K Action, as Revealed by the Action of Trypsin

(irreversible/protein synthesis/sugar transport/aminoacid uptake/viability)

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ABSTRACT **The effects of trypsin on *Escherichia coli* cells that have been treated with colicins have been examined. By the use of trypsin, it has been possible to demonstrate that the action of several colicins (E1, E2, and K) proceeds through at least two stages. Stage I is a period after colicin adsorption when trypsin can restore colony-forming ability to a colicin-treated cell. Stage I is followed by a period when trypsin is unable to restore colony-forming ability (stage II). The transition between stage I and stage II follows first-order kinetics, with a rate proportional to the number of killing units of colicin adsorbed.**

A quantitative comparison of the effects of colicin K on colony-forming ability and on several cellular processes indicates that colicin damage to these processes occurs in the stage II period of colicin action and is not subject to reversal by the trypsin treatment that restores viability to cells in stage I. The implications of these findings for an understanding of the mode of action of colicins are discussed.

The idea that colicins act from the surface of bacterial cells has arisen from reports that agents that presumably do not penetrate the outer membrane, such as trypsin (1–4) or colicin-specific antiserum (5), can reverse the effects of certain colicins. In the case of colicin K, it has been shown that some of the colicin-produced damage on *Escherichia coli* B cells can be repaired by prolonged exposure of colicin-treated cells to trypsin (1). Other observations indicate that reversibility of the effects of colicin K by trypsin is limited. Wendt (6) has presented data indicating that trypsin completely reverses colicin K effects on colony-forming ability of *E. coli* K-12 only if the colicin treatment is performed at low temperatures (10° or lower). Barbu and his colleagues have proposed that trypsin rescues only those cells that colicin, although it has been adsorbed, has not yet damaged (7–9).

To clarify this issue it is necessary to compare quantitatively the effects of trypsin on the colony-forming ability of colicin-treated cells with the residual level of a colicin-inhibited function at the time of trypsin addition. A close correlation between the level of viability restored by trypsin treatment and the amount of residual function would indicate that trypsin can restore viability only if it acts before the function in question is affected by the colicin. If the activity of the colicin-affected function increases after trypsin addition, true reversal or repair is indicated.

We have investigated the effects of colicin K and of trypsin on the ability of *E. coli* K-12 cells to accumulate thiomethyl-β-D-galactoside (TMGal) and leucine, and on their ability to incorporate leucine into protein. The results indicate that treatment with trypsin for a limited period of time rescues a class of cells in which these processes have not yet been affected by the colicin.

We have also found that once cells adsorb colicin E1, E2, or K, their rescuability by trypsin declines exponentially with time. The results indicate that each killing unit of colicin adsorbed has a constant probability per unit time of causing a cellular damage that leads to loss of colony-forming ability and cannot be reversed by trypsin treatment.

MATERIALS AND METHODS

Chemicals. Chloramphenicol was obtained from Parke, Davis and Co., Detroit, Mich. [*1*-^{14}C]TMGal and [*U*-^{14}C]-leucine were obtained from New England Nuclear Corp., Boston, Mass. Trypsin (1× crystallized; salt-free) and soybean trypsin inhibitor were purchased from Worthington Biochemicals, Freehold, N.J. Trypsin solutions were prepared fresh daily. All other chemicals used were of reagent grade.

Bacterial Strain, Media, and Growth Conditions. The bacterial strain used throughout this study was LA319 [F$^-$ *lac*$^-$ (*i*$^-$*z*$^-$*y*$^+$) *pro*$^-$ *str*^{r}] from our laboratory collection. LA319 was routinely grown at 37° in Ozeki medium base (10), supplemented with D,L-lactate (0.5%), L-proline (100 μg/ml), and thiamine (3 μM), to a cell density of 100 Klett units (no. 54 filter). One Klett unit corresponds to about 5 × 10^6 cells/ml. Cultures (20 ml) were incubated in 300-ml flasks shaken in a New Brunswick Gyratory Shaker. The cultures were harvested at room temperature, washed once with Ozeki medium, and resuspended in this medium containing lactate and thiamine, at a density of about 2 × 10^9 cells/ml. The washed cultures were stored on ice until used.

Viability assays were performed by plating appropriate dilutions of cultures on LB agar (11), pH 7.0. Dilutions for plating were done in Ozeki medium at room temperature.

Colicin Preparation. Colicin K, prepared by the method of Fields and Luria (12), was supplied by Dr. H. Unsöld. The number of killing units in the colicin preparation was calculated from the equation $S/S_0 = e^{-m}$, where m is the multiplicity of killing units and S/S_0 is the survival ratio. Multiplicities, where expressed, were calculated from platings done after a 5-min incubation of cells with colicin at 27°.

TMGal Accumulation. Cells (5 × 10^8/ml) were incubated in Ozeki medium supplemented with lactate and thiamine for 5 min at 27°. [*1*-^{14}C]TMGal (100 μM; 2.5 Ci/mol) was then added and, at intervals, 0.2-ml aliquots were removed and the cells were collected on nitrocellulose filters

(pore size 0.45 μm; Matheson–Higgins, Inc., Woburn, Mass.). After a 5.0-ml wash with Ozeki medium at room temperature, the filters were glued to planchets. Radioactivity was measured in a Nuclear–Chicago gas-flow counter.

Leucine Incorporation. Cells (5 × 10^8/ml) were incubated in Ozeki medium supplemented with lactate, thiamine, and L-proline (100 μg/ml) for 10 min at 27°. [*U*-^{14}C]Leucine (100 μM; 10 Ci/mol) was then added and, at various time intervals, 0.2-ml aliquots were removed to tubes containing 1.0 ml of 10% trichloroacetic acid in ice. The precipitates were collected on nitrocellulose filters and washed twice with 5.0-ml portions of cold 5% trichloroacetic acid; the filters were glued to planchets and counted.

RESULTS

Initially, we investigated the effects of trypsin on the loss of viability that results from treatment of an *E. coli* culture with colicin K. Culture samples were incubated with different amounts of colicin K for 5 min, and were then diluted 10-fold to stop or reduce further colicin adsorption. At various times after dilution, aliquots from the cultures were either plated directly to measure survival or were incubated with trypsin for 15 min before plating. An increase in the interval between adsorption of colicin K and addition of trypsin resulted in an exponential decrease in the proportion of K-treated cells made viable by trypsin (Fig. 1). Similar experiments with colicins E1 and E2 produced results fully compatible with those with colicin K. These findings indicate that after colicin adsorption, a period exists during which addition of trypsin can restore colony-forming ability to a colicin-treated cell. We designate this trypsin-rescuable period as stage I of colicin action, and the period that follows, when trypsin cannot restore viability, as stage II.

The results in Fig. 1 also show that the half-life of stage I is inversely proportional to colicin K multiplicity. At a multiplicity near 1, the half-life of stage I for K was about 20 min

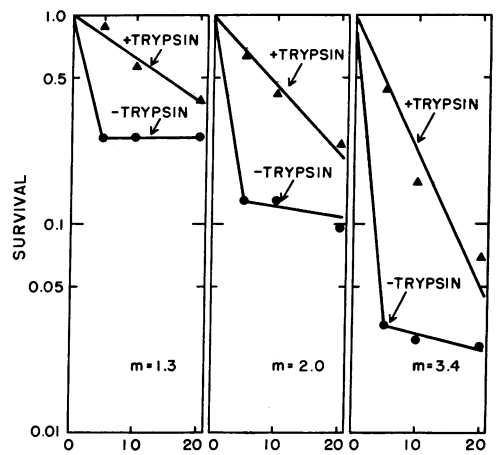

FIG. 1. Effect of trypsin on colicin K-induced loss of viability. LA319 cultures (5 × 10^8/ml) were treated with the concentration of colicin K shown for 5 min at 27°, and were then diluted 10-fold. Colicin K concentration is expressed as multiplicity (*m*), determined as described in *Methods*. At the times after colicin addition indicated, aliquots were either diluted and plated or incubated with trypsin (500 μg/ml, 15 min, 27°) before plating. Survival was determined after overnight incubation of the plates at 37°.

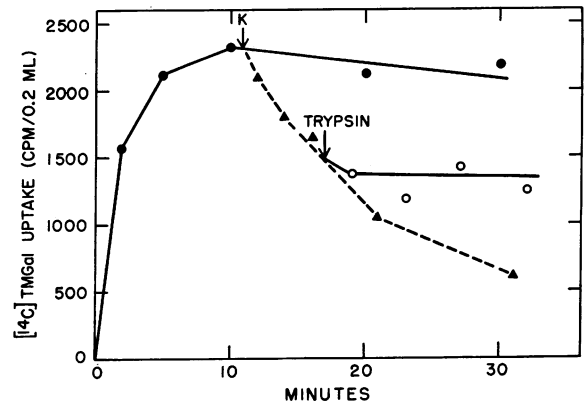

FIG. 2. Effect of trypsin on colicin K-induced TMGal efflux. Uptake of [*1* − ^{14}C]TMGal at 27° was measured. At the indicated time, a portion of this culture was added to a tube containing colicin K. 5 min after colicin K addition, a portion of the colicin-treated culture was added to a third tube containing sufficient trypsin solution to yield a final concentration of 500 μg/ml. Survival measured immediately before trypsin addition was 21%; survival measured after a 15-min incubation with trypsin was 57%.

at 27°. An increase of the amount of colicin 2- or 4-fold decreased proportionately the stage I half-life to about 10 or 5 min, respectively. These results can be explained by the assumption that each killing unit of colicin adsorbed has a constant probability per unit time of undergoing the transition from stage I to stage II. Once this transition occurs, trypsin can no longer restore colony-forming ability.

To determine whether any functional damage to the cell has occurred in stage I of colicin K action, it is necessary to investigate the effects of trypsin on the inhibition of certain cellular functions by colicin K. If such functions can be inhibited by colicin K in stage I, trypsin addition should overcome the inhibition and restore some activity to the function measured. Therefore, we tested the ability of trypsin to reverse colicin K inhibition of the accumulation of β-galactosides (12) and of certain amino acids (13), and its inhibition of protein synthesis (14). The trypsin concentration used was 500 μg/ml, and the incubation time with trypsin was 15 min at 27°. Control tests showed that survival of K-treated cells was maximal by 2 min after trypsin addition. Variation of the trypsin concentration between 200 and 1000 μg/ml did not significantly affect the results.

Colicin K, when added to a cell culture that has reached a steady-state level of TMGal accumulation, promptly causes a net efflux of the galactoside (Fig. 2). Addition of trypsin stopped the TMGal efflux, but did not result in TMGal uptake to the original steady-state level. Instead, a new steady-state level was established, whose ratio to the control level corresponded closely to the fraction of cells that could still form colonies. When TMGal retention and colony-forming ability were followed as a function of the time of trypsin addition to the colicin-treated culture, it was found that the amount of TMGal retained and the colony-forming ability both decreased exponentially, and at the same rate (Fig. 3).

The rate of colicin K inhibition of TMGal uptake is shown in Fig. 4 (curve *A*). At the colicin K multiplicity used, TMGal uptake continued at a decreasing rate for several minutes after colicin K addition. Only after the cells had been exposed

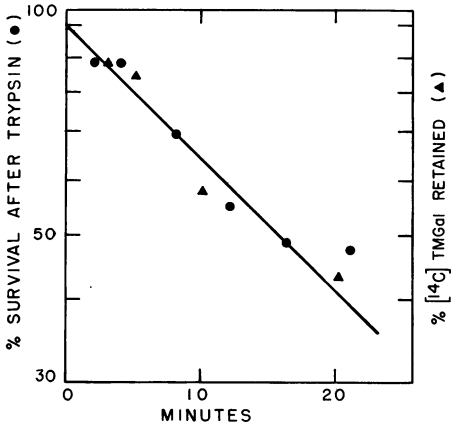

FIG. 3. Ability of colicin K-treated cells to form colonies and retain TMGal when trypsin is added at various times after colicin. Cells were incubated with [1 − ^{14}C]TMGal at 27° until a steady-state level of accumulation was reached (10 min); colicin K was then added. At the indicated times, portions of this culture were either treated with trypsin and plated for survival, or used to measure TMGal retention. Survival without trypsin treatment, measured 5.5 min after colicin K addition, was 28%.

to the colicin for 4 min was net loss of TMGal observed. Addition of trypsin stopped the further progression of colicin K effects on TMGal uptake, and resulted in the establishment of a steady-state level (curve *B*). This steady-state level was similar to that established by cells that had been treated with the same amount of colicin K for an equivalent period of time, and then exposed to trypsin before TMGal uptake was measured (curve *C*). If colicin K had an inhibitory effect on TMGal uptake in stage I, one would have expected some recovery in TMGal uptake after trypsin addition. No such recovery was evident. Moreover, the ratios of the steady-state level (40%) were quite similar to the fraction of colony-forming units that survived the colicin K-trypsin treatment (38%). This finding suggests that the colicin K effects on TMGal uptake occurred in stage II, coincident with the event that led to viability loss, and not during stage I.

From these results we conclude that during stage I of colicin K interaction with cells, the colicin has no detectable effect on the ability of the cells to accumulate TMGal. Rather, it appears that trypsin prevents colicin K from inhibiting TMGal uptake in cells by preventing the transition from stage I to stage II.

The effect of trypsin on colicin K-induced leucine efflux was similar to that obtained with TMGal; addition of trypsin during efflux resulted in the establishment of a steady-state level of leucine accumulation that was proportional to the residual fraction of viable cells (Table 1).

The effects of colicin K on leucine incorporation into protein are shown in Fig. 3. Colicin K caused a progressive inhibition in the ability of a culture to incorporate leucine into protein. At the multiplicity used in the experiment presented, leucine incorporation into protein was completely inhibited only after the cells had been in contact with colicin K for 5–6 min. Addition of trypsin 4 min after colicin K prevented complete expression of the inhibitory potential of this colicin and resulted in a sustained residual rate of protein synthesis about 20% that of the control in this experiment. If trypsin addition was delayed until colicin K inhibition of protein synthesis was essentially complete, little if any restorative effect due to trypsin was noted up to 60 min.

These results are consistent with the idea that trypsin as used in these experiments is unable to reverse colicin K inhibition of either leucine uptake or leucine incorporation into protein once that inhibition has occurred. As with TMGal uptake, it appears that trypsin prevents colicin K inhibition of each of these functions in cells that, at the time of trypsin addition, have not yet entered stage II of colicin K actions. The close correlation between the rates of loss of viability, of capacity to accumulate TMGal or leucine, and of protein synthesizing activity suggests that some step in colicin K action results in all of these cellular functions being damaged within a relatively short period of time.

DISCUSSION

Two main conclusions emerge from this study. The first is that trypsin, acting for several minutes on colicin K-treated *E. coli* K-12, restores colony-forming ability only to those cells that have not been functionally damaged by the colicin. This conclusion is based on two findings: (*i*) once a function has been inhibited by colicin K, the inhibition is not relieved to any measurable degree by the trypsin treatment; and (*ii*) the fractional loss of an affected function due to colicin K corresponds to the fractional loss of colony-forming units. We assume that the residual level of a function after colicin K-trypsin treatment represents the fraction of cells retaining normal levels of that particular function.

These results appear to conflict with those of Nomura and Nakamura (1), who, using *E. coli* B, observed recovery of protein and nucleic acid synthesis after trypsin treatment of cells damaged in these functions by colicin K. Apart from possible differences between *E. coli* strains B and K-12 and growth conditions, an explanation for this discrepancy may lie in the different conditions of trypsin treatment used in the two studies. While we have restricted the trypsin treatment to relatively short times, Nomura and Nakamora (1) found

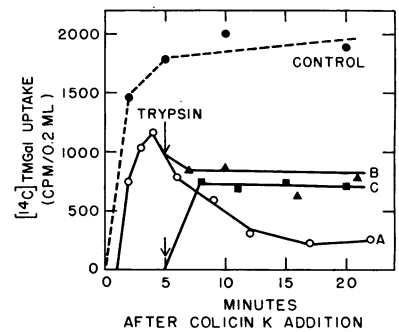

FIG. 4. Effect of colicin K and subsequent trypsin addition on TMGal uptake. (*A*) Cells were incubated with colicin K for 1 min; [1 − ^{14}C]TMGal was then added and uptake was measured. Survival measured 5.5 min after colicin K addition was 5%. (*B*) At the time indicated, a portion of the cells from (*A*) was added to a tube containing sufficient trypsin solution to yield a final concentration of 500 μg/ml. Survival after a 15-min incubation with trypsin was 38%. (*C*) A portion of the cells in (*A*) was exposed to colicin K for 5 min, then trypsin and [1 − ^{14}C]TMGal were simultaneously added. Survival measured immediately before trypsin addition was 5%; survival measured after a 15-min incubation with trypsin was 38%.

significant restoration of inhibited functions only after long periods of incubation with trypsin. A prerequisite for repair or reversal of colicin K-induced functional damage may be a prolonged exposure to trypsin, possibly to permit inactivation of the least accessible colicin molecules. Additional studies are being carried out to clarify this situation.

Nevertheless, the trypsin treatment used in our experiments has revealed that, in the early stages of colicin K action, there are two major classes of colicin-complexed cells—those that at a given moment have not yet manifested physiological damage and can be rescued quantitatively by the trypsin treatment described and those that have sustained functional damage and cannot be rescued by the same trypsin treatment.

These findings agree with the proposal of Barbu and his colleagues (7–9), and substantiate the hypothesis that the colicin–cell interaction goes through at least two successive stages: state I, in which the colicin adsorbs to the cell but does not cause any physiological damage; and stage II, in which the physiological damage occurs. Most adsorbed colicin molecules never reach stage II in their action. Those that do are the "killing units," or potentially damaging colicin molecules. Trypsin reduces the number of potentially damaging molecules on the cell to those that are already in stage II at the time of trypsin addition. Dandeu *et al.* (7) have suggested that the colicin–cell interaction eventually results in the colicin-treated cell reaching a critical state that is unaffected by trypsin. This critical state would be the equivalent of what we have called stage II. Although it has been reported that repair of colicin E2-induced DNA damage follows early addition of trypsin (15), it has not been established that the repair results in restored viability to the E2-treated cells. This point is currently being investigated.

The second conclusion is that the transition from stage I to stage II follows first-order kinetics, with a rate proportional to the mean number of potentially damaging molecules per cell. Each potentially damaging molecule, therefore, has a constant probability per unit time of undergoing the stage I–stage II transition, and thereby causing cell death.

TABLE 1. *Correlation between colony-forming ability and capacity of colicin K-treated cells to accumulate leucine after trypsin treatment*

Minutes with colicin K	Percent survival		[*U*-^{14}C]Leucine accumulated after trypsin treatment	
	− Trypsin	+ Trypsin	cpm/0.2 ml at steady state	Percent of control steady-state level
0	100	96	1900	100
10	4.4	31.4	750	34
20	3.7	11.3	420	16

Cells were incubated with colicin K at 27° for the indicated times. Portions of the colicin-treated cells were then added to tubes containing sufficient trypsin solution to yield a final concentration of 500 μg/ml. After 15 min, the trypsin was inactivated by the addition of soybean trypsin inhibitor (250 μg/ml final concentration). Control cells received no colicin K, but were carried through the trypsin incubation. [*U*-^{14}C]Leucine was then added, and leucine uptake was measured.

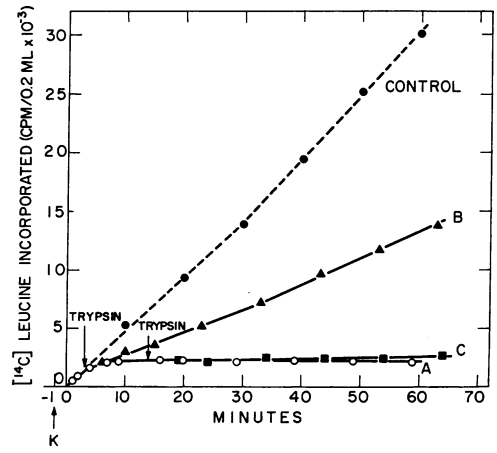

FIG. 5. Effect of colicin K plus trypsin on protein synthesis. The rate of incorporation of [*U* − ^{14}C]leucine into protein was determined as described in *Methods*. (*A*) Colicin K was added to cells 1 min before [*U* − ^{14}C]leucine. Survival at 5.5 min after colicin addition was 2%. At 4 min (*B*) or 15 min (*C*) after addition of colicin K, portions of the colicin-treated cells were transferred to tubes containing trypsin, to yield a final concentration of 500 μg/ml. Survival measured after a 15-min incubation with trypsin was 63% (*B*) and 10% (*C*).

The similar responses to trypsin obtained with colicins K, E1, and E2 suggest that the stage I–stage II transition may be a common feature in the action of many colicins. That the same mechanism brings about this transition for different colicins is suggested by some data. Low temperatures block the stage I–stage II transition of colicins E1 and K (Plate, C., manuscript in preparation). Cells treated with colicins E1 or K (unpublished observations), E2 (refs. 2 and 3; Saxe, L., personal communication), or E3 (16) in the presence of 2,4-dinitrophenol or *p*-trifluoro-methoxycarbonylcyanide phenylhydrazone do not lose trypsin rescuability with time, a suggestion that the stage I–stage II transition is blocked by these compounds.

Little can definitively be said about either of the proposed stages I and II. Stage I probably represents the interval of the initial interaction between a colicin molecule and its specific receptor located in the *E. coli* outer membrane (17). The spatial relationship between the bound colicin molecule in stage I and the components of the outer membrane must be such that at least certain critical regions of the colicin molecule remain accessible to the action of trypsin.

Stage II in colicin action results in cellular damage and loss of viability. The change in response to trypsin in stage II may mean either that the colicin is no longer readily accessible to trypsin, or that once stage II is reached it is maintained even in the absence of the intact colicin molecule. That the damage sustained in stage II of colicin K action is to the cytoplasmic membrane is suggested by the facts that certain transport systems are affected and that isolated membrane vesicles, as well as whole cells, respond to colicin K (18). Since a single killing unit of colicin K in stage II can produce this damage, and since several killing units do not appear to act cooperatively (19), some "spreading" mechanism must exist that distributes the initial effect to all of the systems ultimately affected. Such a mechanism could involve either conforma-

tional changes (20, 21) or chemical modifications of the membrane. The latter possibility has some support in the phospholipid modifications observed after colicin treatment (22). In the case of colicin K, the colicin itself may act enzymatically on the membrane, or it might activate or relocate one or more cellular enzymes, such as the phospholipase A1 recently reported to be located in the *E. coli* outer membrane (23). Recent evidence on the mode of action of colicins E2 (24) and E3 (25, 26) provides grounds for consideration of each of these possibilities for colicin K.

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