THE SURVIVAL OF COLICINE-SENSITIVE BACTERIA WHEN GROWN WITH COLICINE-PRODUCING BACTERIA

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THE production of colicines is a widespread property of the enterobacteria, but it is not known whether this confers an ecological advantage on the producing organisms or is of significance in infections of the bowel, because colicine-producing strains may be isolated from the faeces at the same time as other strains that are sensitive to the colicines produced (Halbert, 1948*b* and *c*; Fredericq and Betz-Bareau, 1950; Vieu, 1964).

These observations and others have been thought to indicate that colicines formed in the intestine are not present in adequate concentration to eliminate sensitive organisms from the same environment (Depoux and Chabbert, 1953; Hamon and Peron, 1959). In contrast, several workers have noted an increase in the number of colicinogenic bacteria isolated from faeces during recovery from certain enteric infections (Halbert, 1948*a*; Fredericq *et al.*, 1949) and have suggested that the presence of colicinogenic bacteria may assist in recovery. Emslie-Smith (1961, 1965) and Branche *et al.* (1963) have claimed that the ability of some strains of *Escherichia coli* to maintain themselves in the intestine may be dependent on their production of colicines.

Colicine-sensitive strains may give rise to mutants, known as colicine-tolerant mutants, which may be either colicine-sensitive or colicine-resistant depending on the conditions of the test (Clowes, 1965; Hill and Holland, 1967; Holland, 1967; Nagel de Zwaig and Luria, 1967; Nomura, 1967; Nomura and Witten, 1967; Holland, 1968), and the appearance of such mutants might account for the survival of colicine-sensitive strains in the intestine.

We investigated the survival of colicine-sensitive bacteria when grown in mixed culture with colicine-producing bacteria, and observed the appearance in some cultures of another type of colicine-refractory organism that was able to survive but not to multiply in the presence of large amounts of colicine.

MATERIALS AND METHODS

Media. The liquid culture medium was Lemco broth containing 10 g of Oxoid Lab Lemco, 10 g of Oxoid Bacteriological Peptone and 5 g of sodium chloride per l of distilled water, adjusted to pH 7.5. For the isolation of colonies this medium was solidified with Oxoid Agar no. 3 at a concentration of 14 g per l. Streptomycin agar was prepared in the same way, but contained streptomycin at a final concentration of 250 µg per ml.

Colicine-producing strains. Three colicinogenic strains were used: *Escherichia coli* no. CA18, CA42 and CA38; these strains produce colicines B, E₂ and E₃ respectively, ferment lactose and are streptomycin-sensitive.

Colicine-sensitive strains. Two colicine-sensitive strains were used: *E. coli* strain B/TKM, which is sensitive to all colicines (the "universal indicator strain"), and *E. coli* no. C600/21,

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which is sensitive to colicines B, E₂ and E₃, resistant to streptomycin and does not ferment lactose.

Colicine-resistant strains. Colicine-resistant mutants were derived from the two sensitive strains as follows: 0.5 ml of a 24-hr culture of the sensitive strain, 2.0 ml of the colicine and 1.0 ml of Lemco broth were mixed and incubated at 37°C for 24 hr. A volume of this culture, usually 0.2 ml, was mixed with 1.0 ml of colicine and 0.1 ml of Lemco broth and incubated for 24 hr. The organisms isolated from this culture were colicine-resistant.

Colicinogenicity and colicine-sensitivity of all strains was determined by the method of Fredericq (1948).

Viable counts. Duplicate serial ten-fold dilutions of each sample were made in chilled isotonic saline up to 10⁻⁶, and 0.1 ml of each dilution was spread on well-dried Lemco agar and streptomycin agar plates. After overnight incubation at 37°C the total viable count was calculated from the colony count on Lemco agar and the sensitive-strain count from the growth on streptomycin agar.

Preparation of cell-free colicines. The colicinogenic strains were grown in Lemco broth under aerobic conditions at 37°C for 48 hr. The cells were deposited and the supernatant fluids sterilised by the addition of chloroform (Senior, Richardson and Emslie-Smith, 1970). Sterile colicine preparations were stored at 4°C until required.

Establishment and examination of mixed cultures. Separate cultures of the colicine-sensitive and colicine-producing strains in 5.0 ml of Lemco broth were incubated for 6 hr at 37°C and then centrifuged at 2500*g* for 15 min. The cells were washed once in chilled isotonic saline and then suspended in 5.0 ml of saline. The optical density of the suspensions was determined in a Gallenkamp photocolourimeter with a 470-nm filter, and the required dilutions were made by the addition of sterile saline. The "primary" mixed culture was established by transferring 1.0 ml of the appropriate dilution of each suspension to 30 ml of Lemco broth in a 150-ml conical flask. The mixed culture was incubated aerobically without agitation at 37°C for 48 hr; 1-ml portions were removed after inoculation and after incubation for 3, 6, 24 and 48 hr. The viable count of each strain in each portion was determined, and 20 isolated colonies from the growth on streptomycin agar were tested for colicine-production and colicine-sensitivity. Each portion was also centrifuged at 2500*g* for 15 min, and the supernatant fluid was examined for free colicine. The deposited cells were suspended in veronal buffer at pH 8.0 containing 250 µg of streptomycin per ml. The cells were deposited by centrifuging at 2500*g* for 15 min, and washed three times in cold veronal buffer. In some experiments the washed cells formed the inoculum for a second mixed culture with the same producing strain. In others the cells were finally suspended in buffer containing 5 mg per ml of trypsin. After 10 min. the cells were again washed three times in cold buffer and then suspended in the original supernatant fluid from the mixed culture sample. The viable count of the cells in this suspension was determined at intervals.

In the case of strain no. C600/21, a second series of mixed cultures was prepared in which the sensitive-strain inoculum consisted of survivors from a "primary" mixed culture that had been exposed to streptomycin, washed and resuspended. In some of these experiments the "secondary" mixed cultures were divided into three equal parts by volume after incubation at 37°C for 6 hr. Each part was incubated for a further 6 hr at either 30°C, 37°C or 42°C. The viable count of strain no. C600/21 in each of these cultures was determined by plating out on streptomycin agar.

The size of the inocula in the mixed-culture experiments (see *Results*) is expressed as the number per ml of each organism in the final mixture, as determined by viable counts at 0 hr.

RESULTS

The survival of the universal indicator, E. coli strain B/TKM, when grown with the colicine-producing strains of E. coli no. CA18, CA38 and CA42

There were no survivors of the universal indicator strain after 6 hr in mixed culture with any of the colicine-producing strains, provided that the initial

inoculum of both strains was approximately equal. With a small inoculum of the sensitive strain (approximately 2×10^3 organisms per ml) and a large inoculum of the producing strain (10^8 organisms per ml), there were no detectable survivors of the sensitive strain after only 3 hours' incubation in mixed culture. On the other hand, with a large inoculum of the sensitive strain (10^8 organisms per ml) and a small number of producing bacteria (10^4 organisms per ml), the sensitive strain multiplied normally up to 6 hr, but thereafter the viable count fell and, after 24 hr, only occasional survivors were detected. In this case the supernatant fluid contained detectable colicine only after 24 hours' incubation, whereas free colicine was present in the samples from all other cultures after 6 hr.

Mutants resistant to all three colicines, derived from strain B/TKM, showed normal growth patterns in mixed culture with each of the colicino-genic strains.

The survival of strain no. C600/21 when grown with the colicine-producing strains, no. CA18, CA38 and CA42

The growth of this sensitive strain in mixed culture with the colicine-B-producing strain no. CA18 showed a similar pattern to the growth of the universal indicator strain in the presence of this producing strain. Except when the inoculum of the sensitive strain was large and the number of producing organisms small, mixed culture of the two strains resulted in the elimination of the sensitive strain within 6 hr. When the size of the inoculum of the sensitive strain was greatly in excess of that of the producing strain, survivors of strain no. C600/21 were found in all mixed culture samples. These survivors were fully colicine sensitive, and no free colicine was detected in the supernatant fluids. Colicine-B-resistant mutants of strain no. C600/21 grew normally in the presence of the colicine-B-producing strain.

In contrast to the above findings, the sensitive strain no. C600/21 was not eliminated during mixed culture with either the colicine-E₂- or colicine-E₃-producing strains (no. CA42 and CA38 respectively). When the size of the inoculum of the sensitive and producing strains was approximately equal, the viable count of the sensitive strain after 3 hours' incubation of the mixed culture was considerably less than the original inoculum, but with prolonged incubation there was no further reduction in the viable count of this strain. The results of a typical mixed culture of strains no. C600/21 and no. CA38 are shown in table I and of no. C600/21 and no. CA42 in table II. With a small inoculum of no. C600/21 (approximately 10^3 organisms per ml) and a large inoculum of either no. CA38 or no. CA42 (10^8 organisms per ml) we were able to detect survivors of the sensitive strain in all samples, but the viable counts were very variable. The addition of cell-free colicine to the samples did not reduce the number of survivors. When the inoculum of no. C600/21 was large (approximately 10^7 organisms per ml) and the numbers of either no. CA38 or no. CA42 were small (approximately 10^4 organisms per ml) the viable counts of both organisms increased up to 6 hr, but thereafter the count of the sensitive strain was reduced from 10^{11} organisms per ml to 10^6 per ml at 12 hr and

10^4 per ml at 24 hr. No free colicine was detected in the supernatant fluids from this type of culture and the survivors were fully colicine sensitive.

The colicine-refractory survivors of no. C600/21 in the mixed cultures with producers of colicine E_2 or E_3 were not sensitive to cell-free, high-potency

TABLE I

Viable counts, in mixed cultures at 37°C, of Escherichia coli strains no. C600/21 (sensitive to colicine E_3) and no. C600/21/ E_3 (a colicine-resistant mutant) with strain no. CA38 (which produces this colicine)

Time of incubation (hr)	Number per ml, at the stated times, in mixture of no. CA38 with			
	no. C600/21, of		no. C600/21/ E_3 , of	
	no. CA38	no. C600/21	no. CA38	no. C600/21/ E_3
0	3.12×10^6	7.42×10^6	2.99×10^6	9.23×10^6
3	3.55×10^{10}	5.23×10^4	2.63×10^{10}	1.25×10^8
6	3.91×10^{13}	7.95×10^4	3.78×10^{13}	7.90×10^8
24	4.6×10^{13}	2.25×10^5	2.20×10^{13}	1.10×10^{10}
48	4.1×10^{13}	1.96×10^5	2.13×10^{13}	1.07×10^{10}

TABLE II

Viable counts, in mixed cultures at 37°C, of Escherichia coli strains no. C600/21 (sensitive to colicine E_2) and no. C600/21/ E_2 (a colicine-resistant mutant) with strain no. CA42 (which produces this colicine)

Time of incubation (hr)	Number per ml, at the stated times, in mixture of no. CA42 with			
	no. C600/21, of		no. C600/21/ E_2 , of	
	no. CA42	no. C600/21	no. CA42	no. C600/21/ E_2
0	3.51×10^6	8.67×10^6	3.79×10^6	6.68×10^6
3	3.08×10^9	4.31×10^4	3.81×10^9	1.19×10^9
6	4.17×10^{12}	4.87×10^4	4.56×10^{12}	4.33×10^9
24	4.81×10^{12}	5.16×10^4	3.89×10^{12}	9.81×10^9
48	4.78×10^{12}	4.95×10^4	3.99×10^{12}	9.78×10^9

preparations of the corresponding colicine, but were fully sensitive to similar preparations of the other colicines, that is, colicines E_3 and B or E_2 and B respectively (see fig. 1). Treatment with trypsin of the refractory survivors, including those isolated after 24 hr in mixed culture, destroyed their colicine refractoriness; but comparison of the survival curves of these trypsin-treated cells and of the original strain when exposed to identical concentrations of the colicines shows that the trypsin-treated cells are not killed as quickly as the original cells (see fig. 2).

The colicine-refractory cells were also tested for their colicine sensitivity and colicine production after overnight growth in peptone water. They did not produce colicine, and were sensitive to high-potency preparations of colicines E₂ and E₃ and also to the chloroformed supernatant fluids of the mixed culture samples taken after 6, 24 or 48 hr of incubation. Furthermore, when

FIG. 1.—Survival of *Escherichia coli* no. C600/21 cells refractory to either colicine E₂ or E₃ when treated with colicine B, E₂ or E₃. Open symbols refer to no. C600/21 refractory to colicine E₂ treated with colicines: Δ, E₂; ○, B; □, E₃. Closed symbols refer to no. C600/21 refractory to colicine E₃ treated with colicines: ▲, E₃; ●, B; ■, E₂.

these supernatant fluids were re-inoculated with the original sensitive strain, to a concentration of approximately 10^6 organisms per ml, the colicine concentration was sufficient to destroy these organisms in less than 30 min. The supernatant fluids were without effect on colicine-resistant mutants derived from the sensitive strain.

The survival of colicine-refractory bacteria of strain no. C600/21 when grown with the colicinogenic strains, no. CA38 and CA42

The colicine-refractory survivors of strain no. C600/21 from the mixed cultures with either of the two colicine-producing strains were harvested and then grown in a second mixed culture with the same colicine-producing strain

as in the primary mixed culture. Estimation of the viable count on streptomycin agar showed that the number of bacteria of no. C600/21 was not reduced by the growth of the colicinogenic strain. However, these bacteria were not able to multiply in the presence of the colicinogenic strain or of free colicine. They showed normal growth when inoculated into peptone water or

FIG. 2.—Reversal by trypsin of the colicine refractoriness induced in *E. coli* no. C600/21 by growth with strains producing colicines E_2 or E_3 . Open symbols refer to percentage survival after treatment with colicine E_2 of: Δ , no. C600/21 refractory to colicine E_2 ; $\circ$, E_2 -refractory cells after treatment with trypsin; $\square$, no. C600/21 sensitive to E_2 . Closed symbols refer to survival after treatment with colicine E_3 of: $\blacktriangle$, no. C600/21 refractory to E_3 ; $\bullet$, E_3 -refractory cells after trypsin treatment; $\blacksquare$, no. C600/21 sensitive to E_3 .

when grown in mixed culture with a non-colicinogenic strain of *E. coli*. The results of three typical experiments are shown in table III. As in the primary mixed culture, the survivors of no. C600/21 had not acquired colicinogeny and, after overnight incubation in peptone water, were sensitive to cell-free colicine and also to the chloroformed supernatant fluid from the mixed culture.

The effect of temperature on the survival of colicine-refractory bacteria

The colicine-tolerant mutants referred to in the introduction to this paper became colicine sensitive with change in temperature. In two of the present series of experiments, the mixed cultures were incubated at 37°C for 6 hr and

then divided into three equal parts. One part was incubated at 30°C for a further 6 hr, the second part at 37°C and the third at 42°C. In each case the

TABLE III

Viable counts, in "secondary" mixed cultures, of colicine-refractory survivors of Escherichia coli no. C600/21 with the strain producing the corresponding colicine

Time of incubation (hr)	Expt no.	Number per ml, at the stated times, in mixture of			
		survivors (of no. C600/21 grown with no. CA38) and no. CA38, of		survivors (of no. C600/21 grown with no. CA42) and no. CA42, of	
		survivors	no. CA38	survivors	no. CA42
0	1	9.08×10^6	2.33×10^6	7.32×10^6	3.39×10^6
	2	6.33×10^5	3.31×10^6	2.31×10^6	2.17×10^6
	3	4.11×10^6	3.73×10^6	5.15×10^6	4.03×10^6
6	1	2.12×10^7	5.89×10^{12}	6.89×10^6	4.51×10^{12}
	2	9.19×10^6	4.31×10^{13}	4.57×10^6	6.79×10^{12}
	3	8.32×10^6	5.11×10^{13}	6.66×10^6	1.18×10^{13}
24	1	2.89×10^7	6.77×10^{12}	7.16×10^6	5.93×10^{12}
	2	1.76×10^6	4.93×10^{13}	5.13×10^6	7.11×10^{12}
	3	9.64×10^6	6.32×10^{13}	6.10×10^6	2.05×10^{13}

TABLE IV

Viable counts of strain no. C600/21 when incubated with either no. CA38 or no. CA42 for 6 hr at 37°C and then for a further 6 hr at one of three temperatures

Temperature of incubation	Expt no.	Number per ml of no. C600/21 after second period of incubation at the stated temperature with	
		no. CA38	no. CA42
30°C	1	6.31×10^6	6.52×10^6
	2	5.35×10^5	5.33×10^6
37°C	1	5.93×10^6	6.39×10^6
	2	5.87×10^5	5.02×10^6
42°C	1	6.62×10^6	6.77×10^6
	2	4.93×10^5	4.72×10^6

viable count of strain no. C600/21 was determined by plating on to streptomycin agar at the end of the second period of incubation. The results of these

experiments are shown in table IV. There was no significant difference in the viable counts at the different temperatures.

DISCUSSION

The bactericidal action of colicines (Fredericq, 1948) is well known, but only recently has it been recognised that certain colicine-sensitive bacteria may show a degree of colicine tolerance (Clowes, 1965; Hill and Holland, 1967; Holland, 1967; Nagel de Zwaig and Luria, 1967; Nomura, 1967; Nomura and Witten, 1967; Holland, 1968). These "colicine-tolerant" or "colicine-refractory" mutants resist the action of colicine over only a narrow temperature range outside which they are fully sensitive. Our observations indicate that another type of colicine tolerance can be induced by exposure of exponential-phase colicine-sensitive bacteria to minimal concentrations of colicine. These refractory cells remain viable even in high concentrations of the colicine but do not multiply. They grow normally and revert to colicine-sensitive cells when grown in the absence of colicine. Our investigations support and extend the recent observations of Reynolds and Reeves (1969) on the possible bacteriostatic action of colicine E₂.

The colicine tolerance of these bacteria is due neither to the acquisition of colicinogeny, since they do not produce colicine, nor to loss of the surface receptors for the colicine, since they absorb and are killed by the same colicine after overnight growth in peptone water. Moreover, their ability to resist the action of colicines is not temperature dependent; hence it is unlikely that any of the mechanisms suggested by Holland (1968) as the basis for colicine tolerance will explain the survival of the colicine-sensitive bacteria in our experiments.

Nor is the survival of the colicine-sensitive bacteria due to the development of an equilibrium in which the colicine is totally absorbed by some of the sensitive cells thus leaving no available colicine to kill the rest of the cells. This situation would apply if the numbers of colicine-producing bacteria were small and colicine production was only moderate. We found that free colicine was readily detected in the supernatant fluid of the mixed cultures and was present in amounts adequate to kill the sensitive strain in numbers equal to the inoculum of the original mixed culture. Also, the survivors of the sensitive strain were refractory to the action of high-potency preparations of the corresponding colicine, but remained sensitive to other colicines and regained full sensitivity after overnight growth in peptone. When the colicine-producing organisms were present in much greater numbers than the sensitive bacteria we were still able to isolate colicine-refractory survivors. In the reverse situation no colicine-refractory cells were isolated.

We suggest that during the early phase of mixed culture the exponentially growing sensitive cells absorb all of the available colicine, but that some of the cells absorb less than a lethal amount, so that the killing mechanism is initiated, but not fully developed because either the amount of colicine absorbed or the number of receptors activated is inadequate to bring about all the necessary biochemical changes. The basis of the subsequent tolerance to the colicine

that accumulates during the primary and secondary mixed cultures, and against highly active colicine preparations, is unknown. It is unlikely that it is due to steric hindrance, since the receptors for colicines E_2 and E_3 are very similar, yet bacteria refractory to E_2 remain fully sensitive to E_3 , and vice versa. The absorption of sublethal quantities of colicine may either induce a conformational change in the cytoplasmic membrane or initiate a repressor mechanism that blocks the transmission of the colicine effect to the biochemical target. In any event this type of colicine tolerance must involve the continuing presence of the colicine on the surface of the refractory cells, since treatment of them with trypsin restores their colicine sensitivity. In contrast to earlier observations on the "trypsin rescue" of colicine-sensitive bacteria, including those of Reynolds and Reeves, we find with the colicine-refractory cells that it is possible to reverse the effect of the absorbed colicine by trypsin for up to 24 hr. We agree with the statement by Reynolds and Reeves that trypsin-rescued cells, when compared with normal cells, show a marked decrease in the rate of killing on treatment with the same colicine. This decreased rate of killing, in terms of the model proposed by Hedges (1966), would imply a decrease in the number of available colicine receptors. Since recent work on bacteriocine receptors has shown that they are lipopolysaccharide in nature it is unlikely that trypsin would have any direct effect on them. It is more probable that the reduction in available receptors is due to the retention of the trypsin-inactivated colicine residues at the receptor sites, as suggested by Maeda and Nomura (1966) and Reynolds and Reeves.

SUMMARY

The survival of two colicine-sensitive strains of *Escherichia coli* when grown in mixed culture with each of three colicine-producing strains was investigated. The universal colicine indicator strain, *E. coli* B/TKM, was completely eliminated during mixed culture with each of the colicinogenic strains, whereas colicine-resistant mutants of it showed normal growth. The second colicine-sensitive strain, *E. coli* no. C600/21, was also eliminated by the colicine-B-producing strain, but not by either the colicine- E_2 - or E_3 -producing strains. The bacteria that were refractory to colicine E_2 or E_3 remained viable even in high concentrations of the colicine yet were unable to multiply; they regained full colicine sensitivity after growth in peptone water or when subcultured with a non-colicine producing strain of *E. coli*, but not when grown with a strain producing the same colicine. The refractory cells were non-colicinogenic and free colicine was present in the supernatant fluids of the mixed cultures. After treatment with trypsin to destroy the absorbed colicine the refractory cells regained their colicine sensitivity.

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