STUDIES ON THE MECHANISM OF THE LOSS OF CHLORAMPHENICOL RESISTANCE IN ESCHERICHIA COLI[1,2]

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The mechanism of the acquisition of bacterial resistance to chemotherapeutic agents has been investigated by a number of workers, but few extensive studies have appeared regarding the loss of this acquired character. As yet, it has not been proven whether this loss is caused primarily by mutation and selection or by a transitory physiological adjustment. This report presents data which support the hypothesis that mutation and selection are primarily responsible for the loss of chloramphenicol resistance in a resistant strain of *Escherichia coli*. In addition, preliminary evidence is presented which suggests that more than one mutational step may be involved.

EXPERIMENTAL RESULTS AND DISCUSSION

Loss of chloramphenicol resistance. The chloramphenicol resistant strain used in these studies was developed by a step-wise process from a sensitive culture of *Escherichia coli*, strain B (Merkel and Steers, 1953). The original parent sensitive strain is prevented from growing by 3 μg chloramphenicol[4] per ml of Difco brain-heart infusion broth or agar for 24 hours at 37 C. The resistant strain, however, readily multiplies in brain-heart infusion broth containing 1 mg chloramphenicol per ml in 24 hours at 37 C and in 2 mg per ml in brain-heart infusion agar after 48 hours' incubation.

The data in table 1 show the rapidity with which resistant strains lose their resistance to 1 mg chloramphenicol per ml of broth. These data were obtained by transferring either a loop inoculum from a 24 hour culture grown in 1 or 2 mg chloramphenicol per ml of broth or a colony grown 48 hours in agar with 2 mg antibiotic per ml to 5 ml of antibiotic-free broth. The drug-free subcultures were incubated at 37 C for 24 hours and transferred again to 5 ml of fresh drug-free broth until a series of nine chloramphenicol-free subcultures was produced. The resistance to 1 mg chloramphenicol per ml of the 24 hour subcultures was tested by transferring a loop of the culture to 5 ml of broth which contained that concentration of the antibiotic. The antibiotic containing tubes were incubated 24 hours, and then turbidity readings were taken. Table 1 shows that the loss of resistance, when tested by this method, occurs rapidly whether the original resistant strain was grown in 1 or 2 mg chloramphenicol per ml of either solid or liquid media.

The curve in figure 1 shows that the loss of resistance begins in the first antibiotic-free subculture and that 99 per cent of the resistance is lost after only a few subcultures. The points plotted in this figure represent the resistance of the serial antibiotic-free subcultures when they were tested by a turbidimetric bioassay method (Merkel and Steers, 1953). This bioassay method determines the concentration of chloramphenicol, permitting one-half the maximal growth of the culture. The curve produced is the summary of six similar experiments undertaken over a period of one year.

Mutation to sensitivity. A number of indirect approaches had to be used to show the occurrence of a spontaneous sensitive mutant since the recognized tests for such a mutation could not be applied easily. Table 2 presents plate counts showing that a number of cells in a resistant culture could not produce colonies in agar with 1 and 2 mg chloramphenicol per ml but could grow in drug-free agar. These sensitive cells must have occurred in the very presence of the drug since all the plate counts were of resistant cultures grown

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[4] Kindly supplied by Parke, Davis and Company.

TABLE 1

Average turbidities produced by various antibiotic-free subcultures of the resistant Escherichia coli when they are grown in broth with 1 mg chloramphenicol per ml for 24 hours

NUMBER OF SUBCULTURES IN BROTH FREE OF ANTIBIOTIC	SOURCE OF ORIGINAL INOCULUM		
	24 hr culture grown in 1 mg chloramphenicol per ml of broth (4 experiments)	24 hr culture grown in 2 mg chloramphenicol per ml of broth (3 experiments)	48 hr colonies grown in agar with 2 mg chloramphenicol per ml (4 experiments)
0	32	32	22
1	34	35	23
2	36	34	24
3	33	35	24
4	33	35	26
5	30	23	17
6	23	8	14
7	5	0	5
8	0	0	1
9	0	0	0

All turbidimetric measurements reported in this paper were made on a Klett-Summerson photoelectric colorimeter with a no. 66 filter.

two years constant exposure to at least 1 mg antibiotic per ml of broth.

There are, perhaps, a number of explanations for the results in table 2, but the data in table 3 tend to exclude all explanations except the one which states that a spontaneous sensitive mutant is arising and overgrowing the resistant cells. For example, one possible nongenetic explanation would be that the resistant culture reduces chloramphenicol to the less effective aryl amine (Merkel and Steers, 1953), thus setting up conditions for a nongenetic "deadaptation" which could produce the sensitive cells indicated by the data in table 2. If such a nongenetic system were functioning, then it would be expected that the cells would lose their resistance even more rapidly when cultured in media free of antibiotic. As table 3 shows, this is not the case; resistant cells are still present even after a number of transfers in drug-free broth. The results of the last two experiments presented in table 3 show that the resistant cells are present in trace numbers even after many transfers. The presence of these resistant cells cannot be explained as a carrying

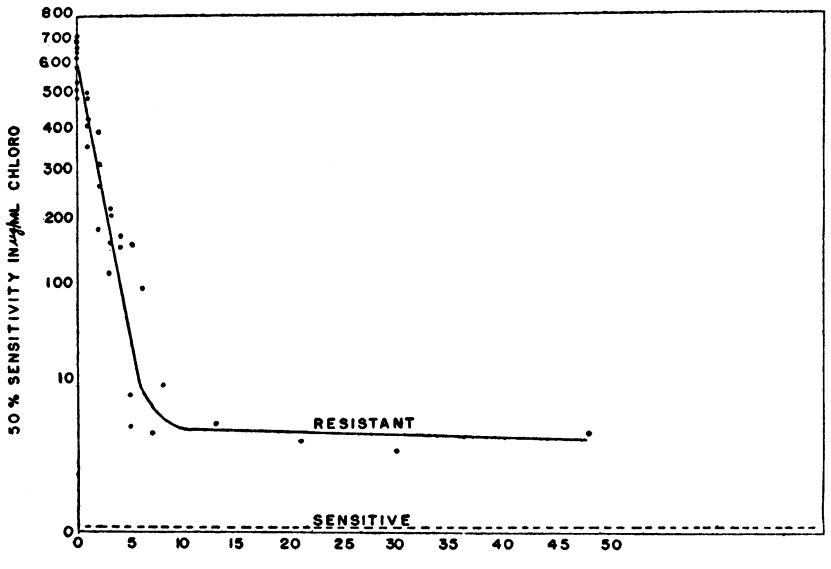

Figure 1. This semilog plot is constructed of points which represent turbidimetric bioassays of various subcultures of the resistant *Escherichia coli* grown in the absence of chloramphenicol.

in broth with 1 mg chloramphenicol per ml. It is difficult to explain by a nongenetic theory why so many sensitive cells are still present even after over of the original resistant cells while making the successive inoculations. For example, in the seventh column of table 3 there are about 11,000

TABLE 2

Comparative average plate counts (48 hr) of various resistant Escherichia coli cultures grown for 18 to 24 hours in broth containing 1 mg chloramphenicol per ml*

GROUP	MG CHLORAMPHENICOL PER ML OF AGAR	RESISTANT CULTURE NUMBER												
		1	2	3	4	5	6	7	8	9	10	11	12	13
A	0	223	184	184	188	89	150	76	189	187	172	168	155	158
B	2	146	139	128	105	40	111	54	86	64	105	81	—	—
C	1	—	—	—	—	—	—	—	162	151	114	146	104	130
Number of plates averaged..		15	15	11	5	4	5	5	3	3	3	3	5	5
		STATISTICAL ANALYSIS												
"Student's"	$P_{(A-B)}$	<0.001	<0.001	<0.001	0.005	0.009	<0.00	<0.001	<0.001	<0.001	0.005	0.003	—	—
t	$P_{(C-B)}$	—	—	—	—	—	—	—	0.003	<0.001	0.35	0.03	—	—
test	$P_{(A-C)}$	—	—	—	—	—	—	—	0.008	0.005	0.004	0.20	<0.001	<0.001

* The plate counts did not change even after 6 days' incubation at 37 C.

No means have as yet been found to control the variation seen from experiment to experiment in the proportion of sensitive and resistant cells occurring in parallel resistant cultures.

resistant cells per 0.1 ml of the fifth subculture. If these cells were carried over from the original resistant culture, then that culture must have had a population of 1.1×10^{15} bacteria per ml. This population figure is based on the assumption that a loop transfer, at the minimum, represents a 1 to 100 dilution (0.05 ml of inoculum in 5 ml of media), and five such dilutions were made in producing the fifth subculture. Since such a population is not possible, the resistant cells present in the fifth subculture must be the progeny of the original resistant cells. Resistance, therefore, is an inherited characteristic. Further, if one attempts to explain table 2 as the result of the varying ages of the cells found in a resistant culture, he must assume also that the disappearance of resistant cells seen in table 3 involves the disappearance of cells of a specific age. Since these above nongenetic explanations are unlikely, the loss of resistance can be best explained as a process in which sensitive mutants arise and overgrow the resistant cells when the medium is free of chloramphenicol.

If sensitive mutants are overgrowing resistant cells, the more sensitive drug-free subcultures would be expected to have improved growth abilities. The typical representative growth curves in figure 2 fit this theory. In addition, it has been observed that the cells from the more sensitive cultures produce the larger colony on agar free of chloramphenicol.

The experiment illustrated in figure 3 was devised to show that the sensitive cells detected by analysis of the data in table 2 are genetically distinct from the resistant cells. Two mechanisms function in this experiment, the first of which involves the bacteriostatic nature of chloramphenicol (Gunnison *et al.*, 1950; Smith, 1951). Chloramphenicol is bacteriostatic at the concentrations used since if it were bactericidal, the

TABLE 3

Decrease in the proportion of cells which can produce colonies in 1 and 2 mg chloramphenicol per ml in agar with an increase in the number of subcultures in antibiotic-free broth

CHLORAMPHENICOL-FREE SUBCULTURES OF THE RESISTANT ESCHERICHIA COLI STRAIN	PER CENT OF 18 TO 24 HR CULTURE'S CELLS PRODUCING COLONIES IN 48 HR (REFERRED TO CONTROL WITHOUT ANTIBIOTIC AS 100)*					NUMBER OF COLONIES RESULTING FROM INOCULA OF 0.1 ML FROM CULTURES WITH A TURBIDITY OF 25†		
	Mg chloramphenicol per ml of agar							
	2	2	1	1	1	0	2	1
	%	%	%	%	%			
0	89	69	89	—	82	+++	++	+++
1	52	0	86	98	91	+++	++	+++
2	37	8.3	80	85	84	+++	+	+++
3	4.4	8.9	83	93	51	+++	+	+++
4	1	0	37	77	35	+++	+	+++
5	0	0	4.1			+++	about 11,000	+++
6			1.3	28		+++		++
7			0	39		+++		+
8						+++		about 14,000
9						+++		about 2,500
10						+++		800
11						+++		160
12						+++		0

* These percentages were obtained by comparing the average count in three drug-free agar plates with the average count of the same culture in 3 agar plates with either 1 or 2 mg chloramphenicol per ml.

† These data involve the comparison of duplicate platings of heavy inocula of the resistant culture in drug-free agar and in agar with either 1 or 2 mg chloramphenicol per ml.

These experiments were done at various times over a period of one year and in all cases involved the same resistant strain of *Escherichia coli*.

+++ full growth as seen on an antibiotic-free plate; ++ diminished growth; + diminished growth with colonies too numerous to estimate.

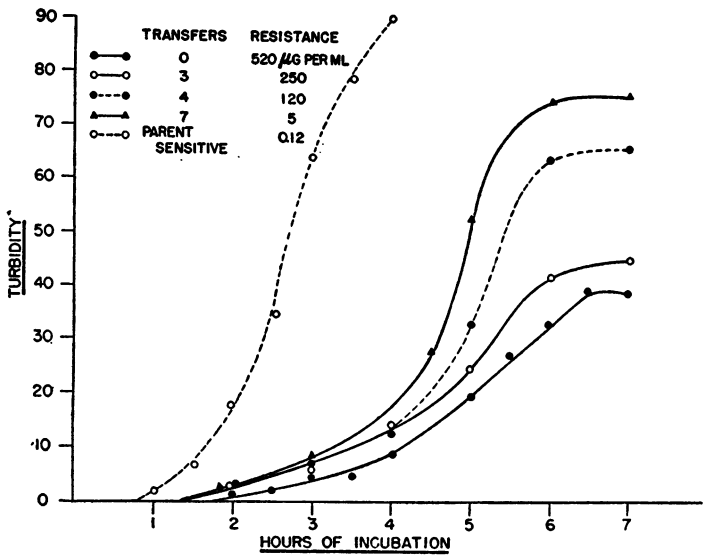

Figure 2. The average turbidity of three tubes was used for each point on these curves. Transfers refer to the number of times the culture had been transferred in the absence of the antibiotic. The resistance was determined by the 50 per cent growth inhibition test.

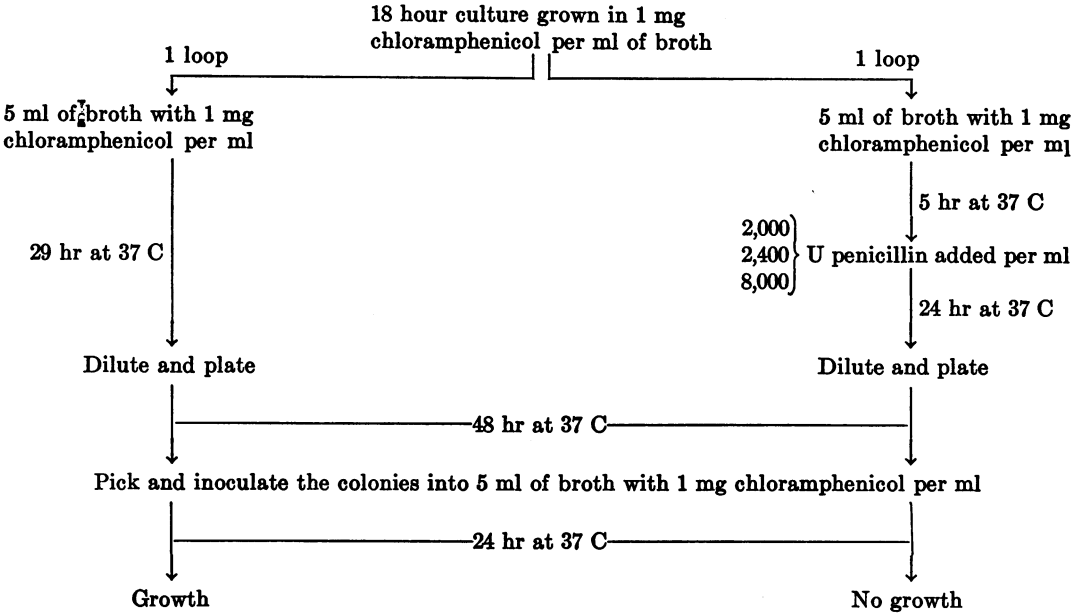

Figure 3. Model of a representative experiment used to separate chloramphenicol sensitive cells from the more resistant cells using both chloramphenicol and penicillin.

sensitive mutants would not have been observed from the very first (tables 2 and 3). Further, there is little likelihood that the data to be discussed (tables 4, 5, and 6) could have been obtained if chloramphenicol were bactericidal. The second mechanism is based on the ability of penicillin to kill dividing cells, leaving resting cells alive (Hobby *et al.*, 1942; Davis, 1950).

If sensitive mutants arise in a chloramphenicol containing culture, they are unable to divide and

can withstand the bactericidal action of penicillin whereas the resistant cells would divide and be

TABLE 4

Viable cells recovered from a resistant culture growing in 1 mg chloramphenicol per ml broth and treated with penicillin for 24 hr at 37 C

UNITS PER ML OF PENICILLIN	TOTAL VIABLE CELLS BEFORE PENICILLIN	TOTAL VIABLE CELLS AFTER PENICILLIN*
800	6.7×10^7 per ml	4.13×10^5 per ml
1,600	6.7×10^7	4.07×10^5
2,400	6.7×10^7	2.56×10^5
2,400	8.5×10^7	1.5×10^5
2,400	9.75×10^7	9.9×10^4
8,000	6.7×10^7	1.03×10^5

* Statistical analysis of these two plate averages shows P to be less than 0.001.

TABLE 5

Ability of colonies produced by cells treated and untreated with penicillin to multiply when transferred to broth containing 1 mg chloramphenicol per ml

UNITS PER ML OF PENICILLIN	NUMBER OF COLONIES PICKED	NUMBER OF COLONIES THAT GREW IN 24 HR IN CHLORAMPHENICOL
0	25	23
0	39	37
2,000	25	1
2,400	25	2
2,400	20	7
2,400	25	1
8,000	40	15
8,000	40	9

	SUMMARY		
	Number growing in chloramphenicol	Number not growing in chloramphenicol	Total
Untreated cells.....	60	4	64
Treated cells.....	35	140	175
Total.....	95	144	

Statistical Analysis of Summary

Chi-square (one degree of freedom)..... 106.4
P less than..... 0.001

killed. Following this reasoning a resistant culture was grown in 1 mg chloramphenicol per ml of broth, and when the bacteria reached their phase of most active growth (5 hours) substantial amounts of penicillin were added. The penicillin was left in contact with the cells for 24 hours; then a dilution was made which lowered the concentration of both antibiotics to insignificant levels. This dilution was plated in agar free of both antibiotics, and the resulting colonies were counted. Table 4 shows that a substantial number of cells survive this treatment.

To show that the above recovered cells were sensitive mutants, the offspring of the cells were tested rather than the individual cells themselves. This was done by comparing the colonies which were produced by cells untreated with penicillin (control) with the colonies resulting from cells

TABLE 6

Viable cells recovered after a 24 hour treatment with 8,000 U penicillin per ml and various concentrations of chloramphenicol

MG CHLORAMPHENICOL PER ML OF BROTH	EXPERIMENT 1	EXPERIMENT 2
1	4.66×10^5 per ml	3.66×10^5
0.8	2.49×10^5	9.8×10^4
0.6	4.35×10^4	2.36×10^4
0.5	—	1.57×10^4
0.4	3.35×10^3	4.45×10^3
0.3	—	3.58×10^3
0	—	0
Population before penicillin	3.9×10^7	3.85×10^7

which were treated. Representative numbers of each type of colony were picked and inoculated into 5 ml of brain-heart infusion broth containing 1 mg chloramphenicol per ml (the concentration in which the culture was able to grow originally). The colonies from the penicillin-free control generally produced growth (as measured by observable turbidity) while the colonies resulting from penicillin treated cells generally could not grow in 1 mg chloramphenicol per ml of broth (table 5). In antibiotic-free broth 99 out of 100 colonies grow regardless of their source.

It must be emphasized that the colonies tested were grown in agar essentially free of both antibiotics. This means that the decreased resistance these colonies show could not have been induced by either antibiotic. Thus, the colonies generally show the degree of resistance that they inherited

from the mother cell. The mother cell, therefore, must have been sensitive due to a genetic difference between it and the highly resistant cells.

In the above experiment large doses of penicillin were used to prevent the possibility of selecting penicillin resistant cells. It was found that the chloramphenicol resistant culture, before or after penicillin treatment, could grow in three or four days in 400 U of penicillin per ml but not in 800 U whether chloramphenicol was present or not. The experiment proper, therefore, used from 3 to 10 times the inhibitory dose of penicillin for this strain. It was found also that penicillin in the concentrations used could sterilize essentially a culture if no chloramphenicol were present.

The possibility of the occurrence of more than one type of sensitive mutant. As yet, no proof can be presented which conclusively shows that more than one sensitive mutant type contributes to the loss of chloramphenicol resistance in *E. coli*. However, much of the data already presented and the results of experimentation still in progress could be interpreted to indicate the occurrence of two or more sensitive mutants with different degrees of resistance. For example, the data presented in table 2 could be interpreted as showing three types of cells in the chloramphenicol containing resistant culture. These types are those able to produce colonies in 2 mg chloramphenicol per ml of agar, those able to grow in 1 mg per ml but not 2, and those unable to grow in either 1 or 2 mg per ml but do reproduce in drug-free agar.

An example of evidence obtained from work still in progress is presented in table 6. This table shows that when the concentration of penicillin is kept constant (8,000 U per ml) and the concentration of chloramphenicol is varied in a broth culture the number of sensitive cells recovered decreases as the concentration of chloramphenicol decreases. This can be interpreted as the result of permitting some of the more resistant sensitive mutants to grow and now be killed by penicillin.

The occurrence of more than one sensitive mutant type would agree with the work of Cavalli and Maccacaro (1950) on the multiloci nature of chloramphenicol resistance in *E. coli*, strain K-12. If a variety of sensitive mutants is occurring, apparently one of the most sensitive mutants must overgrow all those cells of a higher resistance, thus, producing the rapid loss of chloramphenicol resistance observed in a resistant strain of *E. coli*, strain B.

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SUMMARY

The loss of resistance to chloramphenicol in an extremely resistant strain of *Escherichia coli*, strain B, was studied. The evidence presented supports the hypothesis that this instability is caused primarily by the selection of sensitive mutants which overgrow the resistant cells in media free of the antibiotic. Preliminary observations indicate that more than one type of sensitive mutant may be involved.

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