STUDIES ON THE MECHANISM OF THE LOSS OF CHLORAMPHENICOL RESISTANCE IN Escherichia coli

by

Ernest Carl Herrmann, Jr.
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RESISTANCE IN Escherichia coli

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It is now generally believed that various forms of life evolve by a process of mutation coupled with environmental selection. Despite the wide acceptance of this concept the evidence is still indirect and based primarily on what is known to occur today in certain genetic experiments with additional support from paleontology and comparative animal morphology. Direct evidence for evolution has not been obtainable since evolution occurs in multicellular organisms over too long a period of time to be properly observed. Today, however, a new approach is available in using microbial populations where the study of evolution can be telescoped into as few as 24 hours or that time required to grow a large population of cells. With microorganisms the observer can be present from the advent to the decline of a population and properly record any evolutionary changes which might occur.

Microorganisms better than any other form of life can be studied under controlled conditions; this offers the opportunity to change only one specific aspect of their environment and observe their response. Bacteria, like most living things respond less obviously to so-called improvements in their "normal" environment than they do to deleterious changes. The introduction of these harmful changes forces the organism to "adapt" or become extinct. The survivors of these deleterious environmental alterations are of basic evolutionary importance since they usually show the evolution of a new characteristic in the species. If the deleterious factor is one of the newer antibiotics
to understand the occurrence of resistance to antibiotics. As resistance to antibiotics has become widespread, there has been a need for research into the mechanisms of resistance. Some resistance patterns observed in bacteria have been acquired through exposure to antibiotics. This has led to the development of new antibiotics to combat resistant bacteria. However, resistance to these new antibiotics has also been observed. Therefore, it is essential to continue research into the mechanisms of resistance and to develop new strategies to combat antibiotic resistance.
characters from generation to generation. The primary reason for this lack of knowledge is that there is no well-established process of sexual behavior together with the fact that bacterial cytology is just beginning to discern significant facts (6,20). However, there is one method by which bacterial genetics can be studied, that is one based on their mutability, a process in which bacteria are particularly rich. Bacterial genetics must, therefore, rest largely on analogies to mutation in "higher" organisms to which there are many close correlations (20).

Mutation can be defined as a sudden variation in which the offspring differ from their parents in some well-defined character or characters. This characteristic must be passed on to subsequent progeny as an inherited factor otherwise the process is not true mutation. The assumption is that the new character resulted from a change in the basic genetic structure of the organism. In man there is one such mutation called "porcupine man" (3) which apparently occurs only once in two centuries. This is admittedly rare which is typical of most mutations. However, in a bacterial population with upwards of billions of individuals per milliliter of culture medium the rare can be more easily observed.

Large bacterial populations are easily produced in 24 hours and it would be expected that such populations might contain a number of various mutants. The possibility that certain mutants were always present in a bacterial culture was overlooked until investigators learned to manipulate the environment so that the mutant was favored over the "normal" cells. The greater part of the evidence indicates that drug-resistant mutants are present in bacterial populations prior to introduction of the drug. When the drug is introduced in the proper
concentration (sub-lethal) the resistant mutants already present are favored and a new strain results.

The indirect evidence to support the mutation and selection theory is mountainous and at times almost appears to be absolute proof. However, when one asks the fundamental question of how the mutation occurred in the first place, two answers are heard. The school with the greatest support contends that the drug-resistant mutants occur at random and previous to the application of any selecting agent (7, 20, 27), the cause of the mutation being still unknown. In opposition to this view is the theory that the drug has induced the mutation (30). This last view obtains its greatest support from the fact that the drug-resistant mutants can be seen only by applying the specific antibacterial agent, a technical difficulty which has not been overcome. Although induction of mutants is known (35) and many types of chemicals and radiations will produce a variety of mutants there have been no reports of the induction being specific or directed; i.e. induced resistance only to the radiation or chemical being used. As yet, no antibiotic has been studied which acts in the manner of known mutagenic agents.

The mutation-selection theory has found easy application to almost all observations of bacterial variation since it is more flexible than other theories. To illustrate this flexibility we have the example of bacterial resistance to the antibiotic streptomycin. It has long been known that one application of a sub-lethal dose of streptomycin was usually sufficient to select out a mutant which for all practical purposes was completely resistant to the antibiotic (25). This single mutagenic step to complete resistance is explained as only one change
in the cell's genetic structure. This pattern does not prevail, how-
ever, with penicillin, aureomycin and chloramphenicol resistance for in these cases high resistance involves a number of steps. The first mutants selected by these latter antibiotics have a low level of re-
sistance and from these further mutants of higher resistance must be selected by using increased concentrations of the antibiotic.

In explaining the step-wise resistance pattern by the mutation-
selection theory it is assumed that the mutants are rather rare and that a mutant involving two or three or more mutations in the same cell would be exceedingly rare, so much so that its recovery would be ex-
tremely unlikely. Therefore, resistance to those drugs which require more than one mutational step is developed by selecting the first-step mutant and permitting it to produce its own population. From the popu-
lation made up of progeny of the first-step mutant a second-step mutant can now be recovered. This can be repeated until the highest possible resistance is developed. The specific induction theory does not well explain stepwise development of resistance since there is no logical reason for induction being stepwise.

Non-genetic development of drug resistance. Hinshelwood and his associates support an alternate theory to explain acquired bacterial drug resistance (13). This involves a change on the part of the organ-
ism in response to a drug, new nutrient, or other marked environmental change which does not involve a genetic alteration. The exact mechan-
ism of such non-genetic adaptations is not known but in a number of cases the cause might be traced to so-called "adaptive enzymes" (10). Spiegelman, Sussman and Finska (34) describe such "adaptations"
in yeast acquiring the ability to use galactose as a source of energy. The evidence indicates that this adaptation is not due to a genetic alteration (mutation). The enzyme responsible is supposed to occur in minute amounts in the unadapted yeast. On introduction of the new substrate, galactose, the cells increase the production of this enzyme until it is present in the proper amounts to satisfy the organism's requirements. The organism is then said to be "adapted". While this evidence is not conclusive proof of the theory, it is an effective explanation for those adaptations which occur under conditions where mutation has been excluded. Other studies along this line have been done with yeast and acriflavine (9) and with Drosophila treated with extracts of a CO₂ resistant strain (19). A very characteristic feature of the non-genetic adaptation is the loss of the acquired character when the unusual environmental alteration has been removed. A rapid loss of such acquired characters might lead one to call such a phenomenon a non-genetic adaptation. However, this idea is based on the false supposition that a genetic change is permanent in nature.

As yet, there is no convincing evidence that drug resistance can occur to any great degree by a non-genetic adaptation. The most satisfactory method of classifying a bacterial variation as a non-genetic adaptation is by observing the variation occurring under conditions which exclude the possibility of mutation. Such an experiment usually involves study of the variation in a population proved free of any mutants while, at the same time, the bacteria are prevented from dividing so that no new mutants occur. This type of experiment has not been applied with any great success to drug resistance.
All the possible explanations of the occurrence of drug-resistant bacteria have not been exhausted. The possibility exists that both of the foregoing views are in part correct. Eagle and coworkers (8) offer the idea, with supporting evidence, that both genetic and non-genetic adaptation are involved in the development of bacterial resistance to chemotherapeutic agents. Non-genetic adaptation is proposed as the mechanism responsible for low levels of drug resistance and mutation for the high levels.

**Loss of drug resistance.** At present it is generally assumed that the loss of an acquired bacterial character occurs by the same route as its acquisition. This assumption has become so firmly established that apparently no one has felt it necessary to examine experimentally the mechanism of the loss of drug resistance in bacteria. Since mutation and selection have the greatest degree of support as an explanation for the acquisition of drug resistance it follows that the same theory would be most frequently assumed for the loss of resistance (25).

Although the loss of drug resistance in bacteria has been largely neglected the loss of other acquired bacterial characters has recently become the subject of a number of papers. For example, Novick and Szilard (27) studied the forward and reverse mutation to bacterial virus resistance. In this case a number of mutations were involved. Lieb (18) made similar studies on the loss of the acquired requirement for histidine in *E. coli*.

Minshewood (13) has also extended his theory to explain the loss of an acquired bacterial character as a non-genetic adaptation. On
the basis of chemical kinetics he predicts that reversion must occur when the short term adaptation is non-genetic. He explains that the pressure of the drug's presence forces an enzymatic adaptation if life is to be sustained. Hinshelwood also predicts that on removal of the drug the normal enzymes function once again with the adaptive enzyme assuming a secondary role. It might be said that lack of use of the adaptive enzyme results in its "atrophy". This theory fails to explain why many types of drug resistance resulting from a short "training" period are permanent in nature in the absence of the drug.

This dissertation will undertake to show which theory better explains the rapid loss of chloramphenicol resistance. Evidence will be presented that shows this loss is caused by mutation and selection. Data will also be presented to indicate that the mutational pattern is highly complex in nature and probably involves more than one mutational step.
CHAPTER I
THE ORGANISMS

Escherichia coli. The data presented in this dissertation involve in almost every case the strain B of Escherichia coli or strains derived from it. This chloramphenicol sensitive, non-motile strain has been used widely in genetic studies and its characteristics are well known and conform to the criteria outlined in Bergey's Manual (1). This strain is considered sensitive to chloramphenicol since only 3 μg. of antibiotic per ml. of broth prevent observable multiplication for at least 24 hours and the same concentration in agar checks the growth for 48 hours. However, when a more sensitive test was used it was found that as little as 0.2 μg. of chloramphenicol per ml. of broth inhibited the growth of this strain by 50 per cent.

Using this parent susceptible E. coli a strain was developed which was highly resistant to chloramphenicol (22,23). The mode of development of this resistance involved a step-wise process, with only small increases in resistance at each step, a pattern which has been reported in all cases of bacterial resistance to chloramphenicol (2,4). The resistant strain used most often in the experiments reported here was the type which multiplied readily in 1 mg. chloramphenicol per ml. of brain heart infusion broth during 24-hours incubation at 37°C. This same strain produces small colonies in 48 hours in brain heart infusion agar with 2 mg. of antibiotic per ml. The resistant strain, therefore, is more than 333 times as resistant as the parent sensitive strain when grown in broth and better than
By all seven viruses produced that it was still at least fourfold greater than the original test strain used. Nevertheless, the sensitivity of the resistant strain to seven different viruses was still 0.07. Thus, the reason why the resistant strain was still resistant is not clear. Since it is no longer confirmed to the resistant strain, there was some doubt whether the sensitive strain produces the greater colony.

A very easy and obvious conclusion is that the resistant strain between the sensitivity and difference in growth enhancement of the parent and resistant strain in broth. The growth time does not only about one-third as much growth in broth as the sensitive strain. The resistant strain of the parent not observed in the presence of the enrichment and produce the phenotypically resistant strain of the resistant strain from which it arose. The difference is that the resistant parents examined revealed that the resistant were observed. The degree of resistant viruses with the test need to determine. What is 2500 to 3000 times as great as for the parent sensitive strain of broth was required to inhibit the resistant strain by 50 per cent. It was found that between 0.5 and 0.6 ml of chloramphenicol per ml. The maximum growth that the culture produces in a drug-free broth medium. A very easy and obvious conclusion is that the resistant strain is not resistant when compared by a test plate between these two strains. The difference in resistance to resistant in resistant
of the other hand, the E. coli strain that
experiments reported here involve subculturing by the transfer of one
any better growth than small inocula. For that reason the majority of
biota per ml. of broth. It was found that large inocula do not produce
in studying the resistant E. coli strain which grows in 1 mg. of antibiotic.
Variations in the size of the inoculation were of little importance.

Variations in the size of the inoculation were of little importance.

of the two resistant strains.

there is no significant difference between the characteristics of the
to it for comparative purposes. As far as has been determined to date,
poor it was not extensively studied. Although references will be made
be called E. coli. Since the growth of the more resistant strain was so
in broth for well over a year but the growth produced could never
the E. coli strain was cultured on 2 mg. of chloramphenicol per
always poor. This strain was cultured in 2 mg. of chloramphenicol per
in broth to E. coli. In the absence of this concentration, however, the growth
be rather easy to recognize the strain growing in 1 mg. antibiotic per
maximum which can be developed for this species. It has been found that
resistance to 1 mg. of chloramphenicol per ml. of broth is not the

between the separate experiments reported here.

effective chloramphenicol concentration is decreasing with the inoc-
in the light of the above it is most important to remember that the
this reduction in the resistant strain was far more efficient (22, 23). This
has been reported that even though the sensitive strain could produce
the mature form is far less toxic to the bacteria than the nitro form. The
the para nitro group of chloramphenicol to produce the active
in very important differences between the sensitive and resistant

11
grows in 2 mg. chloramphenicol per ml. does require a large inoculum. Usually between 0.1 to 0.3 mls. of a 24-hour culture with at least a 25 turbidity (as measured by the Klett-Summerson photoelectric colorimeter equipped with a number 66 filter) was required to produce observable growth in 24 hours.

All resistant E. coli strains produced better growth in the absence of chloramphenicol which indicates that the drug still has some growth inhibiting properties even against highly resistant bacteria. Further, the poor growth in 2 mg. chloramphenicol even after a year of so-called training at this level indicates that further increases of resistance are unlikely. It could not be proved that the maximum resistance is developed at 2 mg. per ml. since this concentration is the maximum solubility of this drug in the media used.

Micrococcus pyogenes var. aureus. This species was used rarely in the following studies but it is useful in showing a comparison between a stable chloramphenicol-resistant species and an unstable one. It should come as no surprise that the chloramphenicol resistance of this micrococcus is different from that found in E. coli since these two species are so different in all other characters. The micrococcus is spherical while coli is a rod, it is Gram (or gram) stain positive while coli is negative, and it requires more preformed nutrients than the coliform.

The original sensitive M. pyogenes var. aureus was somewhat more resistant from the very outset than the parent sensitive E. coli. Nevertheless, since 5.6 pg. chloramphenicol per ml. of agar completely inhibits the growth of this species it can still be considered a
highly sensitive bacterium. When the more sensitive 50 per cent of
growth inhibition test is used it was found that 0.4 μg. per ml. per-
mittted only 50 per cent of the growth observed in a drug-free control.
In other words the micrococcus is about twice as resistant as the
sensitive E. coli when tested by the latter method.

A resistant strain of this micrococcus was produced by essentially
the same procedure as with E. coli until it could readily grow in 2 mg.
chloramphenicol per ml. in both liquid and solid media. The resistant
micrococcus not only produces a far greater degree of growth in the
presence of the antibiotic than the resistant E. coli but it also
requires a larger inoculum to initiate growth (0.3 ml. of a visibly
turbid culture). The resistant micrococcus in media free of chloram-
phenicol also shows a far superior degree of growth but this result does
not require any special inoculum size. Recent reports also show that
the resistant Micrococcus pyogenes var. aureus cannot reduce the nitro
group of chloramphenicol (22, 23).
CHAPTER II
THE LOSS OF RESISTANCE TO CHLORAMPHENICOL

The literature on bacterial drug resistance usually describes it as a generally stable characteristic. It has been assumed that when the resistance is lost rapidly it is due to incomplete "training" or perhaps development of resistance to too low a level (38). The loss of resistance to be described in this report does not fall into either of the above categories. It is believed that this strain has received more than enough training since it has been cultured over two years in 1 mg. chloramphenicol per ml. of media and for over a year in 2 mg. of the antibiotic per ml. with no stabilizing of the resistance. It is also felt that development of too low a level of resistance is not the answer since this strain appears to be as resistant as it can be made. The reason it is believed that the maximum resistance has been developed is that this strain grows so poorly even after training for one year in 2 mg. chloramphenicol per ml. Certainly it shows little inclination to improve its resistance up to the date of this writing. As will be shown the strain growing in this higher concentration still loses its resistance as readily as the strain of lower resistance.

There are a number of methods by which the loss of chloramphenicol resistance can be studied in E. coli; however, it should be noted that the loss appears to vary with the method used. Table I illustrates the results from one method which tests the loss of resistance of the resistant strains to only one concentration of the antibiotic. Column 1 of this table shows the number of subcultures in antibiotic-free
broth that are required before the culture can no longer produce observable turbidity in 1 mg. chloramphenicol per ml. of broth after 24 hours incubation at 37°C. The technique for obtaining these data involved first growing the resistant *E. coli* in 1 mg. chloramphenicol per ml. Difco brain heart infusion broth (5 ml.) for 24 hours at 37°C. An inoculum is transferred from this 24-hour culture by a wire loop to 5 ml. of broth which contains no antibiotic. This culture was grown for 24 hours and again transferred to drug-free media. Repeating this process eventually produces a set of serial subcultures free of chloramphenicol. The resistance of the drug-free subcultures was tested by transferring a loop of culture to 5 ml. of broth which now contains 1 mg. chloramphenicol per ml. The antibiotic containing cultures were incubated for 24 hours at 37°C and then turbidity readings were made on the photoelectric colorimeter. The data from this technique show how many transfers in drug-free broth are required before the strain can no longer produce growth in 1 mg. antibiotic per ml.

Column 2 of table 1 illustrates essentially the same type of experiment except that in this case the culture used to begin the series of antibiotic-free subcultures was grown in 2 mg. chloramphenicol per ml. of broth. The loss of resistance, however, was still tested by using only 1 mg. per ml. As the data show it matters very little whether the resistant strain was resistant to 1 or 2 mg. chloramphenicol per ml. it still loses resistance to 1 mg. per ml. in essentially the same number of drug-free subcultures.
TABLE 1

Turbidities produced by various antibiotic-free subcultures of resistant
strains when they are grown in broth with 1 mg. chloramphenicol per ml.

<table>
<thead>
<tr>
<th>Number of subcultures in broth free of antibiotic</th>
<th>SOURCE OF ORIGINAL INOCULUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>2. <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>3. <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>4. <em>Staph. aureus</em></td>
</tr>
<tr>
<td></td>
<td>24-hr. culture grown in 1 mg. chloramphenicol per ml. broth</td>
</tr>
<tr>
<td>0</td>
<td>30 37 32 28</td>
</tr>
<tr>
<td>1</td>
<td>32 31 33 41</td>
</tr>
<tr>
<td>2</td>
<td>36 35 34 37</td>
</tr>
<tr>
<td>3</td>
<td>34 33 34 31</td>
</tr>
<tr>
<td>4</td>
<td>36 33 34 30</td>
</tr>
<tr>
<td>5</td>
<td>31 30 29 29</td>
</tr>
<tr>
<td>6</td>
<td>8 24 27 31</td>
</tr>
<tr>
<td>7</td>
<td>0 6 0 13</td>
</tr>
<tr>
<td>8</td>
<td>0 0 0 0</td>
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<td>9</td>
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<td>10</td>
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<td>11</td>
<td></td>
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<td>12</td>
<td></td>
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<td>13</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td></td>
</tr>
</tbody>
</table>

* After 24 hours incubation

All turbidimetric measurements reported in this paper were made on a Klett-Summerson photoelectric colorimeter with a 66 filter.
In column 3 of table 1 the same method of testing resistance was used but a different type of culture was used to start the serial subcultures. In this experiment a single 48-hour old colony was picked via a wire loop from brain-heart infusion agar with 2 mg.

chloramphenicol per ml. and transferred to the drug-free broth to begin the serial "back" transfers. Here again the result is the same but there is additional significance to this experiment as will be shown below.

When well separated colonies are picked from an agar plate the general assumption is that the colony is a pure culture since it arose from a single mother cell. This would imply that the loss of resistance could not have been caused by a contaminating bacterium which may have accidently found its way into the resistant stock culture. Further, this experiment shows that changing from liquid to solid media does not alter the rapid loss of resistance. Finally, the most important point in regard to the data in column 3 involves the possibility that sensitive E. coli cells from the original parent strain might have been carried along with the resistant cells through hundreds of transfers. If such sensitive cells were carried along then they would, of course, cause a loss of resistance when the culture was grown in drug-free broth. However, when using the single colony technique such possible sensitive cells are eliminated from the picture.

In column 4 an entirely different chloramphenicol-resistant species is tested as to the loss of its resistance. The experiments were done exactly as before using a culture of Micrococcus pyogenes var. aureus grown for 24 hours at 37°C in broth with 2 mg.

chloramphenicol per ml. As the data show resistance of this species is
The evidence that the parent sensitive strain is not responsible for the

sensitive strain (broken line). The fact that the presence

level of resistance is still 60 times greater than that of the parent
takes the lower level of resistance for 45 or more transferences but this

of the phenomenon. After & transferences the curve levels off and remains

stability of this strain is lost in about 7 transferences in broth free

The curve in Figure 1 shows that about 99 per cent of the re-

over a period of one year.

stress of the parent strain determined in the strain experiments performed

produces in a drug-free broth medium. The curve in Figure 1 is con-

phenoxybenzonic acid only until the maximal growth of the culture

(27). The end-point of the method is the concentration of cytostatic

proposed by Josien and Gallierth (12) and modified by Mekes and Steene.

The average resistance is arrived at by a plot of each assay method.

the case the average resistance of the mutant culture was observed.

been various serial drug-free subcultures were again tested and in

in the experiment.

low level to which the resistance eventually falls. In this case, the nature of the loss of the

nitet of the mother of the mutants resistant to chloramphenicol may be another method was used to show the

eventually the loss of the resistance to chloramphenicol is rapid in the very

since this loss may be gradual in nature and may begin in the very

taking place in the loss of resistance to chloramphenicol resistance in 0.001 M

The evidence so far presented tells very little of what is really

been reported for the species after 24 drug-free subcultures (24).

6 transferences in drug-free broth. However, loss of resistance in

rather stable since it never loses resistance to 2 me. per ml. In

19
Figure 1. This semi-log plot is constructed of points which represent turbidimetric bioassays of various subcultures of the resistant *E. coli* grown in the absence of chloramphenicol.

loss of resistance for if it were then the resistance would have fallen much lower. This point will be examined in detail in Chapter IV.

It was also observed that the loss of chloramphenicol resistance occurred equally well when the strain was transferred on a solid
medium free of chloramphenicol. When the resistant strain was transferred in a liquid synthetic medium the loss of resistance was somewhat delayed, in fact no loss occurred for the first seven transfers. After 78 transfers in the synthetic medium the resistance had been lost to a large degree but not to as low a level as seen in brain heart infusion broth. This might be explained as a case in which the synthetic medium is slightly more favorable to the resistant strain so that it takes a longer time for sensitive mutants to overgrow the resistant cells. Unfortunately, this point was never fully examined. Merkel et al. (21, 23) have also found that an E. coli strain "trained" in 2 mg. chloramphenicol per ml. produces a remarkably similar curve to the one seen in figure 1. Apparently one of the most constant and reliable characteristics of the resistant E. coli strain is its loss of this resistance.

Table 2 offers still a third method for observing the loss of chloramphenicol resistance. Since both the first and second methods can be considered as measuring the average resistance of the culture, this third method is used to show the resistance loss of the individual cells in the culture. To obtain the data seen in the first five columns of table 2 the various serial subcultures of the resistant strain were diluted to a few hundred cells per ml. This dilution was mixed with agar in a culture dish and incubated until full size colonies were produced (48 hrs.). Colony counts on plates which 1 and 2 mgs. chloramphenicol per ml. were compared to the counts from the same culture on drug-free agar. The table shows the percentage of cells which can produce colonies in the antibiotic containing plates as compared to the total count in the drug-free plates.
**TABLE 2**

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

<table>
<thead>
<tr>
<th>Subcultures in the absence of chloramphenicol</th>
<th>Per cent of 18 to 24-hr. resistant cultures cells producing colonies in 48 hrs. (as compared to antibiotic-free plates)</th>
<th>No. of colonies resulting from inocula of 0.1 ml. of undiluted cultures (25 turbidity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mg. CHLORAMPHENICOL PER ML.</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>89%</td>
<td>69%</td>
</tr>
<tr>
<td>1</td>
<td>52%</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>77%</td>
<td>83%</td>
</tr>
<tr>
<td>3</td>
<td>4.4%</td>
<td>8.9%</td>
</tr>
<tr>
<td>4</td>
<td>1%</td>
<td>0%</td>
</tr>
<tr>
<td>5</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>6</td>
<td>1.3%</td>
<td>23%</td>
</tr>
<tr>
<td>7</td>
<td>0%</td>
<td>39%</td>
</tr>
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<td>8</td>
<td>1%</td>
<td>0%</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+++ full growth as seen on an antibiotic-free plate
++ diminished growth
+ diminished growth with colonies too numerous to estimate
The last two experiments presented in this table involve plating the various serial drug-free subcultures without dilution. This means many millions of cells are plated in each culture dish. When plates with and without chloramphenicol are compared it is seen that the number of cells able to produce growth on a drug-containing plate decreases as would be expected. However, the resistant cells do persist longer than might be supposed. The fact that resistant cells do last in trace amounts for so many transfers favors the mutation theory and will be discussed in detail in Chapter III.

It will be noticed that both 1 and 2 mgs. of antibiotic were used in the experiments presented in table 2 and the overall picture for both is the same. Raising the drug concentration merely permits observing the loss of resistance sooner. A point that is most important is the difference in the proportion of cells resistant to 1 and 2 mgs. This will be discussed in Chapter IV where it will be shown that this result in all likelihood is due to a variety of mutants of different resistances.

In summary it can be said that an E. coli strain highly resistant to chloramphenicol fails to retain its resistance when cultured in either solid or liquid brain heart infusion medium. Since this loss is comparatively rapid it would lead many persons to postulate a non-genetic adaptation as functioning here. Although most mutations are rather stable this does not mean unstable ones are not known. The task ahead is to resolve these two points of view.
CHAPTER III

THE MECHANISM OF THE LOSS OF CHLORAMPHENICOL RESISTANCE

As yet, there is no satisfactory direct means by which spontaneous mutation to the chloramphenicol-sensitive state can be proved. For that reason the evidence to be offered will be of an indirect nature. No single experiment in this dissertation proves mutation; however, all the evidence together scarcely leaves an alternative explanation. There is one method, devised by Newsombe (26), for directly proving spontaneous mutation to bacterial resistance to viruses and drugs but this method cannot be applied to the loss of resistance. An indirect approach used by Demerec (7) to prove spontaneous bacterial mutation to penicillin resistance also could not be applied to the problem presented here.

The data presented in table 3 are examples of the indirect nature of the evidence which favors the mutation theory. These data give a number of comparative plate counts in the presence of 1 and 2 mgs. chloramphenicol per ml. of broth. As the table illustrates there is a statistically significant difference between the colony count in antibiotic-free agar and agar containing chloramphenicol which permits one to say that all resistant cultures are made up of a variety of cells of different resistances.

In anticipation of some possible objections which might be raised, a discussion is now presented on the rationale of plating a culture grown in 1 mg. chloramphenicol per ml. of broth into 2 mg. per ml. of agar and using the result as evidence for any theory. It might well be said that it would be natural, when going from the
lower antibiotic concentration to the higher, for a large propor-
tion of the cells to be found sensitive to the higher concentra-
tion of drug. However, as table 3 shows a large percentage of
the culture's cells are resistant to 2 mg. chloramphenicol per ml.
of agar. In addition, the culture grown in 1 mg. antibiotic per
ml. of broth can be streaked on an agar plate with 2 mg. chloram-
phenicol per ml. and confluent growth will result in 48 hours.
It can also be seen from table 3 that plate counts in 1 mg. per
ml. are significantly lower than counts in drug-free agar but the
heterogeneous nature of the resistant population is still more
obvious if 2 mg. per ml. are used in the agar.

The data in table 3 also indicate that there is a sensitive
mutant or mutants occurring in a resistant culture even before the
antibiotic is removed. This last fact is absolutely required if
mutation and selection of these sensitive cells is occurring. It
should be noted that the data in this table can also be used to
postulate the arising of more than one sensitive mutant type; how-
ever, this point will be covered in Chapter IV.

If the data in table 3 are taken alone there is more than one
possible explanation of them. However, if the data in table 2 and
3 are viewed together only one explanation is likely, that of
spontaneous mutation to the chloramphenicol-sensitive state. One,
of course, might say that the difference seen in the resistances
of various cells by plating in the drug-free agar may be due to
the differences in the ages of the cells. However, if one does
postulate this idea he must carry it to its ultimate end and also
TABLE 3

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Mg. chloramphenicol per ml.</th>
<th>Resistant Culture Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>223</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>146</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

"o. of plates averaged 15 15 11 4 5 5 3 3 3 5 5

Statistical Analysis

<table>
<thead>
<tr>
<th>&quot;Student's&quot;</th>
<th>P(A-B)</th>
<th>&lt;0.001</th>
<th>&lt;0.001</th>
<th>&lt;0.001</th>
<th>&lt;0.001</th>
<th>&lt;0.001</th>
<th>&lt;0.001</th>
<th>0.003</th>
<th>-</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P(C-B)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>test</td>
<td>P(A-C)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.008</td>
<td>0.005</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* The plate counts did not change even after 6 days incubation at 37°C.
use it to explain table 2. As is seen in this table the resistant cells disappear when the culture is grown a number of times in the absence of the drug. If resistance is related to age then the data in table 2 would indicate that cells of a specific age are disappearing. This explanation is unacceptable since all cultures used were at least 18-hours old and all must have contained cells of every age from 0 to 18 hours.

Another non-genetic explanation for the data in table 3 is based on the fact that the resistant culture reduces chloramphenicol to the less toxic aryl amine (23). This reduction would, of course, lower the effective concentration of the antibiotic to such a point that a non-genetic "deadaptation" could produce the sensitive cells indicated by the results presented in table 3. But, if a non-genetic system were functioning then it would be expected that the cells would lose their resistance even more rapidly when cultured in media free of the antibiotic. As table 2 shows, this is not the case; resistant cells are present in trace amounts even after many transfers in drug-free media.

Another explanation of the sensitive cells seen in table 3 is that the drug itself is inducing the return to sensitivity. This, of course, does not fit the theory (30) since loss of resistance is postulated to occur in the absence of the inducing drug. The data, however, show that it occurs in the presence of chloramphenicol.

These theoretical difficulties are not met if the mutation and selection theory is applied to the evidence so far presented.
One can postulate the occurrence in every resistant culture of one or more sensitive mutant types. The removal of the drug would select in favor of the sensitive cells and they would overgrow the highly resistant cells producing a sensitive culture.

At this point it would be best to define the resistant cells and the sensitive mutants in the light of the evidence so far presented. When speaking of resistant cells only those able to grow in 1 mg. chloramphenicol per ml. of broth are considered. There may be a few cells of greater resistance but the experiments are neither concerned with nor affected by such a possibility. Further, it is assumed that the resistant cells can also produce colonies in agar with 2 mgs. chloramphenicol per ml. Those cells unable to grow in the above concentrations are considered to be sensitive mutants.

The evidence presented so far shows that sensitive cells occur in a resistant culture growing in chloramphenicol-containing media. The next experimental approach was devised to show that these sensitive cells are genetically distinct from the resistant cells. The experiment outlined in figure 2 utilizes two mechanisms to separate and differentiate the sensitive mutants from the resistant cells. The first mechanism involves chloramphenicol's bacteriostatic nature (11, 31). It is believed that chloramphenicol must be bacteriostatic in the concentrations used since if it were bactericidal toward the sensitive mutants they would not have been observed from the very first (table 2 and 3). Further, there is little likelihood that the data to be discussed could have been obtained if
18 hour culture grown in
1 mg. chloramphenicol per
ml. of broth

1 loop

5 ml. broth with 1 mg
chloramphenicol per ml.

5 ml. broth with 1 mg.
chloramphenicol per ml.

1 loop

5 hrs. at 37°C

29 hrs. at 37°C

Dilute and plate

2 000

2400 U. penicillin added per ml.

8000

Dilute and plate

48 hrs. at 37°C

Pick and inoculate colonies into 5 ml. of broth with 1 mg.
chloramphenicol per ml.

24 hrs. at 37°C

Growth

No growth

Figure 2. Model of a representative experiment used to separate
chloramphenicol-sensitive cells from the more resistant cells
using both chloramphenicol and penicillin.

chloramphenicol were bactericidal. The second mechanism is based
on that property of penicillin which permits it to kill dividing
cells alone, leaving resting cells intact (5, 14, 17 and 18).

In the light of the above mechanisms when a sensitive mutant
arises in a chloramphenicol-containing medium it fails to divide
but is still alive. When penicillin is introduced it kills the
dividing or chloramphenicol-resistant cells leaving a residue of
cells unable to divide, that is, cells sensitive to the concentra-
tion of chloramphenicol being used. To show this result a resistant
culture was grown in 1 mg. chloramphenicol per ml. of broth and per-
mitted to grow to a point of most active multiplication (5 hours).
At this point penicillin was introduced (either 2000, 2400, or 3000 U.
per ml.) because penicillin is most efficient in killing actively
dividing cells. These extremely high concentrations of penicillin
are left in contact with the cells for 24 hours at 37°C to be sure
any cells which are able to divide will be killed. After 24 hours
the culture was diluted to a point where the concentration of both
antibiotics was lowered to an insignificant level. This dilution
was plated in drug-free agar and the resulting colonies counted.
Table 4 shows that a rather large number of cells survive this
vigorous treatment.

TABLE 4

Viable cells recovered from a resistant culture growing in 1 mg.
chloramphenicol per ml. of broth and treated with penicillin for

24 hrs. at 37°C.

<table>
<thead>
<tr>
<th>Units per ml. of penicillin</th>
<th>Total viable cells before penicillin</th>
<th>Total viable cells after penicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>$6.7 \times 10^7$ per ml.</td>
<td>$4.13 \times 10^5$ per ml.</td>
</tr>
<tr>
<td>1600</td>
<td>$6.7 \times 10^7$</td>
<td>$4.07 \times 10^5$</td>
</tr>
<tr>
<td>2400</td>
<td>$6.7 \times 10^7$</td>
<td>$2.56 \times 10^5$</td>
</tr>
<tr>
<td>2400</td>
<td>$8.5 \times 10^7$</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>2400</td>
<td>$9.75 \times 10^7$</td>
<td>$9.9 \times 10^4$</td>
</tr>
<tr>
<td>3000</td>
<td>$6.7 \times 10^7$</td>
<td>$1.03 \times 10^5$</td>
</tr>
</tbody>
</table>

Note: these are 2-plate averages the significance of which far
exceeds what is required of such data ($p < 0.001$).
The fact that cells survive the combined penicillin-chloramphenicol treatment indicates they are sensitive. A further step was taken to show conclusively that these were actually sensitive mutants. To obtain the data shown in table 4 the cells surviving the combined antibiotic treatment were plated in drug-free agar. The colonies that resulted, each being the progeny of a particular cell, would have inherited the chloramphenicol resistance of that single cell. When these offspring were tested as to their resistance to chloramphenicol it was found they were sensitive since they no longer produced visible growth in broth containing 1 mg. chloramphenicol per ml. However, when the colonies from cells untreated by penicillin were tested they could grow in 1 mg. chloramphenicol per ml. of broth. Table 5 summarizes a number of experiments utilizing the combined antibiotic technique and the results show that penicillin can separate sensitive mutants from a resistant culture.

It must be emphasized that the colonies tested were grown in agar free of both antibiotics which means the characters they show could not have been induced by either antibiotic. Thus, the colonies generally show the 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 resistant cells. This reasoning also accounts for the resistance of the colonies that resulted from cells not treated by penicillin. It must be repeated that these sensitive mutants are detected in the very presence of chloramphenicol which argues against the induction of resistance or sensitivity by the drug.
### 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.**

<table>
<thead>
<tr>
<th>Units of penicillin per ml. used</th>
<th>Number of colonies picked</th>
<th>No. of colonies that grew in 24-hrs. in 1 mg. chloramphenicol per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>2000 0</td>
<td>39</td>
<td>37</td>
</tr>
<tr>
<td>2400 2400</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>2400 2400</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>2400 2400</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>2400 2400</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>8000 8000</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td>8000 8000</td>
<td>40</td>
<td>9</td>
</tr>
</tbody>
</table>

**Summary**

<table>
<thead>
<tr>
<th></th>
<th>Number growing in chloramphenicol</th>
<th>Number not growing in chloramphenicol</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>60</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>Treated cells</td>
<td>35</td>
<td>140</td>
<td>175</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>144</td>
<td>182</td>
</tr>
</tbody>
</table>

**Statistical Analysis of Summary**

Chi-square (one degree of freedom) ...................... 106.4

P less than ............................................ 0.001
There are a number of points regarding the technique of the penicillin experiment which require a fuller exposition. In the first place one might question the efficiency of the colony-picking technique. It was found, in this regard, that when the colonies were transferred to broth with no antibiotic all produced growth irrespective of the source of the colony. This means that the colony picking technique was efficient and also that all colonies were viable.

Another significant question is whether the penicillin experiment selects penicillin resistant cells which just happen to be chloramphenicol sensitive. This is not the case here since from the very beginning very large doses of penicillin (up to 8000 U. per ml.) were used to prevent selection of penicillin-resistant cells. If this E. coli strain could mutate in one step to a resistance to 8000 U. penicillin per ml. it would be the first occurrence of such a phenomenon. It was found in addition, that the cells either before or after the penicillin treatment were unable to grow in as little as 800 U. per ml. with or without added chloramphenicol. These cells could produce a little growth after three or four days in 400 U. penicillin per ml. which is rather common for E. coli strains. In the light of the above facts it appears that from 3 to 10 times the inhibitory dose of penicillin for this strain was used in the experiment outlined in figure 2. The most convincing evidence which argues against selection of penicillin-resistant cells is the fact that the penicillin sterilizes a culture if no chloramphenicol is present. This means that the
cells selected are determined by the resistance to chloramphenicol and not to penicillin.

In looking again at the data in table 5 it is possible to explain the growth from penicillin-treated cells which resulted in a few cases in broth with 1 mg. chloramphenicol per ml. These few positive cultures where negative cultures should have occurred simply showed that mutation is a two-way street. What seems to have occurred is that sensitive colonies produce a few resistant mutants. The production of resistant cells from sensitive cells would, of course, have to be true for otherwise how could the original resistant strain have been produced? The fact that there are a few positives gives greater support to the mutation theory than if no positives had occurred at all.

When the control experiments in table 5 are examined it is found that a few colonies failed to produce growth in 1 mg. chloramphenicol per ml. of broth when all should have grown. This can be explained on the basis that any resistant culture contains a few sensitive mutants. The few negatives which are seen in the control cultures further confirm the presence of sensitive mutants in a resistant culture which contains chloramphenicol.

So far the evidence indicates that sensitive mutants arise in a resistant E. coli culture. In order that such sensitive mutants render the culture sensitive they must overgrow the resistant cells in media free of chloramphenicol. The evidence presented in table 2 certainly supports this idea. In addition the growth curves presented in figure 3 show that the more sensitive culture can grow
The very presence of chlorophenol, if it were also shown
called to be occurring in a chlorophenol-resistant culture,
in summary it can be said that the evidence showed sensitizatio
The resistance was determined by the 50% Growth Inhibition Test.
the culture had been transferred in the absence of the antibiotic.
the number of times *transfer* refers to the number of times
The average turbidity of these cultures was used for each

at various times in the past two years.
Typical representation of many growth-curves which were plotted
These growth-curves are
that as the resistant strain is transferred in the absence of chloramphenicol the number of sensitive cells increase while the resistant cells decrease. These sensitive mutants were separated from the resistant cells and shown to pass on the sensitivity to their offspring. In all biology there is only one mechanism for passing a character from the parent to the progeny and this involves a genetic transfer. Therefore, the only conclusion that can be drawn is that the character of sensitivity is genetically determined and results from mutation. The sensitive mutants apparently arise in a random fashion and overgrow the resistant cells when chloramphenicol is removed from the medium.
CHAPTER IV

THE MUTANTS INVOLVED IN THE LOSS OF CHLORAMPHENICOL RESISTANCE

Although the mutation-selection theory fits all the observations made in the study of the loss of chloramphenicol resistance, there must be further experimentation to explain fully all the data. In the first place chloramphenicol resistance occurs by a stepwise process; this is strong evidence for the idea that a number of mutations were involved in the acquisition of high levels of resistance. The work of Cavalli and Maccacaro (2) is also excellent evidence for the multiloci-nature of mutations to chloramphenicol resistance in E. coli. Since a number of gene loci seem to be involved in resistance then it should come as no surprise that a number of mutants exist in the same resistant culture and that these mutants differ from each other in their degree of resistance. This chapter will present evidence for the possibility of a variety of sensitive mutants occurring in a resistant E. coli culture.

The first point which requires clarifying is whether the parent sensitive strain plays a role in the loss of chloramphenicol resistance. The parent sensitive E. coli gave rise to the resistant strain and it would seem natural that the reverse might also be true in going from resistance to sensitivity. The evidence, however, shows the parent sensitive strain never appeared in all the numerous transfers that were made of the resistant strain in chloramphenicol-free broth. On re-examining figure 1 it can be seen that
the resistance never fell to the same level as that of the parent sensitive strain even after 48 transfers in the absence of the drug. This evidence was further supported by the discovery that if the parent sensitive cells had occurred the culture would have returned to complete sensitivity in at least three transfers, not 48. This evidence was collected by mixing 25 parent sensitive cells with 25 million resistant cells. This mixture, which favored the resistant cells by a ratio of a million to one, was transferred in drug-free broth and within three transfers the culture had the same resistance as the parent sensitive culture. This means that the sensitive cells easily overgrew the resistant cells within three transfers. Since the resistant culture, when transferred alone in drug-free media, never returned to the low level of resistance exhibited by the parent sensitive culture it must be assumed that the reappearance of the parent sensitive cell is not involved in the loss of chloramphenicol resistance. It was also found, in studying cells from the 33rd "back" transfer, that these cells did not show the characters of the parent sensitive strain even though this transfer is only slightly resistant.

It now appears that the mutant or mutants responsible for the loss of chloramphenicol resistance have characters which lie somewhere between those of the resistant strain and the parent sensitive strain. The next question that arises is how many types of these mutants are there and what are their characteristics?

In referring again to table 3 it can be seen that the data show the possibility of more than one type of mutant present in a
resistant culture. The data here tabulated indicate three types of cells: 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 to produce colonies on drug-free agar. Since only two concentrations of the antibiotic were used to obtain the data in table 3 it became apparent that many more types might have been observed if more concentrations had been used. One method of testing the above possibility involved the use once more of the penicillin experiment with appropriate alterations.

Using the same rationale and techniques as previously described for the penicillin experiment it is assumed that when 1 mg. chloramphenicol per ml. of broth was used those cells resistant to this concentration will be killed by the penicillin. The cells which live through the combined treatment are therefore sensitive to 1 mg. per ml. Now if 0.8 mg. or 0.6 mg. chloramphenicol per ml. is used with penicillin then mutants sensitive to these concentrations should be obtained providing such mutants exist. As the data in table 6 show there is a strong possibility that such varieties of mutants do exist. It will be noted that for every level of chloramphenicol used there was a significant drop in the number of cells recovered. In other words as the level of chloramphenicol is lowered a greater number of more resistant mutants can now grow and be killed by the penicillin.

Further attempts were made to show the true nature of the mutants present in a resistant culture and in drug-free cultures
TABLE 6

Viable cells recovered after a 24-hour treatment with 8000 U. penicillin per ml. and various concentrations of chloramphenicol

<table>
<thead>
<tr>
<th>Mg. chloramphenicol per ml. of broth</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.66 x 10^5 per ml.</td>
<td>3.66 x 10^5 per ml.</td>
</tr>
<tr>
<td>0.8</td>
<td>2.49 x 10^5</td>
<td>9.8 x 10^4</td>
</tr>
<tr>
<td>0.6</td>
<td>4.35 x 10^4</td>
<td>2.36 x 10^4</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>1.57 x 10^4</td>
</tr>
<tr>
<td>0.4</td>
<td>3.35 x 10^3</td>
<td>4.45 x 10^3</td>
</tr>
<tr>
<td>0.3</td>
<td>-</td>
<td>3.58 x 10^3</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Population before penicillin</td>
<td>3.9 x 10^7</td>
<td>3.85 x 10^7</td>
</tr>
</tbody>
</table>

Derived from a resistant culture. One approach involved expanding the experiment presented in table 3 to include not only a wider range of antibiotic concentrations but also "bask" transfers of the resistant culture. Table 7 presents data from such an approach.

To obtain the data for table 7 the various cultures were diluted and then plated in agar with various concentrations of chloramphenicol and in agar without any drug. The drug-free plates are the controls which show the total viable cells per ml. in the culture being analyzed. The plates with the drug were compared with the drug-free plates and it was seen that at certain drug-levels
Representative experiments illustrating the possible separation of resistant groups found in a resistant culture or cultures derived from a resistant culture, using agars with a variety of chloramphenicol concentrations. (In per cent of total population as compared to antibiotic-free plate counts).

| Exp. | Subcultures in antibiotic-free broth | Mg. chloramphenicol per ml. of agar | 1.8 | 1.7 | 1.6 | 1.4 | 1.2 | 1 | 0.8 | 0.7 | 0.6 | 0.4 | 0.2 |
|------|-----------------------------------|-----------------------------------|-----|-----|-----|-----|-----|---|-----|-----|-----|-----|-----|-----|
| 1    | 0                                 |                                   | 3.7%| 51% | 67% | 59  | 55  | 59 | 55  | 55  | 55  | 55  | 55  |
| 2    | 4                                 |                                   | 33  | 45% | 59  | 59  | 55  | 55 | 55  | 55  | 55  | 55  | 55  |
| 3    | 1                                 |                                   | 68  | 68  | 68  | 68  | 68  | 68 | 68  | 68  | 68  | 68  | 68  |
| 4    | 2                                 |                                   | 56  | 55  | 55  | 55  | 55  | 55 | 55  | 55  | 55  | 55  | 55  |
| 5    | 1                                 |                                   | 56  | 56  | 56  | 56  | 56  | 56 | 56  | 56  | 56  | 56  | 56  |
| 6    | 2                                 |                                   | 0   | 0   | 0   | 0   | 0   | 0  | 0   | 0   | 0   | 0   | 0   |

| Exp. | Subcultures in antibiotic-free broth | Mg. chloramphenicol per ml. of agar | 1.8 | 1.7 | 1.6 | 1.4 | 1.2 | 1 | 0.8 | 0.7 | 0.6 | 0.4 | 0.2 |
|------|-----------------------------------|-----------------------------------|-----|-----|-----|-----|-----|---|-----|-----|-----|-----|-----|-----|
| 1    | 0                                 |                                   | 68  | 72  | 90% | 86% | 93% | 93%| 93% | 93% | 93% | 93% | 93% |
| 2    | 7                                 |                                   | 5   | 8   | 13  | 13  | 13  | 13 | 13  | 13  | 13  | 13  | 13  |
| 3    | 6                                 |                                   | 0   | 0   | 37  | 37  | 37  | 37 | 37  | 37  | 37  | 37  | 37  |
| 4    | 0                                 |                                   | 72  | 76  | 87  | 95% | 103 | 103| 103 | 103 | 103 | 103 | 103 |
| 5    | 3                                 |                                   | 71  | 71  | 71  | 71  | 71  | 71 | 71  | 71  | 71  | 71  | 71  |
| 6    | 2                                 |                                   | 65  | 65  | 65  | 65  | 65  | 65 | 65  | 65  | 65  | 65  | 65  |
| 6    | 3                                 |                                   | 9   | 9   | 9   | 9   | 9   | 9  | 9   | 9   | 9   | 9   | 9   |

| Exp. | Subcultures in antibiotic-free broth | Mg. chloramphenicol per ml. of agar | 1.8 | 1.7 | 1.6 | 1.4 | 1.2 | 1 | 0.8 | 0.7 | 0.6 | 0.4 | 0.2 |
|------|-----------------------------------|-----------------------------------|-----|-----|-----|-----|-----|---|-----|-----|-----|-----|-----|-----|
| 2    | 7                                 |                                   | 47  | 47  | 47  | 47  | 47  | 47 | 47  | 47  | 47  | 47  | 47  |
| 5    | 6                                 |                                   | 13  | 13  | 13  | 13  | 13  | 13 | 13  | 13  | 13  | 13  | 13  |
| 6    | 2                                 |                                   | 32  | 32  | 32  | 32  | 32  | 32 | 32  | 32  | 32  | 32  | 32  |
| 6    | 3                                 |                                   | 84  | 84  | 84  | 84  | 84  | 84 | 84  | 84  | 84  | 84  | 84  |

| Exp. | Subcultures in antibiotic-free broth | Mg. chloramphenicol per ml. of agar | 1.8 | 1.7 | 1.6 | 1.4 | 1.2 | 1 | 0.8 | 0.7 | 0.6 | 0.4 | 0.2 |
|------|-----------------------------------|-----------------------------------|-----|-----|-----|-----|-----|---|-----|-----|-----|-----|-----|-----|
| 3    | 6                                 |                                   | 28  | 28  | 28  | 28  | 28  | 28 | 28  | 28  | 28  | 28  | 28  |
| 5    | 6                                 |                                   | 41  | 41  | 41  | 41  | 41  | 41 | 41  | 41  | 41  | 41  | 41  |
| 6    | 2                                 |                                   | 79  | 79  | 79  | 79  | 79  | 79 | 79  | 79  | 79  | 79  | 79  |
| 6    | 3                                 |                                   | 84  | 84  | 84  | 84  | 84  | 84 | 84  | 84  | 84  | 84  | 84  |

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the count decreases. For example, from the top line of the data in table 7 it can be seen that 67 per cent of the cells grew in agar with 1.6 mg. chloramphenicol per ml. and 51 per cent in 1.8 mg. when compared to a drug-free plate count. However, in plates with 2 mg. per ml. the counts dropped to 3.7 per cent. These data can be interpreted to mean a type of cell existed which was resistant to 2 mg. chloramphenicol per ml. of agar but these cells constitute only 3.7 per cent of the culture. On the other hand 51 per cent of the culture could grow in 1.8 mg. per ml., which means a second type existed that was resistant to 1.8 but not to 2 mg. per ml. It will be noticed that significant drops in count did not occur at every level of the drug used. This indicated that only certain concentrations of the antibiotic separated the various types of cells found in a resistant culture. From table 7 these concentrations appear to be 2, 1.6, 1.2, 0.4 and 0.2 mg. per ml. These same concentrations also show the limit of the various ranges of the different resistant types. Table 7 is so constructed that the data indicating a specific mutant type are grouped together. Thus the evidence for the possibility of mutant type II and resistant type I existing in the same culture is seen in the first five lines of the table. The next series of data (lines 6 to 12) show the evidence for a separation between mutant types II and III. It is to be noticed that after the ranges for each mutant type had been defined the large number of drug concentrations were discarded in favor of a few representative concentrations. Granted that the data in table 7 and the explanation of
The absence of the antibiotic was transferred to the culture filtrate of the culture filtrate (Figure 4).
Figure 4 presents a number of representative experiments which show the types of cells found in various drug-free subcultures of the resistant E. coli. Those numerals which are followed by a question mark indicate the possibility that these types are present but cannot be detected by the plate count technique.

Both table 7 and figure 4 indicate that there may be as many as 6 different types of cells involved in the loss of chloramphenicol resistance. Further, it can be seen that the least resistant type of cell gradually replaces the more resistant types. One by one the cell types of higher resistance are overgrown by a cell type of a lower resistance until the culture becomes rather sensitive when compared with its original resistance. This ability of the more sensitive cell to overgrow the more resistant cell is confirmed by the growth curves seen in figure 3.

The data presented in this chapter indicate that there may be more than one type of sensitive mutant arising in a chloramphenicol-resistant culture. The same data also show the stepwise manner by which the most sensitive mutant eventually replaces the more resistant cells. It is readily admitted that more experimentation is still required to prove the nature and numbers of the various mutants which appear to occur in a resistant culture; however, it seems at least justifiable to say that more than one type of sensitive mutant is involved.
CHAPTER V

CONCLUSION

The evidence presented in this dissertation shows the loss of chloramphenicol resistance in \textit{E. coli} as resulting from a mutation or mutations of the resistant to the sensitive cell. How and why such a mutation occurs is still unknown; however, this chapter will discuss a few possible explanations of how the mutation might have occurred.

It is now known that a resistant culture of \textit{E. coli} multiplies in the presence of 1 mg. chloramphenicol per ml. of broth and in the process produces sensitive mutants for reasons still unknown. The sensitive mutant cells are favored in broth or agar with no antibiotic and they overgrow the resistant cells lowering the average resistance of the culture to a rather low level. Even though these mutants appear in large numbers in the culture which contains chloramphenicol the resistant cells are still favored in such a medium and the high resistance of the culture is maintained. Apparently more than one mutant type is produced in the resistant culture which results in a very complex picture of mutation, a picture not easily proved.

The large number of mutant cells which occur in the presence of chloramphenicol is unusual in that mutants are relatively rare. However, in a chloramphenicol system it is known that reduction of the drug to the aryl amine produces a
But these would be more difficult to produce and hence result in these
mutations would require a number of simultaneous genetic changes.

The very sensitive mutants require from only one genetic change would be the most
mutant which requires more than one genetic change. The sensitive
mutant occur which be due to the difficulty of producing these

The fact that more mutants of the heterozygous than of the homozygous

\[ \text{MUTANT III, etc.} \]

\[ \text{RESISTANT CELLS} \]

\[ \text{MUTANT II} \]

\[ \text{MUTANT I} \]

in the following manner:

or most of them in the following manner:

of sensitive mutant is that the resistant cell the the occurrence of the various types

another explanation for the occurrence of the various types

\[ \text{RESISTANT CELLS} \]

\[ \text{MUTANT II} \]

\[ \text{MUTANT I} \]

in a stepwise manner:

of course the mutation occurs in a stepwise manner:

resistant cell but rather from the precedure sensitive mutant

other sensitive mutants do not come directly from the latter

and in the target number could well indicate that the

the fact that the more resistant of the sensitive mutants occurs

creases their numbers above what is normally seen in mutation

medium in which the sensitive mutants can divide and thus in
It is believed by some that when a large variety of mutants occur in great numbers that this is not a true nuclear mutation but a mutation of "cytoplasmic genes" (28). The cytoplasmic gene theory holds that all the genetic material is not in the nucleus of the cell but some is scattered in the cytoplasm (9, 32 and 34). This theory could easily explain the variety of mutants seen in a chloramphenicol-resistant E. coli culture. If the loss of one cytoplasmic gene for each mutation is the reason for the occurrence of five sensitive mutants seen in table 7 then there would be five such genes responsible for complete resistance to the antibiotic. When the cell divides then each of these genes also divides so there are ten such genes just before the two daughter cells separate from each other. But what happens if these cytoplasmic genes are not equally distributed to the two daughter cells? The most likely answer would be that the daughter cells would not be of equal resistance. If the new daughter cells received the full complement of five genes then they would be fully resistant. On the other hand if one or more of the genes were not equally distributed to each of the daughter cells than the cell with less than five genes would be more sensitive. The accident of division which would result in the loss of two genes would seem rarer than that which caused the loss of one gene. For the accident to result in the loss of all five genes would seem to be extremely rare. The fact that the most sensitive mutant occurs in the fewest numbers in a resistant population might be thus explained. Of course, if one daughter cell loses a gene than the other daughter cell would
have one too many. This would either result in a cell of higher resistance or a cell fully resistant. Since these cytoplasmic genes are postulated to be the controlling mechanism for a little used adaptive enzymes the loss of such enzymes would not be fatal to the cells in their normal environment.

If, on the other hand, the sight of the mutation is in the nucleus of the cells then the genes responsible for chloramphenicol resistance must be very unstable and change to some other chemical configuration rather easily. Since very little is known of the chemistry of the gene there is very slight evidence to support this idea. At the present writing the real cause for mutation does not seem to be close to solution.

Practical implications of the loss of chloramphenicol resistance. The experiments reported here using both chloramphenicol and penicillin indicate that this combination could cause unfortunate results when used in therapy. Since penicillin acts best on rapidly growing bacteria any substance such as chloramphenicol which prevents bacterial division thereby interferes with penicillin’s killing action. After both antibiotics have been excreted from the body the infective bacterium may well return to its deadly work.

The data in this dissertation show how easily E. coli loses resistance to chloramphenicol and other reports show the same result with strains of *Staphylococcus pyogenes* var. *aureus* (24). Thus it would appear that intermittent therapy might easily prevent the persistence of such infective bacteria for when a
resistant strain occurred then perhaps a discontinuation of the chloramphenicol therapy would result in the recurrence of a sensitive strain. If this be so then a return again to the chloramphenicol therapy might well find the once resistant bacterium sensitive.

From the work presented here and elsewhere (22, 23) there seems to be little to fear from chloramphenicol-resistant bacteria since they are produced with difficulty. In addition, when bacteria do become resistant they seem to be less metabolically efficient. This would materially aid the body in overcoming the infection.

By far the greatest single practical contribution this work might make is to help solve the problem of the prevention of drug resistance in some infective organisms. Although there is no evidence in this dissertation which would indicate the mechanism by which drug-resistant mutants arise, it is the author's hope that perhaps the data presented may lead some other worker to the final solution.
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Name in full: Ernest Carl Herrmann, Jr.

Permanent address: 3431 Dupont Avenue, Baltimore 15, Maryland

Degree to be conferred: Doctor of Philosophy Date: June 6, 1953

Date of Birth: January 1, 1925

Secondary Education: Baltimore City College

Collegiate Institutions Attended Dates Degree Date of Degree
University of Maryland (College Park) 1946-50 B.S. June, 1950
University of Maryland (Baltimore) 1950-51 M.S. Aug., 1951
University of Maryland (Baltimore) 1951-53 Ph.D. June, 1953

Publications:


Positions held:
Veteran World War II 1943-1946 (Bos'n Mate Second Class USN)
Laboratory Assistant, National Dairies Research Laboratories
Laboratory Assistant, Western Maryland Dairy
Bressler Research Fellow, University of Maryland
Research Fellow under an Atomic Energy Commission grant to the University of Maryland

(Prospective position) Research Associate, Department of Microbiology, Rutgers University, New Brunswick, New Jersey