

RELATIONSHIP BETWEEN "CHLORAMPHENICOL REDUCTASE ACTIVITY" AND
CHLORAMPHENICOL RESISTANCE

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To my WIFE and SON

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INTRODUCTION

The present-day widespread use of antibiotics poses two outstanding problems. The first of these is the direct or indirect effects of the antibiotics on the cellular functions of the body. The second problem involves the complications of bacterial resistance to the antibiotic developed on exposure of bacteria to the antibiotic. An approach to both problems should include the determination of the mode of action of the antibiotic and the elucidation of the nature of the antibiotic-resistance mechanisms in bacteria. It is felt that the solution of one of these problems may hold the key to the solution of the other problem.

The present studies were undertaken in an attempt to describe the nature of chloramphenicol resistance developed in strain (B) of Escherichia coli and strain (1A) of Micrococcus pyogenes var. aureus. The magnitude of the original problem necessitated a more concentrated effort utilizing only one of these species, Escherichia coli (B).

Coffey and coworkers (1) have performed in vitro studies on bacterial resistance to chloramphenicol and have found that the resistance increased in a graduate manner as the chloramphenicol concentrations in the growth-media were increased. The resistance of a strain of E. coli was raised 125 fold, and this resistance was found to be relatively stable. Coffey's group was unable to show any correlation between increased enzymatic activity and increased resistance.

A fairly extensive study on the enzymatic reduction of chloramphenicol has been made by Smith and Worrel (2). These studies,

however, involved only chloramphenicol-sensitive strains of bacteria. Their data tend to implicate a dehydrogenase mechanism in the reduction of chloramphenicol, but no attempt was made to determine what effect the enzymatic reduction had on the chloramphenicol resistance mechanism.

A considerable amount of work has been done to locate the site of action of this antibiotic, but, as in the case of studies on most of the other antibiotics, the results generally obtained leave us with two interpretations: the results are either conflicting and vary with varying conditions, or the results are all good but the correlating factors which would crystallize these data are missing.

It is not possible, nor is it the desire of this author, to cite all the experiments implicating certain modes of action of this particular antibiotic. The widely scattered results might even imply multilocal action. It is helpful, however, to list a few of the experiments to demonstrate the diverse nature of chloramphenicol action, which tends to complicate the chloramphenicol-resistance picture.

Egami, et al. (3), using cell-free bacterial extracts to carry out the reduction of chloramphenicol, have submitted evidence which suggests that the enzyme system responsible for this reduction may be identical with the nitrite reductase system.

Studies on the mode of action of chloramphenicol at therapeutic levels by Smith, Worrel and Swanson (4) indicated that this antibiotic inhibited bacterial esterases. Distinct responses were observed at various concentrations of chloramphenicol. At low concentrations the drug had no effect on the esterase activity of E. coli. With higher concentrations a slightly inhibitory effect was observed. This was

followed by marked activation of the esterase activity. In the fourth concentration range there was a marked inhibition of both growth and esterase activity. No attempt was made to determine whether or not the inhibition of esterase activity was a primary reaction.

On the basis of antimetabolite studies with chloramphenicol on a strain of E. coli, Woolley (5) postulated that chloramphenicol acts as an inhibitor of a vital process in which phenylalanine is transformed into a metabolite essential for cell multiplication. Woolley's work can be criticized on the grounds that he worked at relatively low concentrations of antagonist and antibiotic ($2\gamma/ml$), and did not obtain a complete picture since phenylalanine would not antagonize higher concentrations of chloramphenicol. This factor does not discount the possibility that Woolley's observations, along with somewhat similar observations by other investigators, may actually represent a primary site of action; but the antagonistic action could only account for a relatively low stage of resistance.

Molho and Molho-Lacroix (6, 7) have also directed their studies on the inhibitory effects and on the enhancing effects of certain structural analogs of chloramphenicol. They apparently have been unable to demonstrate that phenylalanine will antagonize the action of chloramphenicol on E. coli. They have shown, however, that resistance is associated with a decrease in the synthesis of phenylalanine. Molho and Molho-Lacroix take the view that the resistance produced in E. coli apparently involves the development of a new enzyme system which can effect the hydrolytic cleavage of chloramphenicol, thus inactivating it. This theory of the development of resistance needs considerably more

study, since chloramphenicol apparently is inactivated in a number of different ways in different organisms (8). The conditions under which resistance is developed would, to a large extent, determine the nature of the inactivation processes. Accordingly, it has been reported (8) that *E. coli* preferentially inactivates chloramphenicol by first reducing the nitro group to an amine group.

Other investigators have probed farther ahead, and have observed the end effects of this antibiotic on protein synthesis (9). These investigators have reported that chloramphenicol affects the formation of adaptive enzymes in one instance (10), and in another (11) chloramphenicol causes the accumulation of mononucleotides in bacterial cells. It is my opinion that results of this nature only tend to show the end effects of chloramphenicol action; and, although the problem may be complicated because it apparently touches on protein synthesis, the more basic steps in the mode of action and in the development of resistance require more thorough investigation. Such a study may eventually contribute a small link to the complex problem of protein synthesis.

Metabolite studies in relation to antibiotics are usually designed to uncover a specific metabolic function which is adversely affected by the presence of the antibiotic. Development of resistance to what could now be called the "antimetabolite" would involve either the development of a new metabolic pathway bypassing the antibiotic effect, or the production of enzymes to inactivate the antibiotic, or a combination of both of these activities.

The present paper attempts to demonstrate a correlation between the enzymatic reduction of chloramphenicol and the development of

chloramphenicol resistance. It was decided to approach this problem first by qualitatively following the development of resistance, and secondly by a more specific study of a chemical reaction which is measurably altered by the manifested resistance. The latter study was designed eventually to be directed toward the determination of the primary site of action of this antibiotic. The present paper, therefore, contains only one phase in the study of the in vitro effects of chloramphenicol on bacteria.

Although no specific attempt has been made to define the inherent nature (adaptive or selective) of the resistance which was developed, certain of the data can be interpreted as suggesting the function of both basic mechanisms.

MATERIALS

Chloramphenicol (Chloromycetin*)

The actinomyces responsible for the production of chloramphenicol was originally isolated from a soil sample by Dr. Burkholder's laboratory at Yale University. Eventually the organism was shown to be Streptomyces venezuelae.

The isolation and biological determination of chloramphenicol have been carried out mainly in the Parke, Davis and Company laboratories (12), and independently by Gottlieb, et al. (13) at the University of Illinois.

* Parke, Davis & Co. trade name.

Crystalline chloromycetin has been found to be active in vitro against most Gram-negative bacteria and moderately active against most Gram-positive bacteria including Mycobacterium tuberculosis. It is relatively inactive against Clostridia, Pseudomonas, yeast and fungi. In vivo the crystalline compound has been reported to be active against Rickettsia and moderately active against Klebsiella and Shigella. It has been found to be relatively inactive in vivo against pneumococci and streptococci. More recently the known effective range of this antibiotic has been expanded considerably.

The chemical structure of the chloramphenicol molecule has been determined by the Parke, Davis & Company research group (14 - 16) to be D- (-)-threo-2-dichloroacetamido-1-p-nitro-phenyl- 1,3-propanediol.

Chloramphenicol is a relatively stable neutral compound having a sharp melting point (150.1°C), soluble in many organic solvents, but sparingly soluble in water. It is optically active, $[\alpha]^{25}_D + 19^\circ$ in ethanol and -25.5° in ethyl acetate. It contains carbon, hydrogen, nitrogen, oxygen, and non-ionic chlorine. The molecular weight is about 310. The empirical formula is $C_{11}H_{12}Cl_2N_2O_5$.

Media

Brain heart infusion (Difco)

Monod's salt-glucose medium* (17)

Sorensen's buffer solution*, $Na_2HPO_4/KH_2PO_4 = 4/1$

"Chloramphenicol reductase medium", (note Table 1)

* Reagent Grade chemicals.

TABLE I

Composition of "Chloramphenicol-Reductase" Medium
(Nitrogen-Free)

		<u>Molar conc.</u>
Na ₂ HPO ₄	-----	5.4 x 10 ⁻²
KH ₂ PO ₄	-----	3.2 x 10 ⁻²
MgSO ₄ ·7H ₂ O	-----	8.0 x 10 ⁻⁴
CaCl ₂	-----	9.0 x 10 ⁻⁵
FeSO ₄ ·7H ₂ O	-----	2.0 x 10 ⁻⁶
Glucose	-----	2.0 x 10 ⁻³
Chloramphenicol	-----	3.1 x 10 ⁻⁴

Apparatus

All measurements of growth were made on a Klett-Summerson photoelectric colorimeter using the red, #66 filter.

pH measurements were made with a Beckman, Model G, pH meter.

Polarographic results were obtained with a manually operated Fischer Electrode which was equipped with a mercury stand-tube, saturated calomel cell, KCl-agar bridge, an electrolytic cell prepared by cutting the length of a 13 x 100 mm test tube to approximately 50 mm, a constant temperature water bath, maxima suppressors, commercial nitrogen, and pyrogallol to remove the oxygen that may be in the nitrogen tank.

EXPERIMENTAL

Development of Chloramphenicol Resistance

Chloramphenicol resistance was developed in strain (B) of Escherichia coli and in strain (1A) of Micrococcus pyogenes var. aureus

by three different procedures in the case of E. coli and two procedures in the case of M. pyogenes. One procedure involved a slow process in which the parent strains of E. coli and M. pyogenes were transferred (standard loop) daily into brain heart infusion medium or Monod's salt-glucose medium (E. coli only) containing a certain concentration of chloramphenicol for one week. At the end of the week the resistances of the cultures were determined by the Army Medical Department Research and Graduate School adaptation* of the Joslyn and Galbraith (18) turbidimetric assay method. In the present experiments the Klett-Summerson colorimeter was used to measure turbidities, and the total volume of solution used in the assay tubes was reduced to 5 ml. Relative resistances are reported as the amount of chloramphenicol which will inhibit the growth of a culture 50% as compared to the growth of that same culture in the absence of the antibiotic. This is referred to as the 50% inhibition concentration and is expressed in γ/ml .

The culture which was used to determine resistance at the end of a week of daily transfers was then inoculated into an increased amount of chloramphenicol and the above process was repeated.

In the course of the above development procedure it was decided to "force" the resistance to a high level by making daily, large increases in the chloramphenicol concentrations in brain heart infusion medium. This was done with both species being studied. A similar approach was used in the later stages of the present experiments to study the first step in the development of resistance of E. coli (B).

* Printed document.

One other procedure was used in the development of chloramphenicol resistance in E. coli (B). That was to take an E. coli culture from the regular "training" procedure (slow increases in chloramphenicol) and subculture this in brain heart infusion medium containing a constant amount of chloramphenicol (40 γ /ml) for approximately 13 weeks.

Determination of "Chloramphenicol Reductase Activity"

The particular culture for which the reductase activity was to be determined was transferred to 20 ml of brain heart infusion medium and allowed to grow for 15-18 hours in the absence of chloramphenicol. Each culture was harvested by centrifugation from the brain heart infusion medium and washed two times with cold Sorensen's buffer at pH 7.35 or pH 7.42. After the last washing the cells were resuspended in 6 ml of the so-called "chloramphenicol reductase medium", or in brain heart infusion medium containing 100 γ /ml chloramphenicol, at a turbidity of 50. The cultures were kept in ice baths until all dilutions were complete. These cultures were then placed in a water bath at 37°C along with a control tube containing only the "chloramphenicol reductase medium" or brain heart medium plus chloramphenicol. These tubes were then incubated for various periods of time. At the end of the specific incubation periods the turbidities were again checked; the solutions were chilled in ice water and the majority of the cells were removed by centrifugation at 5000 RPM for 10 minutes. The amount of chloramphenicol remaining in the supernatant solutions was then determined either by the turbidimetric bioassay method of Joslyn and Galbraith (18), or by the polarographic method described in the next section. The percent reduction of chloramphenicol was then calculated by comparison with the concentrations in the control tubes.

Polarographic Method

The determination of the chemical structure of chloramphenicol indicated that the compound contained a p-nitro group. Since organic nitro compounds are reducible at the dropping mercury electrode (19), the possibility of applying the polarographic technique for the routine determination of chloramphenicol in culture broths arose.

The polarographic procedure used to determine chloramphenicol has been developed independently by the author after the earlier initiation of a similar procedure by Hess (20). Hess has made a general survey of the polarographic behavior of both chloramphenicol and the hydrolysis product, 1-p-nitrophenyl-2-amino-1,3-propanediol, and has also presented a method for the routine analysis of chloramphenicol using the polarographic technique. Since the present procedure contains several modifications of the Hess procedure, it will be described below.

After removal of the majority of the bacterial cells from the culture medium or from the "chloramphenicol reductase medium" by centrifugation as described in the previous section, the pH of 3 ml portions of the supernatants were adjusted to 7.2. This is the pH value at which the standard curves have been obtained. The pH of the solutions were adjusted by adding minute amounts of 1:1 HCl or 30% NaOH. In this manner the final volumes were not appreciably altered.

The test solutions were then poured into a specially made test tube (13 x 50 mm) and then placed in a holder in a constant temperature bath ($25^{\circ} \pm 0.5^{\circ}\text{C}$). One drop of methyl red was added as a maxima suppressor in the brain heart infusion solutions, and one drop of basic fuchsin was found to be an effective maxima suppressor for the synthetic solutions. Oxygen-free nitrogen was then bubbled through the solutions for 5 minutes to remove the dissolved oxygen.

A Fischer manually operated Electrode was used to obtain all of the polarographic curves. A drop time of 3 seconds was always used. No attempt was made to determine either the diffusion current or the value for $i^{2/3}t^{1/6}$, since all values are reported on a comparative basis with respect to standard values obtained with known concentrations of chloramphenicol under exactly similar conditions. The voltage range generally employed was restricted to -0.1 to -0.8 volts vs. the saturated calomel electrode.

Several uncontrollable factors probably entered into the above method due to the accumulation of certain metabolic products in the growth media, but these factors apparently did not cause serious alterations of the diffusion current.

From time to time the polarographic results were compared with the turbidimetric bioassay procedure and good agreement was always obtained. Since the intact chloramphenicol molecule is necessary for antibacterial activity, the above comparison indicated that the reduction of the intact chloramphenicol molecule was being measured by the polarographic technique and not the hydrolytic product of chloramphenicol.

RESULTS

When chloramphenicol resistance was developed in strain (B) of Escherichia coli in Monod's synthetic medium and in strain (1A) of Micrococcus pyogenes var. aureus in brain heart infusion medium by the slow stepwise procedure the resistance generally increased in a gradual manner. E. coli (B) in brain heart infusion medium showed a more rapid increase in resistance than it did in Monod's synthetic medium, but the increase

went in somewhat stepwise manner. Fig. 1 illustrates these general trends as resistance was developed.

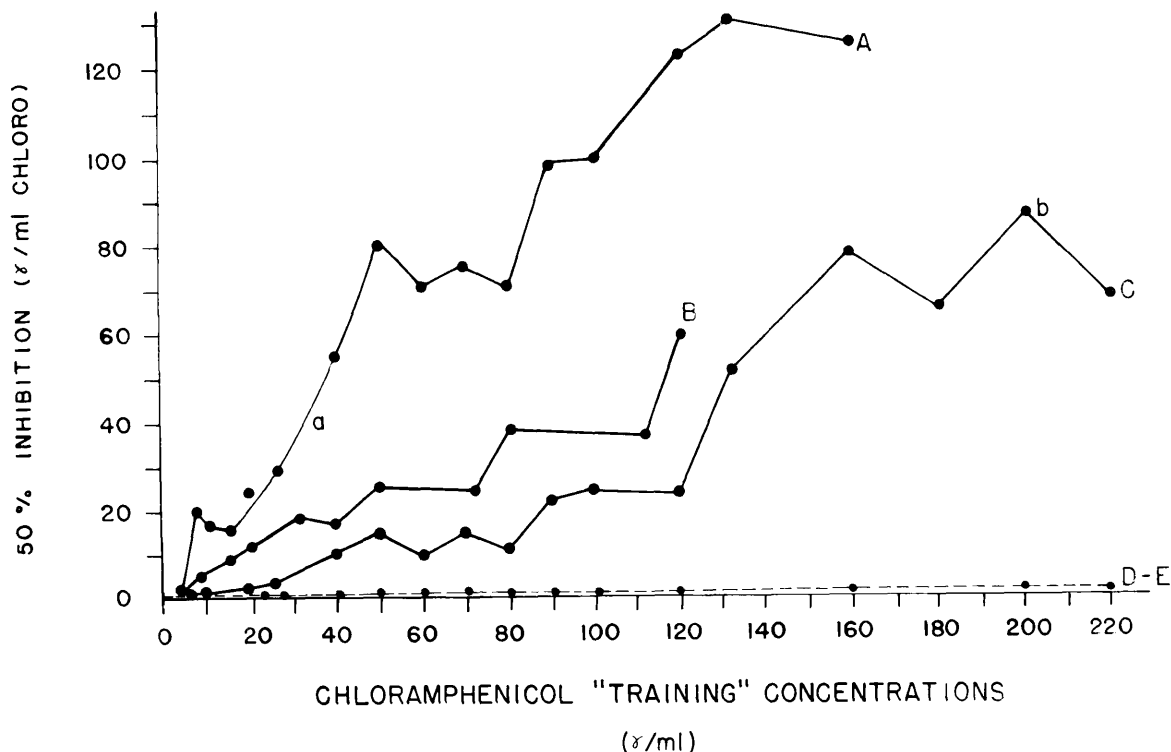


Fig. 1. Development of chloramphenicol resistance.

- A, E. coli (B) "trained" in brain heart infusion medium.
 B, E. coli (B) "trained" in Monod's synthetic medium.
 C, M. pyogenes var. aureus (1A) "trained" in brain heart infusion medium.
 D & E, Curves for the parent sensitive strains fall too close to the abscissa to be differentiated. Actually E. coli (B) maintains a 50% inhibition of approx. 0.1 γ /ml and M. pyogenes var. aureus (1A) maintains its 50% inhibition of approx. 0.5 γ /ml throughout the entire "training" period when not in contact with chloramphenicol.

Note: "Training" refers to the procedure used but is not necessarily meant to imply an adaptive mechanism.

Resistance was developed more easily in E. coli than in M. pyogenes, and chloramphenicol resistance was more readily produced in brain heart infusion medium than in Monod's synthetic medium.

In each case control culture of the parent sensitive strains were treated exactly as the resistant cultures with the one exception that the controls were never brought into contact with chloramphenicol during the transfer procedures.

By disregarding the regular "training" procedure at point (a) in the case of E. coli and at point (b) in the case of M. pyogenes (note Fig. 1) and transferring inocula from these cultures by the "forced training" procedure it was possible to obtain cultures which would grow in 2 mg/ml of chloramphenicol (maximum solubility). The values for the resistances of these cultures, in terms of 50% inhibition concentrations, were 800 to 1000 γ /ml and 96 to 150 γ /ml for E. coli and M. pyogenes respectively. The values for the 50% inhibition of the "highly" resistant organisms cannot be stated with the degree of accuracy which is desired. The resistances at these points were not very stable, and in order to maintain the resistance at a high level it was necessary to transfer these cultures at least every 48 hours into fresh brain heart infusion media containing 2 mg/ml of chloramphenicol. Storage of these cultures on nutrient agar slants, with or without the addition of chloramphenicol, caused a rapid loss of resistance. As will be demonstrated later, a relatively rapid loss of resistance can be effected by subculturing the resistant cultures in brain heart infusion medium which does not contain any added chloramphenicol. The nature of this instability in resistance has been the subject of an investigation by another member of our research

group. A preliminary report on a possible interpretation of the instability has been presented by Herrmann (21) at the 1952 meeting of the Society of American Bacteriologists.

Varying conditions such as glucose concentration, O₂ and CO₂ pressures, and the presence of certain nutrients apparently cause rather extreme fluctuations in both the development and the loss of resistance.

A third procedure was introduced in the development of chloramphenicol resistance in E. coli. In this procedure an inoculum was removed at point (a), Fig. 1, and was then transferred daily in brain heart infusion broth containing a constant concentration of chloramphenicol (40 γ /ml) for 90 days. By this method the resistance was found to fluctuate about a constant level (60 γ /ml) but would not increase above this level until the chloramphenicol concentration was increased.

E. coli "trained" in Monod's synthetic medium followed much the same pattern obtained for M. pyogenes in brain heart infusion medium.

Both strains, whether transferred in the brain heart infusion broth or in Monod's synthetic medium, exhibited a pronounced decrease in growth (even in the absence of the antibiotic) as the resistance was increased. This is demonstrated in Fig. 2.

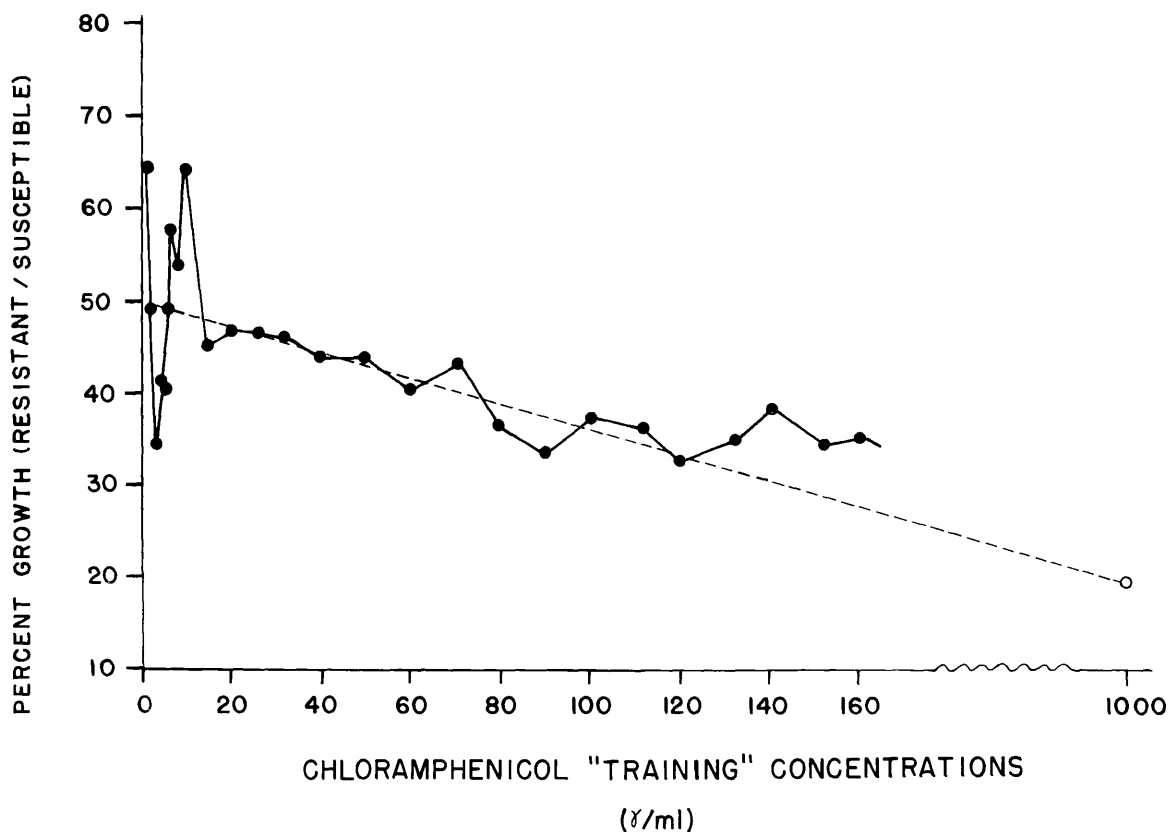


Fig. 2. Decrease of *E. coli* (B) growth with increasing concentrations of chloramphenicol during the "training" procedure. The growth is expressed as the percent of the average of 7 daily turbidity readings of cultures transferred in a particular concentration of chloramphenicol in brain heart medium compared to similar averages for the parent susceptible culture transferred in the absence of the antibiotic.

As resistance was developed certain morphological changes took place in the organisms. A general phenomenon observed with both species is a marked increase in the size of the individual cells, and a tendency toward pleomorphism. In addition, resistant *M. pyogenes* cells lose their tendency to form clusters and generally exist in the liquid media as single or paired organisms.

Another phenomenon which is very characteristic of the development of resistance is the tendency for the resistant cells to coagulate in the liquid media and settle to the bottom of the culture tubes.

Comparative biochemical studies on the resistant E. coli (1000) (E. coli (B) "trained" to grow in 1000 γ /ml of chloramphenicol) and on the parent sensitive strain of E. coli indicated that the development of resistance had caused changes in the metabolic activities of the resistant cultures. In most instances, however, these changes appeared to be only quantitative with perhaps a few exceptions in which the lags in the activity of the resistant culture were so great as to make the differences appear to be qualitative. This apparently was the case in the fermentation of maltose and dulcitol which appeared to be qualitatively different in the resistant and parent sensitive strains. The instability of the resistant cultures probably contributed to the difficulty of obtaining very conclusive qualitative data. There was never any assurance that the results which were observed in these comparative studies were due to fully resistant organisms or to partly resistant or sensitive organisms in the cultures. The comparative studies which were made have been reported elsewhere (22).

A characteristic which apparently had the potentiality for varying with varying resistance, and which would lend itself conveniently to experimental procedures, was the ability of E. coli to reduce the nitro group on the chloramphenicol molecule (2) ("chloramphenicol reductase activity"). The possibility existed that an increase in this enzymatic activity might contribute to the development of resistance.

It was originally thought that if the "chloramphenicol reductase activity" was the primary factor in resistance, a detectable amount

of reduction should occur in the culture tubes used to determine resistance (sensitivity tests). Since the sensitivity tests were usually stopped when the control tube without chloramphenicol reached a turbidity of 60, the chloramphenicol concentration which just permitted half of this growth was exposed to a relatively small amount of cells for a relatively short period of time. A turbidity of 30 meant that the chloramphenicol-cell ratio and the chloramphenicol-cell contact time were rather low, and by the technique first employed (turbidimetric bioassay (18)) no measurable reduction of chloramphenicol was observed until the cultures reached their maximum growth stage.

With the introduction of the polarographic technique for the determination of chloramphenicol it appeared as if the reduction of the chloramphenicol could be detected at an earlier stage.

Fig. 3 summarizes these observations.

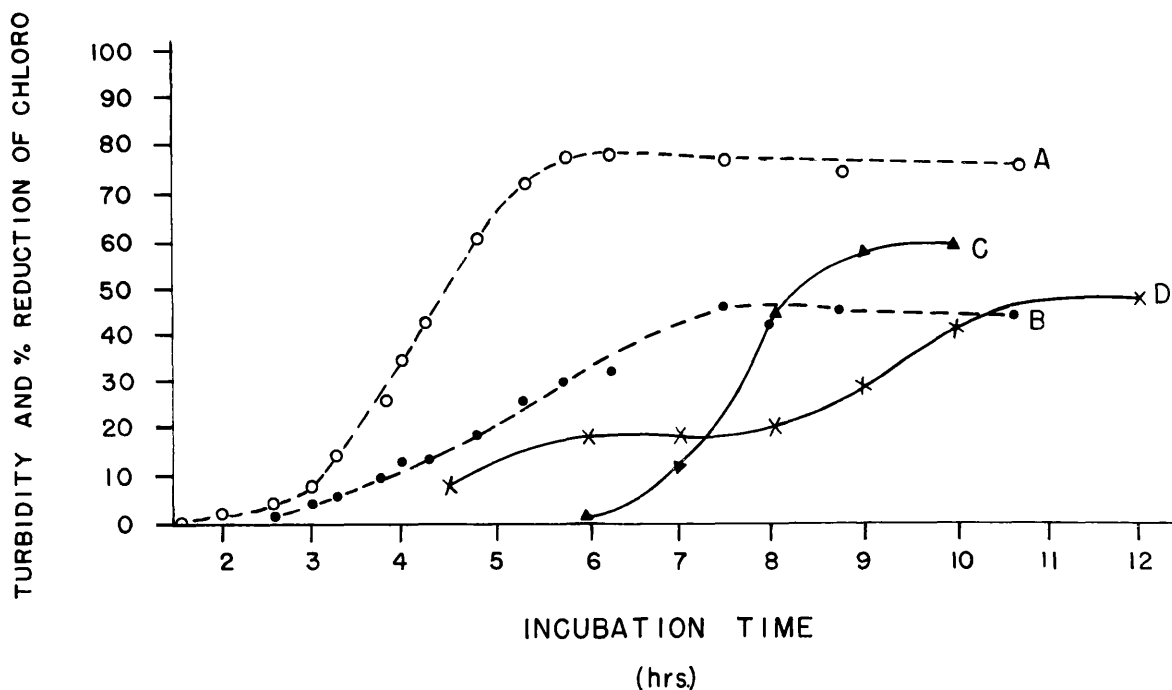


Fig. 3. "Chloramphenicol reductase activity" of E. coli (100) (E. coli (B) "trained" to grow in 100 γ /ml chloramphenicol.).

- A, Growth curve for E. coli (100) in brain heart infusion medium; no chloramphenicol.
 B, Growth curve for E. coli (100) in brain heart infusion medium; 100 γ /ml chloramphenicol in the medium.
 C, % reduction of chloramphenicol as measured by the bioassay method.
 D, % reduction of chloramphenicol as measured by the polarographic method.

It appears as if the maximum reduction is not attained until the culture is 8 to 10 hours old. This could lead to the assumption that the reduction of chloramphenicol by E. coli was merely a secondary process.

There was, however, another possibility the likelihood of which was increased by the results which indicated that the reduction was either an intracellular process, or at least a reaction which required the

presence of bacterial cells. This was the possibility that small-scale, cellwise reduction was occurring continually in the partly resistant cultures growing in the presence of chloramphenicol, but the reduction could not be detected until either a larger amount of cells was present in the chloramphenicol media, or the cells were left in contact with the chloramphenicol for longer periods of time. Only then would the cultures measurably reduce the chloramphenicol.

With this possibility in mind (small-scale reductions), three experiments utilizing whole cells were planned which, it was hoped, would essentially magnify the reactions occurring with small inocula. These involved studies on the effects of cell mass or cell numbers, culture age, and chloramphenicol-cell contact time on the "chloramphenicol reductase activity".

These experiments were performed in both "growing" media (brain heart infusion broth) and in "non-growing" media ("nitrogen-free" synthetic, "chloramphenicol reductase medium"). The use of the "growing" media introduced a number of undesirable variables into the experiments. The most obvious variable was the effect of culture growth during the incubation periods when the cells were in contact with the chloramphenicol. Since the resistances varied, the growth rates varied from culture to culture. This led to misconceptions of the reductase activities of certain of the partially resistant cultures.

To eliminate this variable "nitrogen-free" media were employed. The medium first used was Monod's synthetic salt-glucose medium without the addition of ammonium salts. The glucose concentration in this medium was adjusted to 0.3 M. Using this medium only very small amounts of reduction were observed (5-15%).

Grant Smith's data (2) indicated that there was an optimal glucose concentration for maximum reductase activity of a chloramphenicol-sensitive strain of E. coli. However, within the range of his experiments, variations in the reductase activity were not great. This was not true in the case of the resistant E. coli strains developed in the present experiment. The curves in Fig. 4 demonstrate the importance of utilizing an optimal concentration of glucose to get maximum reductase efficiency. This is especially true for the chloramphenicol resistant organisms.

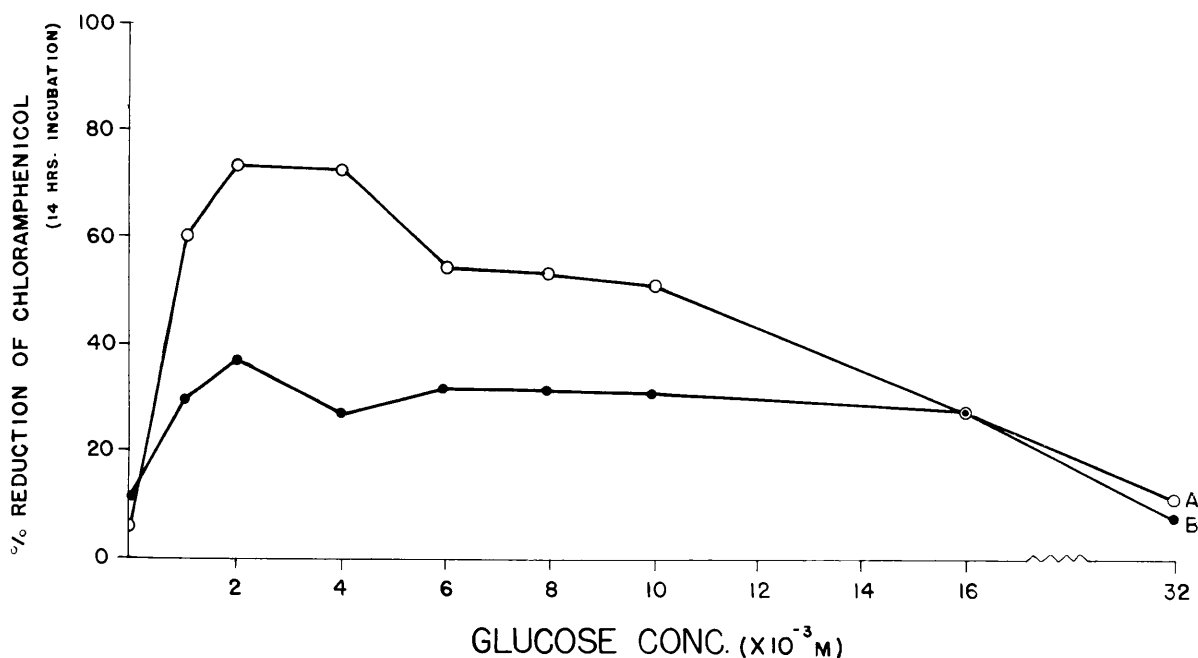


Fig. 4. Effect of glucose concentration of the "chloramphenicol reductase activity" of the partly resistant E. coli (100) A, and on the parent sensitive E. coli (B) B.

The curves in Fig. 4 were obtained with 6.5 hour, washed cultures of E. coli (B) and E. coli (100) after adjusting the turbidities to 50 in 6 ml. of synthetic medium containing a constant amount of chloramphenicol (100 γ /ml) and varying amount of glucose. Polarographic determinations of the chloramphenicol remaining in the media were made after 14 hours of additional incubation at 37°C. It is apparent from the curves that high glucose concentrations inhibit the reductase activity of the resistant culture. Maximum activity was obtained with 0.002 M to 0.004 M glucose for the resistant culture, E. coli (100). Glucose apparently does not inhibit the sensitive cultures until the glucose concentration exceeds 0.02 M. At high glucose concentrations the reductase activities are either about the same, or the activity of the parent sensitive cultures extends slightly above the resistant cultures.

Fig. 4 also demonstrates the increased "chloramphenicol reductase activity" of the resistant culture at the optimal glucose concentration as compared to the parent sensitive culture.

pH optima were determined for each of the above cultures in synthetic media containing 0.002 M glucose. Both strains exhibited maximum reductase activity between pH 7 - 8.

At this point it was decided that henceforth the "chloramphenicol reductase medium", which has been described earlier, would be used for all of the determinations of "chloramphenicol reductase activity" where the growth factor was to be eliminated.

Results recorded in some of the experiments below in which both the "growing" and "non-growing" media were utilized illustrate the effect which culture growth had on the reductase results.

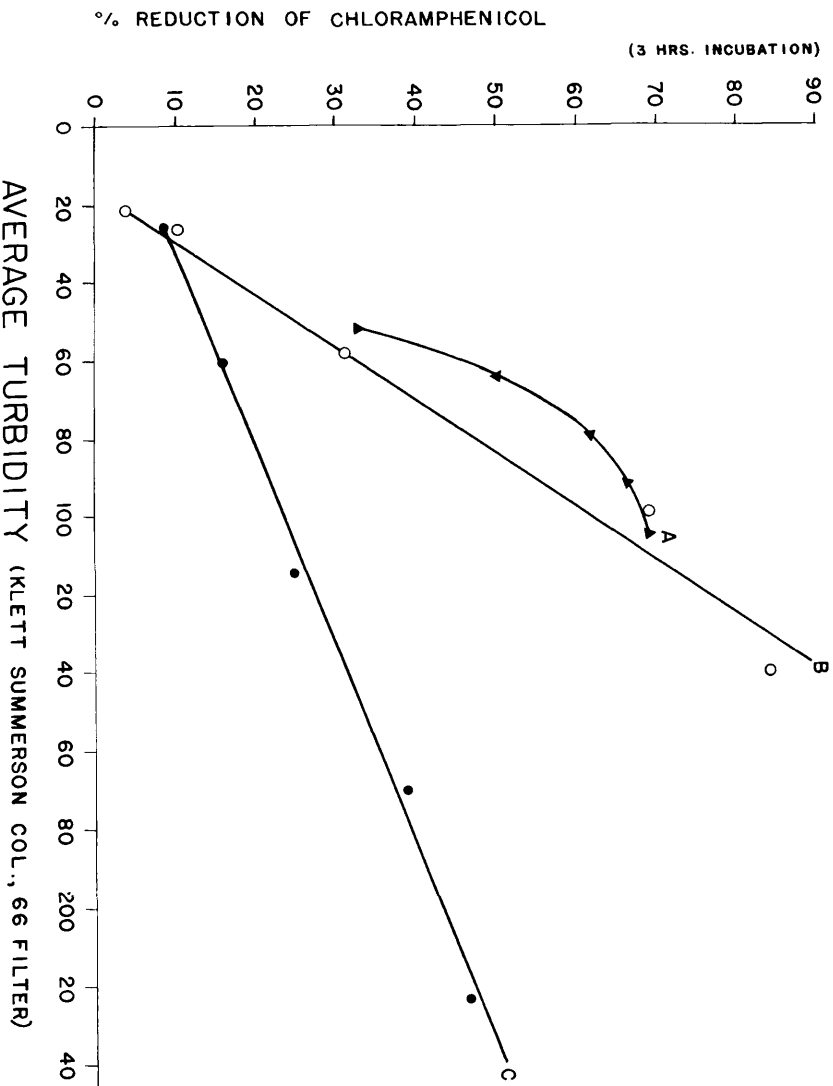


Fig. 5. Effect of cell concentrations or cell numbers on the "chloramphenicol reductase activity".

- A, E. coli (100) in brain heart infusion medium; 18 hour culture.
 B, E. coli (2000) in the "non-growing", "chloramphenicol reductase medium"; 16.5 hour culture.
 C, E. coli (B) in the "non-growing", "chloramphenicol reductase medium".

In each of the above experiments the cultures of each strain were diluted to the various turbidities shown on the graph; using the desired chloramphenicol medium; these cultures were then incubated an additional 3 hours and the percent reduction of the chloramphenicol was determined by the polarographic method.

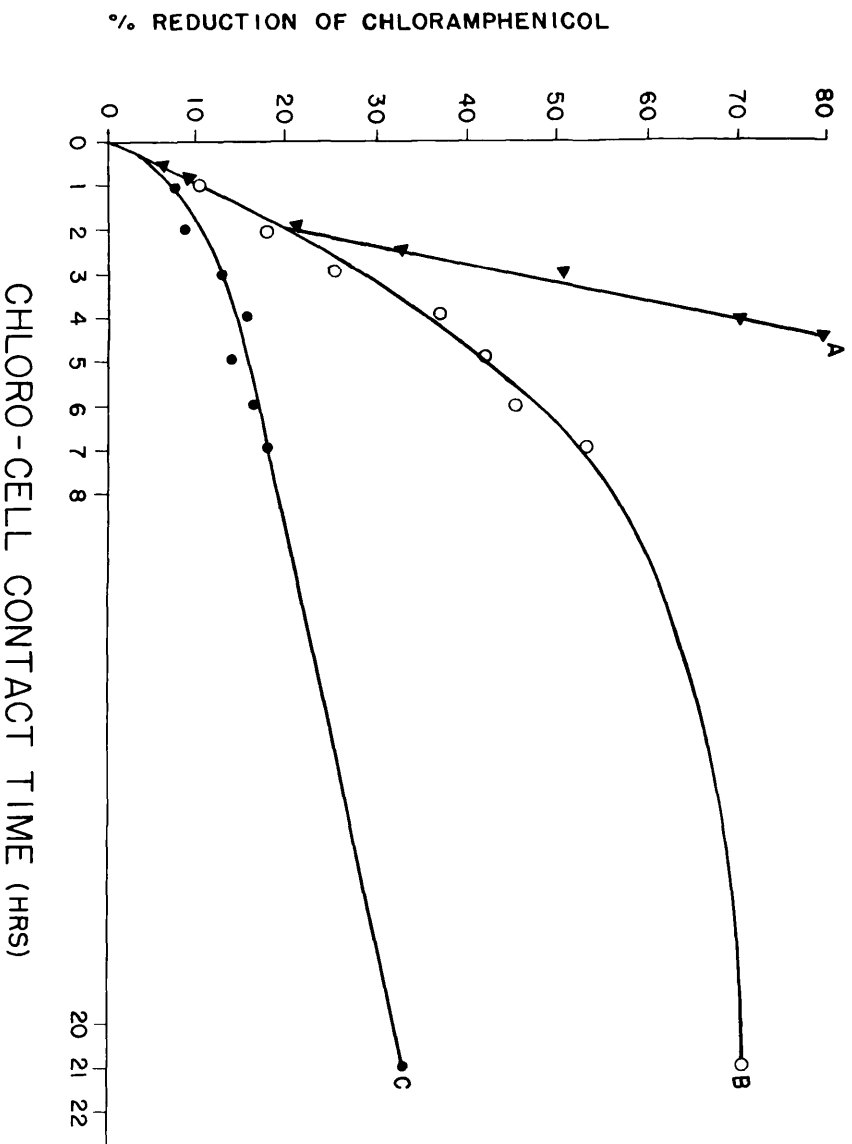


Fig. 6. Effect of chloramphenicol-cell contact time on "chloramphenicol reductase activity".

A, *E. coli* (100) in brain heart infusion medium; 6 to 10 hour cultures.
 B, *E. coli* (2000) in the "non-growing", "chloramphenicol reductase medium"; 15 hour culture.

C, *E. coli* (B) similar treatment as B.

TABLE II

Effect of Culture Age on "Chloramphenicol Reductase Activity"

Culture age BHI medium	E. coli (2000)*		E. coli (100)#
	50% Inhibition γ /ml chloro	% Reduction	% Reduction
2	620	29.4	71.6
3	—	—	65.3
4	—	29.4	71.6
6	670	46.2	80.0
8	—	—	74.0
10	—	—	86.0
12	—	—	71.0
24	300	36.5	43.0
48	280	38.8	—
72	23	20.6	—

* 6 hours chloramphenicol-cell contact time in nitrogen-free synthetic medium.

4.5 hours chloramphenicol-cell contact time in brain heart infusion medium.

The results demonstrated in Table II and in Figs. 5 and 6 can be summed up as follows: the reductase activity can be increased by (1) increasing the cell concentrations, (2) extending the chloramphenicol-cell contact time, and (3) apparently within certain limits the reductase activities do not vary appreciably with age; beyond this range the reductase activities and resistances fall off.

An attempt was then made to see if there was a correlation between the development of resistance and the increase of "chloramphenicol

reductase activity". The results in Table III indicate that the first step in chloramphenicol resistance is associated with a comparable rise in the "chloramphenicol reductase activity".

TABLE III

Relation Between Gain of Chloramphenicol Resistance*
and "Chloramphenicol Reductase Activity"

14 hrs.		Chloro-cell contact time at 37°C				6 hr. (Back transfer-a)	
50% Inhib. γ/ml	% Red.#	6 hrs.		3 hrs.		50% Inhib.	% Red.
		Inhib.	Red.	Inhib.	Red.		
0.13	26.0	0.16	17.8	0.16	9.1	0.57	66.0
0.15	22.3	0.18	28.4	0.19	10.5	0.55	74.5
<u>0.26</u>	<u>64.1</u>	0.28	43.0	0.28	19.2	<u>0.41</u>	<u>66.4</u>
0.46	57.5	<u>0.48</u>	<u>59.7</u>	0.46	43.4	0.31	48.8
0.66	62.1	0.57 ^b	60.2	<u>0.64</u>	<u>55.2</u>	0.27	26.7
1.04	55.6			1.25	55.7	0.20	13.6
1.20	58.3			2.20	58.0	0.14	18.7

* E. Coli (B) "trained" in brain heart infusion medium.

% reduction of chloramphenicol by approx. 1.0 mg of cells (turbidity = 50) in "chloramphenicol-reductase" (nitrogen-free) medium.

The resistance was developed by transferring the sensitive E. coli culture into brain heart infusion medium containing increasing amounts of chloramphenicol and incubating for 18 hours. At the end of 18 hours, the cells were harvested by centrifugation from the highest concentration of chloramphenicol permitting fair growth (turb. >20); diluted to a turbidity of 25 with Sorensen's buffer (pH - 7.42). Five

tenth of a ml. of this dilution was then inoculated into 20 ml. of fresh brain heart infusion broth without chloramphenicol. This culture was then incubated for 15 hours at 37°C; harvested, washed two times with cold buffer, and resuspended in the "chloramphenicol reductase medium" at a turbidity of 50. The cells were left in contact with the chloramphenicol media for the periods of incubation indicated on the table. At the end of that period the cells were removed by centrifugation and the amount of chloramphenicol remaining in the solutions was determined in the usual manner.

Sensitivity tests were performed on all of the above 15-hour cultures by the method described earlier.

Table III also illustrates a similar relationship between the loss of resistance on subculturing a slightly resistant culture in the absence of chloramphenicol and the loss of "chloramphenicol reductase activity".

It should also be pointed out in this same table that the maximum reductase activity, under the conditions of the experiments, was reached at a relatively low resistance; and the maxima reached apparently depended on the resistance of the culture and the chloramphenicol-cell contact time. An attempt to raise the maximum amount of reduction, using the same amount of cells (turb. = 50) and the same chloramphenicol-cell contact time (6 hours), by carrying out the 6-hour incubation period under strict anaerobic conditions was unsuccessful. The same amount of reduction was obtained as with the cultures growing under normal conditions. Apparently the normal conditions used approached those of anaerobiosis, since aeration of the media in which chloramphenicol

reduction was supposed to occur almost completely inhibits the reductase activity of a growing culture of E. coli (100). With the results obtained thus far, it appears as if the only means for increasing the reductase activity is to either increase the amount of cells or extend the chloramphenicol-cell contact time.

Through a period of 7 to 9 daily transfers it has not been possible to develop any resistance in E. coli using Monod's medium with either 0.01 or 0.02 M glucose. Normally Monod's medium contains 0.03 molar glucose.

Chloramphenicol resistance has been developed in strain (B) of E. coli to the point where the organisms can grow in 2 mg/ml of chloramphenicol (E. coli (2000)). The reductase activity of E. coli (2000) generally appeared to be slightly lower than some of the more sensitive cultures; however, it was felt that the apparent decrease in reductase activity was merely the result of the coagulation phenomenon. The resistant cultures begin to coagulate after about 20 minutes of incubation in either the "chloramphenicol reductase medium" or in Sorensen's buffer or brain heart infusion broth. Among other things, the coagulation tends to reduce the active surface area of the cells, and can probably account for the reduced activity of these cultures as compared to the more sensitive cultures which do not coagulate. Intermittent shaking (not enough to be considered aeration) tends to give the resistant cultures an apparent increase in the reductase activity.

On subculturing of the highly resistant culture (E. coli (2000)) both resistance and reductase activity dropped off rapidly, but a stage was reached where the resistance remains above that of the parent

sensitive cultures, yet the "chloramphenicol reductase activity" actually falls below that of the parent sensitive cultures. In some of the earlier experiments this break between resistance and reductase activity occurred between the fourth and the fifth back-transfers in the absence of the antibiotic. Since the conditions of the experiments over the period of one year could not be controlled with the desired degree of certainty, it is understandable that a factor as indefinite as the number of loop subcultures to attain a certain step in the loss of resistance and the loss of reductase activity could not be predicted with any degree of accuracy. Since the highly resistant strains have been continually transferred in the presence of the drug to maintain the resistance, it is possible that the resistance was more stable when the last experiments were performed as compared to the very earliest experiments. However, the general relationship between "chloramphenicol reductase activity" and resistance is maintained for certain stages in the development and the loss of resistance in E. coli. The loss of resistance and reductase activity on subculturing E. coli (2000) cultures in brain heart medium minus chloramphenicol is illustrated in Table IV.

TABLE IV

Correlation of Loss of Chloramphenicol Resistance*
with Loss of "Chloramphenicol Reductase Activity"

No. of back- transfers of <u>E. coli</u> (2000)	I (14 hrs)		II (14 hrs)		III (6 hrs)	
	50% Inhib. γ /ml	% Red.#	50% Inhib.	% Red.	50% Inhib.	% Red.
0	1100	66.1	920	46.3	950	53.1
1	—	—	800	57.7	—	—
2	870	64.8	710	64.8	—	—
3	890	68.5	430	55.6	—	—
4	550	64.8	225	52.9	—	—
5	19.3	45.5	25	27.4	200	42.4
6	22.5	43.0	10.8	17.6	—	—
7	9.1	26.7	8.4	17.6	21.2	37.9
10	—	—	7.4	21.7	13.7	12.7
15	—	—	—	—	12.5	15.5
<u>E. coli</u> (B)	—	—	0.1	33.1	0.1	19.5

* loss of resistance by the highly resistant strain E. coli (2000) when subcultured in brain heart infusion medium without chloramphenicol.
% reduction of chloramphenicol by approx. 1 mg of cells (turbidity = 50) in the "chloramphenicol-reductase" (nitrogen-free) medium.

In one particular experiment which is not shown on the table the break between the reductase activity and resistance did not occur until the tenth back-transfer. The break occurred when the culture had dropped in resistance to a 50% inhibition of 10 to 12 γ /ml. The break always occurred at this stage of resistance. The resistance of this

particular culture remained the same even after 28 transfers in the absence of the antibiotic. This result has been repeated a number of times, and would tend to show that this particular stage in resistance is relatively stable.

DISCUSSION AND CONCLUSIONS

It has been possible to develop chloramphenicol resistance in strain (B) of Escherichia coli and strain (1A) of Micrococcus pyogenes var. aureus when grown in either a natural medium (brain heart infusion) or in a synthetic medium (Monod's). It was noted in Fig. 1, however, that the rates at which resistances were developed, and possibly the nature of the resistances, varied with the media used and with the particular species used.

The differences observed in the development of resistance in E. coli as a result of the variations in the composition of the growth media indicate a possible lack of some preformed nutrients in the synthetic media. The absence of these nutrients may have several results; (1) they may slow down the metabolic activity of the organisms to the point where the mechanism for overcoming the chloramphenicol is also slowed down; (2) the lack of certain nutrients may either inhibit or prevent the production of necessary genetic material; (3) the inhibition of the production of certain specific metabolites; and (4) an effect which has not been too thoroughly investigated in the present paper, the possible effect that these nutrients might have in creating better conditions for the reduction of chloramphenicol.

The differences observed in the development of resistance in the two species studied is quite interesting. Again referring to Fig. 1, resistance was developed with considerably more difficulty in the strain of M. pyogenes in brain heart infusion medium as compared to the development of resistance in E. coli in the same medium. One outstanding difference has been detected between the two species used. That is the fact that E. coli has the ability to inactivate some of the chloramphenicol by a reductive process, while M. pyogenes lacks the ability to reduce the chloramphenicol under the same conditions. As resistance is developed in E. coli its reductase ability also increases. On the basis of results obtained with a growing culture of M. pyogenes in 2 mg/ml of chloramphenicol, which showed that there was little or no reduction of chloramphenicol even after 20 hours of incubation (even though considerable growth had taken place), it is concluded that the enzymatic reduction of chloramphenicol does not enter into the resistance picture for M. pyogenes. This one factor alone may account for the different rates of development of resistance observed with the two species studied.

Since it was not possible to demonstrate an increase in the reductase activity in cultures "trained" in Monod's synthetic medium, this may explain why the curves for the development of resistance in E. coli in synthetic medium is quite similar to the curve obtained with M. pyogenes in brain heart infusion medium.

The implication here is that several mechanisms are possible for the development of chloramphenicol resistance, and E. coli when grown in brain heart medium apparently has the ability to utilize other mechanisms in addition to those present in M. pyogenes, or in a strain of E.

coli which had been grown in a synthetic, salt-glucose medium. Further evidence has been obtained which would tend to support the idea that several mechanisms are involved in the development of chloramphenicol resistance in E. coli. This will be discussed later.

Associated with the development of resistance is a continual decrease in culture growth as the resistance is increased (note Fig. 2). Morphological observations always indicated an increase in the size of the cells which have been exposed to chloramphenicol for some length of time. These two factors, decrease in culture growth and increase in size, indicate that chloramphenicol is affecting cellular division, possibly at some phase of protein synthesis (9). This phenomenon of increased cell size in the presence of antibiotics has been reported in the case of penicillin-treated cells (23, 24), and undoubtedly has been observed with other antibiotics. Generally, the lack of growth coupled with increased cell size has been attributed to interference in protein synthesis.

To say that chloramphenicol interferes with protein synthesis does not imply that the specific site of action of chloramphenicol is known, since protein synthesis is an end observation of all bacterial anabolism. This concept also does not contribute much to the determination of the problem of the development of resistance unless the resistance which is developed is only the result of a change in some protein structure in the cells.

In general, the development of resistance in E. coli is associated with a decrease in its biochemical reactivity; but the instability of the chloramphenicol resistance, coupled with the possibility that the resistance is developed in a stepwise manner, does not permit any definite

conclusions to be drawn from comparisons of the sensitive strains with the highly resistant strains. The uncertainty of drawing inferences from end results usually makes comparative studies of this nature undesirable unless accompanied by other supporting data.

The demonstration by Smith and Worrel (25) that E. coli inactivates chloramphenicol primarily by reducing the nitro group on the chloramphenicol molecule to an amine group suggested the possible role of a "chloramphenicol reductase" in the development of resistance. The "chloramphenicol reductase activity" could either be one of the primary functions in the resistance mechanism, or it could merely be the secondary results of developed resistance. If E. coli was able to grow in the presence of chloramphenicol simply because it had the ability to inactivate the drug by reduction, growth in a concentration of chloramphenicol which was normally inhibitory should be associated with a marked reduction of the chloramphenicol. Also, if the reductase activity is associated with resistance, variances in resistance should accompany variances in the reductase activity. Neither of these points could be clearly demonstrated in preliminary experiments, primarily because of certain technical difficulties involved in the experimental procedures.

Under ordinary circumstances, in which a sensitivity test was begun with a relatively small inoculum (0.1 ml. of a culture diluted to a turbidity of 25 per 5 ml. of media), reduction of chloramphenicol could not be detected until the culture was almost out of the log phase (6-8 hours in the case of E. coli (100); note Fig. 3). This would lead to the assumption that the reduction of chloramphenicol was merely a secondary reaction.

The possibility that the reduction of chloramphenicol was taking place continually in the inoculated cultures in a cellwise manner, and was not being detected until the cell numbers increase to a point where a measurable amount of chloramphenicol was being reduced, could not be excluded. This possibility became more significant when it was shown that chloramphenicol reduction apparently was an intracellular process.

Since no techniques were available to study the single-cell reactions, experiments were designed which, it was hoped, would tend to magnify the single-cell reactions so that they could be studied with the techniques at hand. This, of course, was done with the reservation that the results obtained with large numbers of cells may not necessarily merely enlarge the cellwise reactions, but may also show additional effects due to intercellular reactions. One of the most pronounced effects of large numbers of cells is to cause the lowering of the oxygen tension in the media. This possible effect has been excluded since strict anaerobic conditions applied to either a small inoculum (turbidity =10), or to the normal amount (turbidity = 50) of resistant cells in the "chloramphenicol reductase medium" did not produce a greater amount of reduction than that obtained under ordinary conditions.

The results indicated very clearly, all other conditions being equal, that more reduction of chloramphenicol could only be obtained by either increasing the number of cells in the chloramphenicol media, or by allowing the cells to remain in contact with the chloramphenicol for longer periods of time (note Figs. 5 and 6).

Table II indicates that the reductase activity of E. coli

cultures of various ages are practically similar over a wide range. Very old cells begin to lose their reductase activity and their resistance. From the data obtained with E. coli in the "chloramphenicol reductase medium" it would appear as if the very young cultures do not possess maximum reductase activity. This may be quite true; however, several factors have to be taken into account when considering the latter result. In order to obtain enough very young cells it was necessary to use larger amounts of growth media (100 ml compared to the usual 20 ml). This may have introduced enough variation in the growth conditions to affect the results, but a larger source of error was probably due to the uncontrollable co-centrifugation of a large amount of fine black material from the large amounts of brain heart medium. This black material could not be washed out thoroughly, and consequently the turbidity readings on the very young cultures were slightly higher than the true values for these cell suspensions. This would tend to give us less of the younger cells in the "chloramphenicol reductase medium" as compared to the older cells which were harvested from much smaller amounts of media.

Nevertheless, even if these factors are not considered, the reductase activity does not vary greatly between the 2- and 12-hour cultures, and all of the cultures have considerably more activity than the parent sensitive cultures.

From the data presented, it therefore appears that the possibility of continual reduction of chloramphenicol in the chloramphenicol media is very good. The only reason chloramphenicol reduction is not observed when growth starts is most likely the inability of the investigator to measure these minute amounts of reduction. Reduction of

chloramphenicol can well be considered as one of the primary mechanisms in the resistance of E. coli toward chloramphenicol.

Some evidence which would also tend to support the above conclusion is the fact that even the smallest resistant colonies have the ability to noticeably reduce methylene blue or resazurin in nutrient agar plates, and other investigators have shown a correlation between the reduction of methylene blue, e.g. (8), and the reductase activity.

If the ability of E. coli to reduce chloramphenicol is one of the primary mechanisms by which it becomes resistant, then any change in resistance of cultures should follow a similar change in the "chloramphenicol reductase activity" of those cultures. This was indeed true in almost every instance. The reductase activity of the resistant cultures was observed to be two to three times as great as that of the parent sensitive cultures.

To see if there was a direct correlation between the development of resistance and the "chloramphenicol reductase activity" the development procedure was repeated for E. coli in brain heart infusion as described in the previous section. At each stage in the resistance, as measured by the usual procedure, the reductase activity was also determined. The development procedure was also reversed (subculturing in the absence of the antibiotic) for a slightly resistant culture. A representative number of these experiments were shown in Table III. In every experiment involving the development of resistance to the first "stage" a direct correlation is observed between the resistance and the reductase activity. This same correlation exists at the high levels of resistance (Table IV), and can be observed in the experiments involving

the loss of resistance and reductase activity as the highly resistant cultures are subcultured in the absence of the antibiotic.

All this indicates that there is a correlation between the development and loss of both resistance and reductase activity. However, two factors which have a direct bearing on the nature of the resistance should be pointed out. The first of these is the fact that the maximum reductase activity is reached at a relatively early stage in the development of resistance, yet the resistance of the cultures can be driven up to a much higher stage. Maximum reductase activity can be obtained at various levels of resistance, depending on the chloramphenicol-cell contact time. Secondly, on subculturing the partly resistant E. coli in the absence of antibiotic from the first "stage" in resistance both resistance and reductase activity fall off in about the same pattern as that by which they were developed. When the highly resistant E. coli (2000) was subcultured in the absence of the antibiotic the loss of resistance and reductase activity paralleled each other for a certain number of back-transfers; but a point was reached where the resistance remained above that of the parent sensitive culture, yet the reductase activity fell down below the normal sensitive level. In other words, the transition from the high resistance (50% inhibition of about 1000 γ /ml) to sensitivity exhibits a divergence of resistance and reductase activity at a point above the first "stage" in resistance.

These data can be interpreted to mean that chloramphenicol resistance is developed in this strain of E. coli by two or more methods at different stages; and that the loss of resistance and the loss of reductase activity follow the same general pattern; however, some stages

(particularly around 10 to 15 γ /ml) block the continual drop to sensitivity on subculturing in the absence of chloramphenicol. Since maximum resistance is reached at such an early stage, and since this particular stage (10 to 15 γ /ml) maintains its resistance even when subcultured numerous times (at least 28) in the absence of chloramphenicol, this particular breaking point might represent a genetic step in the development of resistance.

It seems a little more than coincidental that the point at which maximum reductase activity is attained, or where the reductase activity-resistance relationship is broken on transferring in the absence of the antibiotic, is also approximately the same point at which a sharp break was obtained in the early development picture (note Fig. 1).

It was not the intent of this paper to determine whether the developed resistance and the reductase activity were due to selective, inductive, or adaptive responses of E. coli to chloramphenicol. However, the data presented strongly suggests that a combination of both genetic and adaptive functions account for the chloramphenicol resistance developed in this strain of E. coli. A picture can be formulated in which adaptation operates to build the resistance up to a certain level, then a genetic step enters which allows the organisms to adapt to still higher concentrations of chloramphenicol. These processes can be occurring individually or in conjunction with each other, and the number of "steps" or "stages" may be many or few.

The data which have been presented lend themselves to the interpretation that there is a direct relationship between resistance and "chloramphenicol reductase activity" in several "stages" of

resistance, but the reductase activity cannot account for all of the resistance which can be developed in this strain of E. coli. The direct correlation between resistance and reductase activity is interrupted at certain stages in the development of resistance. The "chloramphenicol reductase activity" appears to function as a primary mechanism in the development of resistance in E. coli.

SUMMARY

Chloramphenicol resistance has been developed in strain (B) of Escherichia coli and strain (1A) of Micrococcus pyogenes var. aureus by several procedures employing a natural and a synthetic medium. The patterns of the development of resistance are graphically recorded. Data are presented which illustrate the possible role of "chloramphenicol reductase activity" in the primary mechanism by which E. coli becomes resistant to chloramphenicol. This feature has only been demonstrated for E. coli in brain heart infusion medium and apparently does not enter into the M. pyogenes resistance picture or into the results obtained with E. coli in a synthetic medium. A direct correlation has been shown between chloramphenicol resistance and "chloramphenicol reductase activity" in E. coli. Evidence is presented which indicates that several "steps" are involved in the development of chloramphenicol resistance in E. coli, and the "chloramphenicol reductase activity" cannot account for all of the resistance which can be developed.

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