Mechanism of High-level Resistance to Chloramphenicol in Different *Escherichia coli* Variants

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SUMMARY

Mutants resistant to high levels of chloramphenicol can be obtained in *Escherichia coli* B by one or two mutational events. All of 144 high-level resistant mutant clones examined were powerful inactivators of the drug. Growth of this kind of mutants in nutrient media containing chloramphenicol 100 µg./ml. or more depended on the inoculum size, the composition of the medium, and the concentration of the drug. No growth was observed with lactose as sole energy source unless the organisms had been previously induced for β-galactosidase production.

With a strain of *Escherichia coli* K 12 an entirely different type of resistant mutants occurred. High-level resistant derivatives were obtainable only through several serial mutations. None of 36 high-level resistant mutants was able to inactivate the drug. Growth of these bacteria was extremely slow, even in the absence of drug. This resistance was due to a decreased rate of permeation, which probably was non-specific and concerned many species of micromolecules.

INTRODUCTION

Resistance to chloramphenicol may be due to different mechanisms: (1) resistance of the protein synthesizing machinery (Ramsey, 1958); (2) decreased permeation to the site of antibiotic action (Cavalli & Maccacaro, 1952; Okamoto & Mizuno, 1962, 1964; Vazquez, 1963, 1964); (3) inactivation of the drug (Okamoto & Suzuki, 1965; Rananarayana, Rayaulakshmi & Ehaskaran, 1962; Miyamura, 1964; Chabbert & Debruge, 1956; Dunsmoor, Pin & Sherris, 1963).

Studies with *Escherichia coli* B and *E. coli* K 12 have now shown that high-level resistance to chloramphenicol in *E. coli* B is due to inactivation and in *E. coli* K 12 to impaired permeation. Inactivation of chloramphenicol has been effected also with the susceptible wild-type *E. coli* B, though at a slow rate, but could not be demonstrated with *E. coli* K 12. This is apparently a parallel to the well-known fact that penicillinase-producing mutants are never obtained from initially penicillinase-negative bacterial strains.

METHODS

Bacterial strains. (1) *Escherichia coli* B: the wild strain was inhibited on nutrient agar containing chloramphenicol (cm) 2.5 µg./ml.; for the present work a mutant resistant to dihydrostreptomycin 5000 µg./ml. and sulphathiazole 40 µg./ml. was developed;

Cm-resistant mutants isolated were tested for dihydrostreptomycin and sulphathiazole resistance. (2) *E. coli* K 12-W 945 (lactose-, proline-, thiamine-): this strain was inhibited by Cm 4.0 µg./ml. (3) *E. coli* K 12-W 945: with resistance transfer factor (RTF) mediating resistance to dihydrostreptomycin, sulphathiazole, tetracycline and chloramphenicol. The RTF was transferred from a wild strain of *Shigella flexneri* by using the technique of Watanabe & Fukasawa, 1961. (4) A susceptible wild strain of *Shigella boydii* was used for assay of chloramphenicol concentrations.

Drugs. Chloramphenicol (Cm) was obtained from Abic Ltd., Ramat-Gan, Israel. [¹⁴C]Cm (methylene-¹⁴C) was purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England; its activity was stated to be 6.81 mc/mm. Tetracycline HCl: Lederle.

Culture media. (1) Bacto blood agar base (BAB). (2) Bacto tryptose phosphate broth (TP): same solidified with 1.5% Bacto agar is designated TPA. (3) Davis's minimal medium (Davis & Mingioli, 1950) and the same solidified with Bacto Noble Agar are designated DB and DA, respectively. The same minimal media were also used with lactose substituted for glucose (lactose DB, lactose DA). For growth of *Escherichia coli* K 12-W 945, the minimal medium was supplemented with L-proline 50 mg. and thiamine HCl 1 mg. per litre.

Mutation rate was estimated by the method of Luria & Delbrück (1943). In each experiment 20–56 independent cultures grown overnight from an inoculum of about 100 bacteria were plated on DA or BAB with Cm. The mutation rate was calculated from the fraction of clones with no mutants.

Bioassay for chloramphenicol. The samples to be tested were sterilized by heating in a boiling water bath during 5 min. This procedure stopped the enzymic activity. Control samples showed no decrease in the activity of chloramphenicol due to the heating. For chloramphenicol (Cm) assay, a susceptible strain of *Shigella boydii* was used as indicator. Samples of the test samples heated to 37° were added to cultures in the exponential phase (1.7×10^8 – 2.1×10^8 bacteria/ml.). The amount added was calculated to give a final concentration of Cm 2 µg./ml. if no inactivation of the drug had taken place. Turbidity of the cultures was examined with a Klett Summerson photoelectric colorimeter every 20 min. during 1 hr. Control cultures of *S. boydii* in TP medium with Cm were included in each titration. The following concentrations of Cm (µg./ml.) were used in these controls: 0, 0.5, 1.0, 2.0. From these controls a curve of generation time as a function of Cm concentration was drawn. The Cm concentration in the test samples was then determined from the generation time by interpolation.

Uptake of [¹⁴C]Cm. An exponential culture in TP medium was gathered by centrifugation to a concentration of 4×10^{10} bacteria/ml. and [¹⁴C]Cm 2 µg./ml. added. The culture was incubated with slight shaking during 20 min. at 37°, chilled, and the bacteria washed twice with 2 ml. cooled plain TP medium and once with 2 ml. distilled water. After the last deposition of the bacteria by centrifugation, 1 ml. of the supernatant liquid was transferred into a counting vial, and so was the sediment after resuspension. To each sample was added 15 ml. of following scintillator: 6 g. 2,5-diphenyloxazole, 0.3 g. 1,4-bis-2-(5-phenyloxazolyl)-benzene + 100 g. naphthalene in 1 l. *p*-dioxane. The vials were counted in a Packard Tricarb liquid scintillation spectrometer, model 3203. The radioactivity of the intercellular fluid varied between 15% and 25% of the activity of the cell suspension. Uptake of Cm by the bacteria was calculated from the difference between radioactivity of the cell suspension and

the intercellular fluid. Results were compared with radioactivity obtained by arbitrary amounts of [^{14}C]Cm added to vials of bacteria treated as the test samples but without added Cm.

Results were correlated to the wet weight of bacteria, which was determined by centrifugation at 18,000g for 10 min., removing the supernatant fluid and draining by tilting the test-tube on adsorbing paper. One g. wet *Escherichia coli* B corresponded to 6.8×10^{11} bacteria.

RESULTS

Escherichia coli B mutants

Mutation rate was determined in five different experiments using media BAB and DA containing Cm 4.0 $\mu\text{g./ml.}$ The results varied between 5.3×10^{-10} and 6.7×10^{-9} mutations/bacterium/division. The degree of resistance was determined for 635 separate mutant colonies. The mutant clones were first purified by several transfers on agar medium containing Cm 4 $\mu\text{g./ml.}$ Heavy suspensions in broth were then streaked on BAB containing different Cm concentrations. Of the clones 578 were inhibited by Cm 8 $\mu\text{g./ml.}$, 21 clones by 16 $\mu\text{g./ml.}$, and 36 clones (5.7%) by 150–300 $\mu\text{g./ml.}$

One-step high-level resistant mutants were obtained more conveniently by plating the susceptible strain on BAB containing Cm 16 $\mu\text{g./ml.}$ The mutation rate was in this case calculated to be 1.2×10^{-10} mutations/bacterium/division. Of 100 mutant clones examined 100 grew when streaked on BAB with Cm 100 $\mu\text{g./ml.}$ and 8 on 250 $\mu\text{g./ml.}$ High-level resistant mutants were also isolated from low-level resistant clones (Cm 4 $\mu\text{g./ml.}$) by plating on BAB with Cm 16 $\mu\text{g./ml.}$ These mutations occurred at a frequency of about 10^{-9} /bacterium/division. It might be concluded from these experiments that *Escherichia coli* B may obtain low-level as well as high-level resistance to chloramphenicol in a single mutational event.

Inactivation of chloramphenicol by Escherichia coli B. The susceptible *Escherichia coli* B inactivates chloramphenicol but at a very slow rate. This was shown in experiments of the following type. A TP culture was grown to the exponential growth phase and the bacteria concentrated by centrifugation to a density of 1.6×10^9 bacteria/ml. Chloramphenicol was added to Cm 100 $\mu\text{g./ml.}$ and the culture incubated for 18 hr at 37°. Assay of residual Cm showed about 80 $\mu\text{g./ml.}$

On the other hand, 114 one-step and 30 two-step high-level resistant clones all proved to be powerful inactivators of Cm. In the tests with these strains, TP with Cm 100 $\mu\text{g./ml.}$ was inoculated with about 2.0×10^8 bacteria/ml. After 18 hr incubation at 37° and centrifugation the supernatant culture fluid was assayed for Cm; none was found.

The kinetics of Cm inactivation were studied more closely in a few of the one-step high-level resistant clones. Some observations with one of the strains (*Escherichia coli* B/1) will be given. After addition of Cm to 100 $\mu\text{g./ml.}$ to an exponentially growing culture of *E. coli* B/1 in TP, more than 90% of the Cm was inactivated during 30 min. and inactivation was complete after 60 min. (Fig. 1). At this time the growth, which initially was slowed by the Cm, continued at the same rate as in the control culture. With Cm 400 $\mu\text{g./ml.}$, 75% of the Cm was inactivated in 30 min., and after 120 min. inactivation was complete; however, even then growth remained slow (Fig. 2). This problem will be dealt with elsewhere.

Inoculum size and nutrient medium. The minimal inhibitory concentration of Cm

for *Escherichia coli* B/1 depends on the inoculum size and the nutrient medium. This is shown by observations summarized in Table 1. An exponential phase culture in TP was centrifuged, the bacteria washed twice in cold phosphate buffer (pH 7.2; 0.15M) and inoculated on plain TPA as well as on TPA containing various amounts of Cm. With Cm up to 60 $\mu\text{g./ml.}$ the colony counts were as on plain agar. However, with TPA containing Cm 100 $\mu\text{g./ml.}$ growth occurred only when at least 9×10^8 bacteria were seeded, but then growth was confluent as on the control plate with

Fig. 1. Inactivation of chloramphenicol by *Escherichia coli* B/1 and growth in tryptose phosphate medium (TP) with and without Cm 100 $\mu\text{g./ml.}$ Extinction and concentration of residual chloramphenicol are plotted on logarithmic scales against a linear time scale. $\circ$ — $\circ$, Cm concentration; $\bullet$ — $\bullet$, growth of strain B/1 without Cm; $\blacktriangle$ — $\blacktriangle$, growth in presence of Cm 100 $\mu\text{g./ml.}$

a similar inoculum. To obtain growth on TPA with Cm 200 $\mu\text{g./ml.}$ a 1000-fold increase in inoculum size (to 9×10^6) was required. With this inoculum growth was confluent, though no colonies occurred on the plate inoculated with 9×10^5 bacteria. On minimal medium with glucose (DA) an inoculum size of 9×10^6 was required for growth in Cm 100 $\mu\text{g./ml.}$

Of special interest was the observation that on lactose DA+Cm 100 $\mu\text{g./ml.}$ no growth was obtained even with an inoculum of 9×10^7 bacteria/ml. However, when the galactosidase-system was induced by incubation with lactose (1 hr), no difference was observed between growth on lactose and glucose media.

Uptake of [^{14}C]Cm. No significant difference was noticed in the uptake of labelled

chloramphenicol between *Escherichia coli* B and its high-level resistant derivative *E. coli* B/1 (Table 2). The radioactivity/g wet wt bacteria corresponded very closely to the [^{14}C]Cm 2 $\mu\text{g./ml.}$ initially added to the suspension of the bacteria. Even though a part of the reversibly bound Cm might have been removed by washing, it seems that no marked intracellular concentration of label had taken place, and this in spite of the fact that with the high bacterial concentration used (4×10^{10} bacteria/ml.) virtually all the Cm must have been inactivated (compare Fig. 1). It seems therefore

Fig. 2. Inactivation of chloramphenicol by *Escherichia coli* B/1 and growth in tryptose phosphate broth medium with and without Cm 400 $\mu\text{g./ml.}$ ○—○, Residual Cm; ●—●, growth without Cm; ▲—▲, growth in presence of Cm.

that the turnover of Cm molecules was high, so that after 20 min. the same amount of radioactivity was found in the susceptible bacteria as in those which had inactivated about 17 times more Cm than the amount corresponding to the amount of label found.

Cross resistance to tetracycline. No resistance to tetracycline was found in 22 one-step high-level resistant mutants of *Escherichia coli* B. The minimal inhibitory concentration in BAB of tetracycline was 1 $\mu\text{g./ml.}$, both for the mutants and for the susceptible ancestor.

Escherichia coli K 12 mutants

Mutants were isolated by plating on BAB and DA containing chloramphenicol 8–16 $\mu\text{g./ml.}$ Mutation rate varied in different experiments between 4.6×10^{-10} and

1.3×10^{-9} mutations/bacterium/division. High-level resistance was only obtained stepwise. Four mutational events were needed for resistance to Cm 300 $\mu\text{g./ml.}$

Inactivation of chloramphenicol. Inactivation by the susceptible *Escherichia coli* K 12 was examined on a TP culture concentrated to about 1.6×10^9 bacteria/ml. and

Table 1. *Minimal inhibitory concentration of chloramphenicol for Escherichia coli B/1: the effects of inoculum size and growth medium*

Incubation was for 48 hr at 37°.

Inoculum size*	Controls				Chloramphenicol (μg./ml.)							
					100		200		100		100	
					In media†							
	Lactose											
	TP	TPA	DB	DA	TP	TPA	TP	TPA	DB	DA	DB + lactose	DA + lactose
	Growth response‡											
9·0 × 10 ⁷	+	+	+	+	+	+	+	+	+	+	—	—
9·0 × 10 ⁶	+	+	+	+	+	+	+	+	—	+	—	—
9·0 × 10 ⁵	+	+	+	+	+	+	—	—	—	—	—	—
9·0 × 10 ⁴	+	+	+	+	+	+	—	—	—	—	—	—
9·0 × 10 ³	+	+	+	+	+	+	—	—	—	—	—	—
9·0 × 10 ²	+	+	+	+	—	—	—	—	—	—	—	—

* No. of viable bacteria inoculated per tube (10 ml. liquid medium) or per plate (10 ml. solid medium) and distributed evenly on the surface by a cotton-wool applicator.

† TP = Tryptose phosphate broth; TPA = same solidified with agar; DB = minimal medium; lactose DB = same with lactose substituted for glucose; DA and lactose DA = same solidified with agar.

‡ Growth response: +, in tube, pronounced turbidity; on Petri dish, confluent growth; —, no growth.

Table 2. *Uptake of [^{14}C]chloramphenicol by various strains of Escherichia coli*

Sample no.	<i>E. coli</i> B		<i>E. coli</i> B/1		<i>E. coli</i> K 12		<i>E. coli</i> K 12/1	
	CPM*/ 59 mg.† bacteria	$\mu\text{g. Cm}\ddagger$ / g. bacteria	CPM/ 59 mg. bacteria	$\mu\text{g. Cm/}$ g. bacteria	CPM/ 59 mg. bacteria	$\mu\text{g. Cm/}$ g. bacteria	CPM/ 59 mg. bacteria	$\mu\text{g. Cm/}$ g. bacteria
1	6080	2.1	5815	2.0	9461	3.2	1701	0.58
2	5297	1.8	4334	1.5	9141	3.1	1426	0.49
3	5144	1.8	.	.	8614	2.9	1320	0.45

* CPM, counts/min.

† All weights are *wet weight* determined for cultures of the same turbidity values as the samples.

‡ Chloramphenicol (Cm) calculated by comparison with CPM obtained with 0.1 $\mu\text{g. } [^{14}\text{C}]\text{Cm}$ in 59 mg. *E. coli*.

addition of Cm to 100 $\mu\text{g./ml.}$ In 36 high-level resistant derivatives inactivation was examined by growing cultures in TP with Cm 100 $\mu\text{g./ml.}$ After 24–28 hr virtually the entire amount of added Cm was found on assay (95–105%). It was therefore concluded that neither the susceptible ancestor nor its resistant mutants were able to inactivate chloramphenicol.

Properties of high-level resistant mutants. Determination of viable counts of the high-level resistant cultures on plain TPA and on TPA+chloramphenicol gave comparable results. The inoculum size was of no influence on the minimal inhibitory concentration (Cm 350 $\mu\text{g./ml.}$). The markers L-proline, thiamine and lactose were unaltered in the resistant mutants, but the bacteria were more elongated with 2.7 nuclei/bacterium in an early exponential phase culture instead of 1.4 nuclei/bacterium

Fig. 3

Fig. 4

Fig. 3. Comparison of growth of *Escherichia coli* K 12 and its resistant mutant *E. coli* K 12/1 in tryptose phosphate broth medium, with and without chloramphenicol (Cm).

Fig. 4. Inhibition of growth of *Escherichia coli* K 12/1 after pre-incubation in a buffer solution containing chloramphenicol (Cm) 100 $\mu\text{g./ml.}$, ---; without pre-incubation —.

in the susceptible parent strain. All the resistant mutants examined grew very slowly on plain nutrient media as well as on Cm-containing media. In Fig. 3, growth in TP (37°, with shaking) of the susceptible strain is compared with growth of one of the high-level resistant mutants (*Escherichia coli* K 12/1). The shortest generation time was about 30 min. for the susceptible and 100 min. for the resistant strain. Growth ceased at 0.52 extinction for the susceptible strain as compared with only 0.28 for the resistant strain. Addition of Cm 100 $\mu\text{g./ml.}$ to the culture of the resistant strain had almost no effect during the first 90 min.; thereafter the growth slowed down to cease entirely at 0.20 extinction. This might indicate that 'resistance' in *E. coli* K 12/1 was caused by decrease in the permeability of the bacteria to the drug. This assumption was confirmed when the bacteria were suspended in a buffer

solution containing Cm 100 $\mu\text{g./ml.}$ during 120 min. before incubation in Cm+TP medium. Inhibition of growth was in this case immediate and complete (Fig. 4).

Escherichia coli K 12 infected with resistance transfer factor (RTF). Further evidence for the assumption that permeation by chloramphenicol was delayed in *E. coli* K 12/1 was obtained by infecting this strain as well as its ancestor (*E. coli* K 12) with a resistance transfer factor from a strain of *Shigella flexneri*.

Fig. 5. Inactivation of chloramphenicol and growth in chloramphenicol (Cm) 200 $\mu\text{g./ml.}$ in tryptose phosphate (TP) medium by *E. coli* K 12 with resistance transfer factor (RTF) and the tolerant mutant *E. coli* K 12/1 with the same RTF. Growth of *E. coli* K 12 (RTF) in TP without Cm (Δ) and with Cm ($\bullet$). Growth of *E. coli* K 12/1 (RTF) in TP without Cm ($\blacktriangle$), and with Cm ($\blacksquare$). $\circ$, Cm by assay in culture of *E. coli* K 12 (RTF); $\square$, Cm by assay in culture of *E. coli* K 12/1 (RTF). Cm was assayed in samples of the three parallel cultures separately. When any of the values varied more than $\pm 5\%$ from the mean, all three values are shown; in the other cases only the mean is indicated. The curves for growth in presence of Cm indicate the mean for three parallel cultures. In no case were the deviations in extinction greater than 5%.

The curves on Fig. 5 show the results of three experiments with *Escherichia coli* K 12/RTF and *E. coli* K 12/1/RTF. Both strains were able to grow in TP containing Cm 200 $\mu\text{g./ml.}$ Inactivation of Cm is shown in the figure as a 'semi-log' plot of active Cm against time. During the first 120 min. after addition of the drug, more than 90% was inactivated by *E. coli* K 12/RTF, but only 55% by the tolerant

E. coli K 12/1/RTF, though the concentration of the latter organism was slightly greater than of the former. Similar kinetics of Cm inactivation by these strains were observed in an experiment with Cm 100 µg./ml. As inactivation displays itself intracellularly (Miyamura, 1964; and unpublished observation with *Shigella flexneri* and with *E. coli* B/RTF in our laboratory), it must depend on the rate of uptake.

Uptake of [¹⁴C]chloramphenicol. Uptake of [¹⁴C]Cm was apparently a little higher in *Escherichia coli* K 12 than in *E. coli* B. The resistant mutant *E. coli* K 12/1 showed a significantly lower radioactivity (1/6) than its ancestor (Table 2).

Cross-resistance to tetracycline. The minimal inhibitory concentration of tetracycline in TPA was raised from 1 µg./ml. for the parent strain to 20 µg./ml. for all 22 high-level resistant clones of *Escherichia coli* K 12 which were examined.

Table 3. *Properties of high-level chloramphenicol-resistant mutants of Escherichia coli B and of E. coli K 12*

Bacterial strain	Growth rate	Inactivation of Cm	Uptake of Cm	Effect of inoculum size on resistance	Cross resistance to tetracycline
<i>E. coli</i> B/1	As parent strain	Yes	As parent strain	Yes	No
<i>E. coli</i> K 12/1	Decreased	No	Decreased	No	Yes

DISCUSSION

The main properties of high-level resistant mutants of *Escherichia coli* B and *E. coli* K 12 are summarized in Table 3. All the observations can be explained by the assumption that in *E. coli* B resistance is due to inactivation of chloramphenicol, and in *E. coli* K 12 to impaired permeability. Inactivation of Cm by micro-organisms has been reported by several other authors since the first investigation by Smith & Worrel (1949). Recent publications indicate that an oxidative process requiring acetyl co-enzyme A is responsible for inactivation in *E. coli* (Okamoto & Suzuki, 1965) and in *Staphylococcus aureus* (Suzuki, Okamoto & Kono, 1966). The latter authors as well as Miyamura (1964) have presumed that mutants obtained *in vitro* will never be resistant by virtue of an inactivating ability. Our results with *E. coli* B contradict this. No entirely new enzyme can, of course, be formed by a mutant. Accordingly, this kind of resistance was found only in mutants of *E. coli* B of which the parent strain also proved to inactivate the drug, though to a slight degree. A mutation to an effective degree of inactivation might be one in which the enzyme: (1) is formed in a greater amount; (2) has a higher affinity for the substrate (Cm); (3) is constitutive instead of inducible. It should, however, be pointed out that we have not shown that inactivation of Cm by the resistant *E. coli* B was due to the same enzymic system as functions to a slow degree in the susceptible ancestor.

One-step mutations to high-level resistance to streptomycin are commonly observed. As far as we are aware, such single-step mutation to high-level resistance to chloramphenicol has hitherto only been reported for *Mycobacterium ranae* (Szybalski & Bryson, 1954), but no data are available on the mechanism of resistance in this micro-organism.

In our case it was amply manifested that inactivation was the cause of resistance and not simply a consequence of growth in presence of chloramphenicol, as can be

seen from the following facts. (1) After addition of Cm the growth rate was decreased until inactivation was completed (Fig. 1). (2) The minimal inhibitory concentration of Cm depended on the inoculum size. (3) On Cm-containing media an all-or-none growth response occurred (Table 1). On the plate with Cm 200 $\mu\text{g./ml.}$ TPA, for example, confluent growth was obtained after incubation with 9.0×10^6 bacteria/ml. This was not due to the ability of a small fraction of the population to form colonies at this Cm concentration, since no growth occurred when the inoculum size was decreased by only one-tenth.

The inactivating enzymic system must be constitutive because otherwise the chloramphenicol would prevent its synthesis. Further, confirming the report of Merkel & Steers (1953) that glucose is required for inactivation by non-multiplying bacteria, our results show that inactivation depended on the presence of utilizable carbohydrate. On lactose medium no induction of β -galactosidase was possible, because of the Cm present, and without this enzyme no energy source was available for the inactivation. Pre-incubation with lactose unlocked this metabolic knot.

On media containing Cm 100 $\mu\text{g./ml.}$ or more growth depended on very large inoculum sizes; the smallest inoculum which gave growth was 1000 times greater on minimal medium (DA) than on complex TPA medium. Miyamura *et al.* (1962) reported that small amounts of peptone stimulated inactivation by non-multiplying bacteria. The explanation may be that with an initial high concentration of chloramphenicol virtually all protein synthesis was blocked. Cm-inactivation was therefore limited by the amount of inactivating enzyme present in the sample of bacteria inoculated and co-factors in the bacteria and the nutrient media. On the other hand, with Cm 60 $\mu\text{g./ml.}$ each bacterium of the inoculum grew to a colony, indicating that additional enzyme and/or co-factors might be synthesized *de novo*. This concentration of Cm may not completely prevent protein synthesis (Okamoto & Mizuno, 1962).

Uptake of [^{14}C]chloramphenicol is generally several times lower in resistant bacteria than in sensitive organisms of the same species (Vazquez, 1964; Unowsky & Rachmeler, 1966). Our results with *Escherichia coli* K 12 confirm this; but with *Escherichia coli* B no difference between the susceptible ancestor and the resistant mutant was noticed. The intracellular concentration of Cm, calculated per wet wt bacteria, was near that of the medium, proving that no concentration of Cm occurred. It follows from this that entry to, inactivation, and exit from the cell of Cm molecules must occur at a great rate. From Fig. 2 it can be seen that 1.5×10^8 bacteria (1 ml. at a concentration corresponding to 22 Klett units) inactivated 300 $\mu\text{g.}$ Cm, which is 5.6×10^{17} molecules Cm/30 min. or 2.54×10^6 molecules/bacterium/sec.

Hitherto we have not isolated mutants of the chloramphenicol-tolerant kind from *Escherichia coli* B, in spite of the fact that many mutant clones have been examined. With *E. coli* K 12, of which 36 mutants were examined, not one Cm inactivator was found. Decreased permeability to Cm was found in mutants of the latter variant of *E. coli*. That resistance was due to this lowered permeability was shown by the complete inhibition of growth after an incubation period in a Cm-containing buffer solution.

The decreased permeability may be non-specific for Cm. Cross-resistance with tetracycline and puromycin (Reeve & Bishop, 1965) should not uncritically be related to common features in the action of these three antibiotics. It might well be a consequence of decreased permeability for a wide range of micromolecular species. The

markedly decreased growth rate in TP medium as well as in minimal medium might simply be a consequence of an unspecific permeability barrier.

The growth curve of the resistant mutant *Escherichia coli* K 12/1 in TP medium (Fig. 3) indicates that there must have been something more than a simple decrease in the rate of permeation, since growth ceased at a much lower population density than for the sensitive strain.

The practical aspect of resistance to chloramphenicol is outside the scope of this communication. It may, however, be mentioned that inactivation is a far more serious form of drug resistance than is the mechanism observed with *E. coli* K 12, which places the micro-organism in absence of the drug at a competitive disadvantage.

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