Inactivation of Chloramphenicol by Chloramphenicol-Resistant Bacteria

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Virtually all (94.5 per cent of 110) chloramphenicol-resistant strains of *Shigella, Escherichia,* and *Staphylococcus* isolated from clinical cases caused significant inactivation of the antibiotic, but none of the 29 resistant pseudomonads did so. None of the 235 clinically isolated sensitive strains, representing five genera, inactivated the drug. Resistant organisms derived *in vitro* from initially sensitive ones by repeated subculture in increasing concentrations of chloramphenicol had only limited ability to inactivate the antibiotic, but those produced *via* transformation inactivated it almost completely. Such transferred resistance was lost following treatment with acriflavine. Simultaneously, ability to inactivate chloramphenicol was lost.

INACTIVATION OF penicillin by the bacterial enzyme penicillinase has been studied by several workers, but similar observations on other antibiotics are limited. Microbial degradation of chloramphenicol was first described in 1949 by Smith and Worrel (1). Later a direct correlation between chloramphenicol resistance and ability of *Escherichia coli* to destroy the antibiotic was reported (2). However, the degradative activity of the organism did not seem to account for the full degree of resistance that was developed. Subsequently, Chabbert and Debruge (3) noted that resistant strains of *Staphylococcus*, cultured in conventional media, produce a substance that is diffusible in agar and that inhibits the action of chloramphenicol. More recently, Miyamura (4) showed that resistant dysentery bacilli also have the ability to inactivate chloramphenicol, and he described some of the properties of the inactivating substance.

The present paper demonstrates a relationship between resistance to chloramphenicol and ability to inactivate the compound in *Shigella, Salmonella, Escherichia, Pseudomonas,* and *Staphylococcus* isolated from clinical cases in Niigata Prefecture, Japan.

EXPERIMENTAL

Strains Tested.—The isolates tested included 105 strains of several types of *Shigella* (*Sh. flexneri* 1*b*, 5; 2*a*, 21; 2*b*, 10; 3, 45; 6, 8; var. X, 4; var. Y, 1; *Sh. sonnei*, 11), six strains of *Salmonella* (*S. typhi*, 4; *S. paratyphi* B, 2), 107 strains of *E. coli*, 31 strains of *Ps. aeruginosa*, and 125 strains of *Staph. aureus*. Each strain was isolated from a different patient between 1958 and 1961. In addition, *E. coli*, strains B and K-12, were used as representative sensitive parent strains from which resistant strains were developed in the laboratory.

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Test for Chloramphenicol Sensitivity.—The chloramphenicol sensitivity of each strain was determined by the plate-dilution method, using 10 ml. of heart infusion agar per plate. The final concentrations of the antibiotic ranged from 100 to 0.195 mcg./ml. in twofold dilutions. Each plate was inoculated aseptically by streaking with an 18-hour broth culture of the appropriate organism, the seed culture having been prepared previously by inoculating 10 ml. of broth with 0.1 ml. of a 20-hour culture. Plates were observed after incubation for 48 hours at 37°. The minimal inhibitory concentration was taken as the lowest concentration (mcg./ml.) of chloramphenicol required to inhibit visible growth completely.

Organisms were designated sensitive if there was no visible growth on plates containing 50 mcg. or less chloramphenicol per milliliter and were considered resistant if there was no inhibition at a concentration of 50 mcg. or more per milliliter.

Determination of Ability to Inactivate Chloramphenicol.—Organisms to be tested for ability to inactivate the antibiotic were inoculated into 10 ml. of heart infusion broth and incubated at 37° for 18 hours. Then to each culture was added an equal volume of 0.01 *M* phosphate buffer solution (pH 7.0) containing 100 mcg. of chloramphenicol per milliliter, and the tubes were placed in a constant temperature water bath at 43°, at which bacterial inactivation of the drug was maximum. After 20 hours of incubation, the tubes were heated to 60° for 30 minutes, the majority of cells were spun down in a centrifuge (5000 r.p.m. for 20 minutes), and the potency of the residual unreduced chloramphenicol remaining in the supernatants was determined by cylinder-plate assays, using *Bacillus subtilis* PCI 219 as the test organism.

Development of In Vitro Resistance to Chloramphenicol.—Chloramphenicol-resistant strains of bacteria were obtained by two methods.

Increasing Concentration Method.—Bacterial strains highly resistant to the antibiotic were produced from initially sensitive cultures by successively subculturing in progressively increasing concentrations of the drug, as reported by Coffey, *et al.* (5), Cavalli, *et al.* (6), Merkel and Steers (2), and others. In the present study, *Sh. flexneri* 2*a* strain 3195, *Sh. flexneri* 3*a* strain 11, and *E. coli* strains 60 and K-12 were rendered resistant by this technique, subcultures being made at intervals of 2 to 3 days over a period of 3 to 5 months.

Mixed Culture Method.—Drug resistance is easily transferred from *E. coli* to *Shigella* and vice versa following mixed cultivation of an appropriate donor and an appropriate recipient (7, 8). In this study two resistant donor strains and six sensitive recipient strains were used as follows:

Resistant Donor	Sensitive Recipients
E. coli strain H (MIC = 400 mcg./ml.)	(*a*) *Sh. flexneri* 2*a* strain 3195
	(*b*) *S. typhi* strain 375
	(*c*) *S. paratyphi* B strain 63
Sh. flexneri 3*a* strain 1871 (MIC = 200 mcg./ml.)	(*a*) *E. coli* strain 60
	(*b*) *E. coli* K-12
	(*c*) *E. coli* B

Each donor strain was naturally resistant, *i.e.*, it was isolated directly from patients.

One-tenth milliliter from a 20-hour broth culture of the donor and a like amount from a similar culture of the recipient organism were inoculated into a tube containing 5 ml. of heart infusion broth. The mixture was incubated (37°) for 20 hours. Then a loopful of the culture was streaked on a Drigalski agar plate containing 20 mcg. of chloramphenicol per milliliter for selection of resistant organisms. Colonies characteristic of the recipient bacteria were isolated, and the chloramphenicol resistance of the isolates was determined.

Reversal of Acquired Resistance.—Since it has been reported that acriflavine effectively eliminates F factor from *E. coli* K-12 (9) and is applied for elimination of drug-resistance factor in *Escherichia* and *Shigella* (10), the agent was tested.

An overnight broth culture of chloramphenicol-resistant organisms was inoculated into a broth containing a sublethal concentration of acriflavine (5–40 mcg./ml.) and incubated at 37°. After 20 hours, a loopful of the culture was streaked on a nutrient agar plate and then chloramphenicol-sensitive colonies were selected by means of the replica plating method (11).

RESULTS AND DISCUSSION

Naturally Resistant Strains.—The relation between susceptibility and resistance to chloramphenicol of all the strains isolated and their ability to inactivate the drug are shown in Table I. Marked inactivation was observed with virtually all resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus*. Of the 110 strains of these genera studied, 104 (or 94.5%) caused more than 50% reduction of the inhibitory activity of the drug during a 20-hour period of contact. Only members of the genus *Pseudomonas* failed to exhibit a correlation between resistance and ability to impair the antibacterial activity of the antibiotic substantially. None of the 29 resistant strains of *Pseudomonas* tested caused so much as 50% loss of antibiotic activity. Among the 235 sensitive strains (representing five genera and including two pseudomonads) that were studied, only two strains of *Escherichia* (0.85% of the total of all sensitive strains) caused more than 50% reduction in the inhibitory activity of chloramphenicol. Therefore, exempting only the 29 resistant pseudomonads (7.75% of the total 374 strains tested), there appears to be a clear correlation in the organisms studied between resistance to chloramphenicol and ability to inactivate it.

Strains with Resistance Acquired In Vitro.—Resistant organisms obtained *in vitro* by isolation from cultures serially transferred in broth with increasing concentrations of chloramphenicol were tested for their ability to inactivate the drug. The data in Table II show that the ability of organisms to impair the antibacterial activity of chloramphenicol did, indeed, increase with acquisition of resistance as reported by others (2, 5). However, the capacity of such bacteria to reduce the potency of the drug was not so great as that of naturally resistant strains of the same species (compare with Table I).

In other experiments, organisms with "transferred resistance," *i.e.*, rendered resistant *via* the mixed culture method described under *Experimental*, were examined for their ability to reduce the potency of chloramphenicol. The data are shown in Table III. It is noteworthy that in all instances chloramphenicol solutions exposed to the transformed organisms, *i.e.*, those carrying "transferred resistance," lost more than 90% of their antibacterial potency. Comparison of relevant data in Tables II and III suggests that, with respect to the basis for resistance, there is a significant difference between organisms rendered resistant *via* the serial transfer method and those acquiring resistance *via* transformation (mixed culture method). Three examples can be noted. *Sh. flexneri* 2*a*-3195 with initial MIC of 0.39 mcg./ml. had no destructive effect on chloramphenicol, but when rendered resistant it was capable of inactivating the drug. However, the degree to which it did so depended on the method used to induce resistance. Organisms rendered resistant *via* transformation (Table III) did not acquire a degree of resistance so high as those

TABLE I.—RELATION BETWEEN SUSCEPTIBILITY AND RESISTANCE TO CHLORAMPHENICOL AND ABILITY OF BACTERIA ISOLATED FROM PATIENTS TO INACTIVATE THE ANTIBIOTIC

Organism	Susceptibility[a]		Number of Strains — Chloramphenicol Inactivation,[b] % 0–25 %	26–50%	51–75%	76–100%
Shigella	Resistant	45	0	3	10	32
	Sensitive	60	57	3	0	0
Salmonella	Sensitive	6	6	0	0	0
Escherichia	Resistant	44	0	2	8	34
	Sensitive	63	39	22	2	0
Pseudomonas	Resistant	29	23	6	0	0
	Sensitive	2	2	0	0	0
Staphylococcus	Resistant	21	0	1	6	14
	Sensitive	104	93	11	0	0

[a] Resistant: no inhibition of growth at chloramphenicol concentration of 50 mcg. or more per milliliter. Sensitive: inhibition of growth at 50 mcg. or less per milliliter. [b] Per cent reduction of chloramphenicol in broth culture of the microorganism. Initial concentration of antibiotic = 50 mcg./ml.

developing resistance during serial transfer (Table II), but they developed a far greater capacity for inactivating the drug. An analogous phenomenon can be seen in *E. coli* 60. *E. coli* K-12 developed the same degree of resistance regardless of the method used to induce it, but strains with transferred resistance (Table III) exhibited greater increase in ability to inactivate chloramphenicol than did strains that developed resistance during serial transfer. Resistance acquired *via* transformation in the absence of drug (mixed culture method) we designate "transferred" to distinguish it from the "selective" type of resistance developed by continuing exposure to increasing concentrations of the antibiotic during a long series of serial transfers.

Reversal of Acquired Resistance.—It seemed desirable to ascertain whether resistance acquired *via* transformation could be reversed and, if so, whether ability of the organisms to inactivate chloramphenicol would simultaneously be lost. When chloramphenicol-resistant *E. coli* 60 derived from the sensitive strain *via* transformation (Table III) was treated with acriflavine, there was substantial loss of resistance and complete loss of ability to inactivate the drug (Table IV).

The data presented above suggest that there are at least two different mechanisms involved in bacterial resistance to chloramphenicol. It seems clear that the resistance of some organisms can be attributed largely, if not entirely, to an inherent (or transferred) ability to inactivate the antibiotic, whereas in other organisms, *e.g.*, naturally resistant *Pseudomonas*, inactivation is not a significant factor in resistance. It may be suggested that in the latter instance resistance depends on the biochemical resourcefulness or versatility of the species.

When the normal or usual pathway from substrate to synthesized metabolites essential for growth and reproduction is blocked, some strains which can circumvent the roadblock by developing an adequate alternative pathway will be resistant. Figuratively speaking, the organisms will simply go around the obstruction without destroying it; if it is unable to develop a satisfactory biochemical detour and cannot destroy the roadblock, it will be sensitive. On the other hand, a species or strain that cannot develop such a detour route may still be resistant, if it can remove (or smash its way through) the roadblock or, in terms of bacteria and antibiotics, can destroy or inactivate the antibiotic molecule. Of course, it is possible that in some organisms both mechanisms of resistance may operate simultaneously.

From the present studies it seems that the naturally resistant strains of *Pseudomonas* we isolated from patients and the resistant strains of *Sh. flexneri* and *E. coli* developed from initially sensitive populations by the serial transfer technique have the same type of resistance, which stems primarily from what was referred to above as resistance due to biochemical versatility. It is, of course, possible that direct enzymatic attack on the antibiotic molecule may also contribute to the resistance of some of these strains, but such contribution, if any, appears to have minor significance. On the other hand, naturally resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus* isolated from patients and resistant strains of *Sh. flexneri*, *S. typhi*, *S. paratyphi*, and *E. coli* developed *via* the transformation technique seem to owe their resistance primarily to native or transferred ability to destroy the chloramphenicol molecule or otherwise block its antibacterial action. These observations regarding bacterial resistance to chloramphenicol are reminiscent of the patterns of resistance to penicillin found in appropriate organisms.

It is proposed that some strains of chloramphenicol-resistant bacteria produce an enzyme that is analogous to penicillinase. Support for the enzymatic nature of the chloramphenicol-inhibiting factor has been published (4). Briefly, the substance was shown to be active in the pH range 5 to 8 at 40–45° but to be destroyed by heating at 60° for 30 minutes.

TABLE II.—RELATION BETWEEN INCREASE IN CHLORAMPHENICOL RESISTANCE (INDUCED BY SERIAL TRANSFER IN INCREASING CONCENTRATIONS) AND ABILITY TO INACTIVATE THE DRUG

Strain	Original MIC[a]	Original % Red[b]	Resistant MIC[a]	Resistant % Red[b]
Sh. flexneri 2a-3195	0.39	0	200	24
Sh. flexneri 3a-11	1.56	10	100	20
E. coli 60	0.78	7	400	35
E. coli K-12	3.12	5	200	23

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol in broth culture. Initial concentration of antibiotic = 50 mcg./ml.

TABLE III.—RELATION BETWEEN INCREASED CHLORAMPHENICOL RESISTANCE (INDUCED BY TRANSFORMATION *via* MIXED CULTURE METHOD) AND CHLORAMPHENICOL INACTIVATION

Strain	Original MIC[a]	Original % Red[b]	Resistant MIC[a]	Resistant % Red[b]
Sh. flexneri 2a-3195[c]	0.39	0	100	>90
S. typhi 375[c]	1.56	5	200	>90
S. paratyphi B 63[c]	1.56	0	400	>90
E. coli 60[d]	0.78	7	50	>90
E. coli K-12[d]	3.12	5	200	>90
E. coli B[d]	0.39	10	50	>90

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol activity in broth culture. Initial concentration of antibiotic = 50 mcg./ml. [c] Donor: *E. coli* H (MIC:400 mcg./ml.; % red: >90). [d] Donor: *Sh. flexneri* 3a-1871 (MIC:200 mcg./ml.; % red: >90).

TABLE IV.—LOSS OF CHLORAMPHENICOL RESISTANCE AND ABILITY TO INACTIVATE CHLORAMPHENICOL IN RESISTANT *E. coli* 60 TREATED WITH ACRIFLAVINE

Strain	MIC[a]	% Red[b]
Original	0.78	7
Resistant (produced by mixed culture)	50	>90
Resistant (after acriflavine treatment)	3.12	0

[a] Minimal inhibitory concentration of chloramphenicol, mcg./ml. [b] Per cent reduction of chloramphenicol activity in broth culture. Initial concentration of antibiotic = 50 mcg./ml.

SUMMARY

Three-hundred and seventy-four strains of *Shigella*, *Escherichia*, *Salmonella*, *Pseudomonas*, and *Staphylococcus* isolated from patients in Niigata Prefecture were examined for their ability to inactivate chloramphenicol. Most chloramphenicol-

col resistant strains of *Shigella*, *Escherichia*, and *Staphylococcus* exhibited such action, but the sensitive strains and all strains of *Pseudomonas*, whether resistant or sensitive, did not.

In the experiments on resistance developed *in vitro* and the inactivation of chloramphenicol, organisms with resistance transferred by the mixed culture method were far more effective inactivators of chloramphenicol than the resistant organisms developed by growth in progressively increasing concentrations of the drug. A direct correlation was observed between the gain and loss of resistance *in vitro* and the increase and decrease of chloramphenicol inactivation in *E. coli* strain 60.

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Determination of Thickness of Walls of Hard Gelatin Capsules by Radioisotopic Means

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A procedure has been developed for the determination of the thickness of the wall sections of hard gelatin capsules. A chlorine-36 source was mounted in the tip of a stainless steel capsule dipping pin and was used to observe the differences in thickness of the wall sections. Twelve series (replications) of 25 determinations each were performed. The measurements taken included the weight, area, calculated weight per unit area, thickness in inches using a micrometer, and beta count rate. The count rates were plotted against the corresponding weight per unit area (mg./cm.^{2}) and thickness (in.). Regression curves were prepared using least squares calculations. A *t* test was used to compare the individual slopes of the regression curves with the mean slope. The applicability of this method of determining the thickness of wall sections of hard gelatin capsules is discussed.

THE FORMATION of hard gelatin capsules involves the use of stainless steel pins which are dipped into a liquid gelatin medium, after which the formed capsules are slowly dried. Not until the drying process has been completed is gauging of the wall sections currently possible. If the thickness of the drying capsules could be gauged early in the drying process, it would be possible to adjust the process more quickly should it produce capsules which were not within control limits. This would result in a saving of time and less loss of product. Furthermore, an isotopic method of evaluating capsule thickness as an in-process control could conceivably result in an automated line operation.

Some of the more important reported uses of radioisotopes in industry apply the principles of penetration and absorption of the isotopic radiations (1). These principles are generally applied to the area of thickness gauging. Those radioisotopes which emit beta particles are most frequently used. Since they are less penetrating than gamma rays, they may be used for measuring the thickness of thin films, paper, and plastic materials (2). One type of gauging system frequently employed is based on the principle of the change of transmission of beta particles through the medium (3). To measure very thin, low density materials—such as paper—carbon-14, a weak beta emitter, has been used (4). Other beta sources that have been used include chlorine-36, krypton-85, strontium-90, and thallium-204 (5–7). Iodine-131 was used to measure the thickness of thin films in the vicinity of 1 μ (8).

The purpose of this study was to select and evaluate a radioisotope to be used for the thickness gauging of wall sections of hard gelatin capsules based on the principle of beta particle absorption.

EXPERIMENTAL

The usual method of expressing the thickness of thin materials by radioisotopic means is in terms of weight per unit area (mg./cm.^{2}). From a study of No. 000 hard gelatin capsules, it was found that the average weight per unit area of capsule sections was 14.12 mg./cm.^{2}. Small pinpoint sources of two pure

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