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RESTORATION OF DRUG-RESISTANT BACTERIA TO SENSITIVITY TO CHEMOTHERAPEUTIC DRUGS

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I. BACKGROUND

Throughout history, military operations have been hampered by outbreaks of communicable diseases among troops. Many times, entire operations had to be abandoned; one recent example of this is the invasion of Taiwan from mainland China, planned in 1949, which had to be called off because of a massive outbreak of schistosomiasis which the assembled Red Chinese troops acquired while practicing landing maneuvers on inland lakes in Fukien Province. Some idea of the magnitude of this military health catastrophe can be derived from the fact that the Communist Chinese government purchased at that time, 300,000 doses of an antischistosomal drug from a pharmaceutical house in Germany (1). Even numerically insignificant occurrences of communicable diseases such as epidemic hemorrhagic fever during the Korean War or meningococcal meningitis in training camps can have effects on the morale of troops.

The past 60 years have seen the development of chemotherapeutic drugs for the cure of many protozoal and bacterial infections. These advances in combination with preventive medical measures gave rise to an almost limitless optimism concerning the prospect of control and ultimate eradication of major communicable diseases. The introduction of sulfonamides, penicillin and streptomycin some thirty years ago was, however, soon followed by the emergence of drug-resistant mutants of disease-producing bacteria. The emergence of drug-resistant Plasmodium falciparum around 1960 (2) and the ensuing "malaria problem" for our forces in SE Asia dissipated a sense of complacency which had accompanied decades of steady advances in coping with communicable diseases.

As long as drug resistance only was found to arise from spontaneous chromosomal gene mutations, followed by selective propagation of mutant populations under "drug pressure", one could reduce

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the frequency of such occurrences by intelligently devised regimens of drug administration, foremost chemotherapy with drug combinations. One could further expect that established drug-resistant strains of bacteria would be competetively replaced by drug-sensitive wild-type organisms when the drug pressure was removed, i.e. when the drugs in question were shelved for extended periods of time. Finally, one could have hoped that molecular modification of drugs like that of penicillin (3) might become a more general approach to overcoming mutational resistance to single drugs.

Beginning in Japan in the late 1950ies, however, a radically new type of bacterial drug resistance has arisen (4) and has subsequently been encountered in all parts of the world; this involves multiplicities of structurally and functionally unrelated drugs, antibiotic as well as synthetic. Multiple drug resistance, too, has a genetic basis but does not originate in chromosomal mutations; instead it is determined by non-chromosomal genetic entities, the episomes which can carry resistance determinants for several different drugs (4). Such episomes are known as resistance factors or R-factors. In addition to a variety of drug-resistance determinants, they also carry a large segment known as "resistance transfer factor", RTF, which enables R-factors to be transferred into drug-sensitive bacterial cells, not only of identical or taxonomically related species, but to other bacteria which grow in mixed cultural environments such as the intestinal tract. A recent dysentery epidemic in Guatemala was caused by a multiple-resistant strain of Shigella and claimed 15,000 deaths, i.e. 0.4 per cent of the total population. The majority of bacterial infections in SE Asia is caused by multiple drug-resistant strains. Hospital outbreaks of infections caused by multiple drug-resistant bacteria are increasing in number and endanger, specifically, patients whose immune mechanism is impaired. Some fifty per cent of severe burn patients die from uncontrollable bacteremia. Clearly, R-factor-mediated multiple drug resistance has become a problem in military medicine which will probably increase in magnitude. We report here experimental studies aimed at the elimination of R-factor-caused multiple drug resistance.

II. SPECIFIC INTRODUCTION

The F-factor, an episome, can be eliminated from Escherichia coli by culturing this organism in the presence of non-inhibitory concentrations of acriflavine (5), proflavine or acridine orange (6). Watanabe and Fukasawa (7,8) and others (9) reported that certain resistance determinants were eliminated similarly from R-factors of enteric bacteria. Frequencies of elimination or of "curing" as it is sometimes called, were quite low, especially in E. coli. Subsequently it was found that ethidium bromide, an antitrypanosomal drug, also can eliminate resistance determinants from R-factors, harbored by bacteria (10).

Episomes consist essentially of DNA. For example, the F'lac factor of E. coli has been found to be supercoiled, closed-circular DNA (11,12). R-factors were shown in 1966 to be DNA in nature (13,14) and more recent studies have brought out numerous instances in which R-factors were isolated as supercoiled, closed-circular DNA (for example 15,16).

Aminoacridines and ethidium bromide which eliminate R-factors are members of a class of compounds which form intercalation complexes with DNA (17). Our laboratory has studied this phenomenon in detail (for example, 18,19). We have proposed the hypothesis (20) that the ability to eliminate resistance determinants from bacterial R-factors is a group property of DNA complexing drugs and dyes. The subject of this paper is the successful experimental testing of this hypothesis.

III. ELIMINATION OF THE LAC MARKER FROM F'LAC FACTOR

Strain 3876 of E. coli K-12 which contains the F'lac episome was cultured overnight in the presence of subinhibitory concentrations of the DNA-complexing substances listed in Table 1. The bacteria were then plated on Mac Conkey's agar, the total numbers of colonies, as well as the numbers of lac^- colonies, were counted and the frequency of the elimination of the lac^+ marker was expressed as the percentage of lac^- colonies on these plates. Dosage response data for the elimination of lac^+ were converted by a graphic probit transformation to straight lines which were fitted by the method of least squares, according equal statistical weight to each point. From these lines, 50 per cent effective doses, ED_{50}s, were intrapolated.

The dosage-response correlation for the elimination of lac^+ by the DNA-complexing drug, quinacrine, is shown in Fig. 1. The ED_{50} was intrapolated to be 7.2×10^{-5} M. Fig. 2 compares the dosage-response lines for quinacrine, acridine orange, ethidium bromide and chloroquine. For the sake of clarity, individual points have been deleted. The intercepts with the 50 per cent line of the dosage-responses for quinacrine, acridine orange and ethidium bromide lie close to each other, and the slopes of the three lines are similar, hence, the potencies of the compounds were similar. On the other hand, the dosage-response line for chloroquine intercepts the 50 per cent line at a drug concentration of 10^{-3} M, and the slope of the line is flatter, indicating that chloroquine was much less active in eliminating the lac^+ determinant from the F'lac episome.

Table 1 lists the 50 per cent eliminating concentrations for all DNA-complexing compounds tested. For the antischistosomal drug, miracil D, which also has antibacterial and antineoplastic activity, an ED_{50} of 3.3×10^{-3} M was extrapolated; quinine showed an activity trend but extrapolation to an ED_{50} could not be made

with confidence. Methylene blue and p-rosaniline were devoid of activity.

The elimination of the lac marker raised the question of the physical fate of the episomal DNA; Hirota (6) had shown by a genetic analysis that acriflavine and proflavine eliminated the F-factor function from E. coli, but no molecular analysis of the alteration or absence of F-factor DNA upon elimination of its genetic activity was carried out. We labelled the total DNA of the F'lac containing bacteria and of those from which lac had been eliminated by cultivation in the presence of quinacrine by growing mass cultures from single lac^+ and lac^- colonies in the presence of ^{14}C-thymidine. The radioactive DNAs were extracted and subjected to equilibrium centrifugation in a cesium chloride density gradient by established methods (21). The typical result of these analyses is shown in Fig. 3. The DNA from F'lac containing bacteria (Frame A) showed a distinct band of heavy DNA below the very large band of chromosomal DNA (not shown in its entirety); the DNA from lac^- bacteria contained a much smaller band in the same region of the gradient. The persistence of residual circular DNA in the region normally occupied by F'lac episomal DNA is reminiscent of the formation and presence of aberrant DNA in yeast mitochondria whose genetic and biochemical properties are eliminated by DNA-complexing substances (22).

IV. ELIMINATION OF RESISTANCE DETERMINANTS FROM R-FACTOR-HARBORING E. COLI

E. coli RS-2 harbors an R-factor which carries determinants of resistance to kanamycin, chloramphenicol, ampicillin, streptomycin and sulfadiazine. This organism was grown overnight in the presence of subinhibitory concentrations (routinely 10^{-4} M) of the DNA-complexing substances listed in Table 2. The cultures were then divided into aliquots; one was plated on nutrient agar and others on agar plates containing singly the antibiotics listed above or sulfadiazine. The difference between the numbers of colonies on drug-containing agar plates and those on drug-free control plates were expressed as percentages of the numbers of colonies on the latter; these percentages were considered the frequencies of resistance elimination which had been attained. In order to avoid a possible carry-over of traces of eliminating compounds to drug-containing test plates, followed by synergistic growth suppression (which would result in erroneously high elimination frequencies), aliquots of experimental cultures which had grown in the presence of the eliminating compounds (Table 2) were also passed through one subculture in the absence of such compounds and were then plated as described above. Both procedures yielded identical results.

Table 2 lists the results of such experiments. Kanamycin resistance was eliminated by five substances with berberine and

chloroquine showing activity trends. Chloramphenicol resistance was eliminated by 3 substances and ampicillin resistance only by ethidium bromide. Resistance to streptomycin and sulfadiazine was not eliminated. Such differential effect (segregation) has also been observed by others (10) in Salmonellae in which kanamycin resistance but not that to ampicillin was eliminated by ethidium bromide.

For the most active agent, ethidium bromide, we developed dosage-response correlations of the elimination of kanamycin and chloramphenicol resistance which are shown in Fig. 4. The ED_{50}s and slopes of the two dosage-response lines were similar for the elimination of these two resistance determinants.

It is of theoretical importance whether the elimination of resistance determinants by DNA-complexing compounds is a contact effect or requires extended growth of a bacterial culture in the presence of eliminating compounds. To test for these alternatives we exposed the growing RS-2 strain to ethidium bromide for 30 min and produced a large number of plate counts sufficient for statistical analysis of the extent to which kanamycin resistance was eliminated. The number of colonies on drug-free control plates and on kanamycin-containing test plates were not significantly different; we conclude that brief exposure of bacteria to ethidium did not eliminate the kanamycin resistance determinant.

V. ELIMINATION OF A GENTAMICIN R-FACTOR FROM KLEBSIELLA PNEUMONIAE

Gentamicin is an aminoglycoside antibiotic which has been considered the drug of choice in the treatment of systemic infections with Gram-negative bacteria. The drug does not possess a hydroxyl group in position #3 of its methylamino sugar moiety as do its congeners, streptomycin, kanamycin and other aminoglycosides. Since R-factors for streptomycin and kanamycin direct the formation of bacterial enzymes which derivatize this hydroxyl group and, in that manner, inactivate these antibiotics, it was postulated (23) that R-factors for gentamicin were not observed because the antibiotic lacks the prerequisite chemical target group for enzymatic attack. However, gentamicin R-factors subsequently turned up in France (24), and strain 3694 of Klebsiella which we have studied was isolated in an outbreak of gentamicin-resistant infections in Georgetown University Hospital. This strain carries, additionally, determinants of resistance to kanamycin, penicillin, chloramphenicol, tetracycline and sulfadiazine as we found in our laboratory. "Curing" experiments on the elimination of the gentamicin resistance determinant were undertaken by the methods described above with results shown in Table 3. Ethidium bromide, quinacrine and acridine orange eliminated the gentamicin determinant. Berberine, a DNA-complexing alkaloid (19) and mitochondrial mutagen (25) with curative properties in cutaneous leishmaniasis (26) showed border-

line activity but chloroquine, quinine, spermine, methylene blue and p-rosaniline were without effect. The elimination frequencies were similar to those observed for the "curing" of R-factors in E. coli (Table 2).

VI. ELIMINATION OF MULTIPLE DRUG RESISTANCE IN SALMONELLA TYPHIMURIUM

Elimination of resistance determinants is attained with greater frequencies in strains of Salmonella than in E. coli (10). Furthermore, a spontaneous loss of resistance determinants occurs in Salmonella typhimurium LT-2 with unusually high frequencies (27). We undertook, therefore, curing experiments with the LT-2 strain in the hope of attaining higher elimination frequencies and of demonstrating curing by DNA-complexing compounds which had shown little or no activity in the preceding studies with E. coli or K. pneumoniae.

The R-factor of E. coli RS-2 was transferred into Salmonella typhimurium LT-2 by standard methods.* In the isolation of the LT-2 acceptor organisms, after R-factor transfer, use was made of the fact that the RS-2 donor bacterium is a proline auxotroph and fails to grow on defined medium agar plates from which proline is withheld. Since Salmonella typhimurium LT-2, even before R-factor transfer, was found to be insensitive to >25 mg/ml of sulfadiazine, curing experiments on the elimination of the sulfonamide determinant could not be carried out.

All DNA-complexing compounds were used in a concentration of 10^{-4} M and the methods for the determination of elimination of resistance determinants were the same as described above for the experiments with E. coli. The results are shown in the histograms, Figs. 5,6,7 and 8. All eleven compounds tested eliminated the kanamycin determinant from S. typhimurium (Fig. 5); the most active substance was ethidium bromide which produced more than 95 per cent curing. The spontaneous loss of the kanamycin determinant occurred with a frequency of 63 per cent. The chloramphenicol resistance determinant was eliminated by all compounds except methylene blue (Fig. 6). The frequency of spontaneous loss was 50 per cent. Similarly (Fig. 7), all compounds except methylene blue eliminated the ampicillin resistance determinant which was lost spontaneously with the same frequency as the chloramphenicol resistance determinant. Finally (Fig. 8), the streptomycin resistance determinant was eliminated by all compounds except methylene blue and chloroquine; the frequency of spontaneous elimination was 55 per cent. It should be noted that methylene blue actually decreased the frequencies of spontaneous loss of the streptomycin and ampicillin resistance determinants and that chloroquine produced a similar decrease for

*The authors acknowledge the advice of Dr. Louis Baron in the conduct of these transfer experiments.

the streptomycin determinant. To our knowledge, this is the first instance in which certain R-factors seem to be stabilized by DNA-complexing compounds.

VII. DISCUSSION

The present work has shown that sensitivity to chemotherapeutic drugs can be experimentally restored to R-factor-harboring multiple-resistant bacteria at frequencies which optimally involved 95 per cent of entire pathogenic bacterial populations. Our studies progressed systematically from model experiments with non-pathogenic E. coli to the curing of human pathogens, Klebsiella and Salmonella.

The elimination of episomal genetic determinants by DNA-complexing compounds occurred in systematic response to the dosage of these substances. The significance of this lies in the fact that the hyperbolic dosage response correlation [or its linear probit transformation which we have represented] (Figs. 1,2 and 4) indicate that a biological response is the result of the occupancy of specific receptor sites by active compounds according to the law of mass action. In fact, the hyperbolic dosage-response correlation can be derived mathematically from that law (28). Since our test compounds were selected for their group property of forming complexes with duplex DNA, our results suggest that these substances act by occupancy of receptor sites on episomal DNA.

A spontaneous segregation of resistance determinants in Salmonella has been observed by several authors (27, 29). This we have confirmed. Anderson (29) interprets such observations as consequences of asynchronies in the replication of individual resistance determinants during logarithmic bacterial growth. Although R-factors when harbored by E. coli behave as single units of genetic transmission and can be isolated as single circular DNA molecules (16), they can in Salmonella and Proteus dissociate into distinct and physically separable component DNAs (16). It has been proposed (30) that seemingly unitary R-factors represent, in reality, recombinational assemblages of several individually replicating DNA units or "replicons."

This not only explains observations of spontaneous segregation of individual resistance determinants but also our present results of curing which we regard as experimentally enhanced segregation owing to the ability of DNA-complexing drugs to inhibit differentially the replication of different DNA replicons. One differential effect of ethidium bromide on DNA polymerizations by different enzymes, using different DNA templates, has recently been reported (31). A differential inhibition of the replication of resistance determinant DNAs requires extended growth of test cultures in the presence of DNA-complexing compounds and mere contact should not eliminate such determinants. This we have shown with ethidium

bromide for the kanamycin determinant in E. coli.

It has been postulated in our laboratory (18) that substances which interfere with DNA strand separation will inhibit DNA replication in vivo because strand separation is a mechanistic prerequisite for this replication. Our substances which actively eliminated lac^+ or resistance determinants belong into a category of compounds which form complexes with DNA and do interfere with strand separation. Ethidium bromide, chloroquine, proflavine, miracil D (32), quinacrine (17), berberine and quinine (19) become inserted ("intercalated") between base pairs in DNA and stabilize the double helix. Spermine also stabilizes the double helix through electrostatic attraction to both complementary strands. Acridine orange binds to DNA by different modes, depending upon the dye/DNA ratio but it, too, stabilizes the helix (33). Methylene blue and p-rosaniline complex DNA as demonstrable spectrophotometrically. There exists an extensive literature which documents the inhibitions of DNA-depending nucleic acid polymerase reactions in vitro and of nucleic acid biosynthesis in vivo by most of the compounds cited above.

Moreover, intercalation of the substances cited into closed circular DNA has the effect of winding up such DNA into artificial supercoils (32). At the outset of our studies we postulated (20) that such compact and unnatural supercoils of circular episomal DNAs should not merely be incapable of undergoing strand separation but also of interacting with enzymes as necessary for DNA replication. Our present findings are in accord with this hypothesis.

In conclusion then, we have postulated, tested and verified the hypothesis that DNA-complexing substances eliminate resistance determinants form bacterial R-factors and, hence, restore bacterial sensitivity to major chemotherapeutic drugs. Although this was observed to maximally 95 per cent of a population of pathogenic bacteria, the effects were, on the average, not large enough to suggest clinical application, for example, of quinacrine, at this time. Our results, however, suggest the feasibility of a research and development program aimed specifically at discovering "second generation," superior R-factor-eliminating pharmaceutical substances. We are aware of one major drug house abroad which already is actively embarked on such a program, although detailed technical information is, understandably, not available. The ultimate practical aim of such programs must be the development of pharmaceuticals which eliminate R-factor-mediated drug resistance clinically and can be administered to patients in combination with chemotherapeutic drugs in order to effect cure of bacterial infections with drug-resistant bacteria to which drug sensitivity is restored in vivo.

VIII. SUMMARY

Bacterial diseases caused by drug-resistant organisms present major problems in military medicine. Clinical (foremost quinacrine) as well as experimental pharmaceuticals and dyes restored drug-resistant bacteria to sensitivity to chemotherapeutic drugs when this resistance was caused by episomal R-factors. This was first shown in model experiments with Escherichia coli and subsequently with pathogenic Klebsiella pneumoniae and, especially, with Salmonella typhimurium. Maximally, more than 95 per cent of cells of S. typhimurium regained sensitivity to the antibiotic drug, kanamycin, when grown in the presence of ethidium bromide. The mechanism underlying the elimination of resistance determinants is the formation of complexes of active compounds with R-factor DNA followed by selective inhibition of its replication. The fate of the episomal F'lac DNA upon elimination of the lac determinant with quinacrine was studied biophysically. Since R-factor DNA is closed circular in its molecular architecture, the eliminating compounds act not only by stabilizing double-helical DNA to strand separation (which is a prerequisite to DNA replication) but also wind up the DNA circles into artificial supercoils whose biochemical functionality is suspended. Our experimental elimination of bacterial drug resistance suggests the feasibility of undertaking an R&D program with the objective of developing superior drug sensitivity-restoring pharmaceuticals to be administered to patients along with chemotherapeutic drugs for the clinical cure of bacterial infections with drug-resistant organisms.

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TABLE 1

FIFTY PERCENT EFFECTIVE DOSES, ED_{50}S, OF COMPOUNDS ELIMINATING THE LAC$^+$ MARKER FROM *E. coli* 3876

Compound	ED_{50}
Quinacrine	7.2×10^{-5} M
Ethidium bromide	7.5×10^{-5} M
Acridine orange	8.2×10^{-5} M
Chloroquine	9.0×10^{-4} M
Miracil D*	3.3×10^{-3} M
Quinine	†
Methylene blue	†
p-Rosaniline	†

* Extrapolated.
† No 50 percent activity attained.

TABLE 2

FREQUENCIES OF CURING R FACTORS IN *E. coli* RS-2 BY SELECTED COMPOUNDS KNOWN TO BIND TO DNA

Compound 10^{-4} M	Percent Curing of R Factors for: Kanamycin	Chloramphenicol	Ampicillin	Streptomycin	Sulfadiazine
Ethidium bromide	59 ± 4	61 ± 4	15 ± 8	0	0
Quinacrine	25 ± 3	22 ± 8	4 ± 2	0	0
Acridine orange	21 ± 5	22 ± 3	0	0	0
Berberine	9 ± 4	9 ± 4	0	0	0
Quinine	10 ± 3	0	0	0	0
Chloroquine	10 ± 6	0	0	0	0
Spermine	11 ± 2	0	9 ± 4	0	0
p-Rosaniline	0	0	0	0	0
Methylene blue	0	0	0	0	0

TABLE 3

Elimination of Gentamicin R-Factor in Klebsiella pneumoniae

Compound 10^{-4} M	Percent Elimination: Method I	Method II
Ethidium bromide	57	47
Quinacrine	34	28
Acridine orange	20	19
Berberine	trend	trend
Chloroquine	0	0
Quinine	0	0
Spermine	0	0
p-Rosaniline	0	0
Methylene blue	0	0

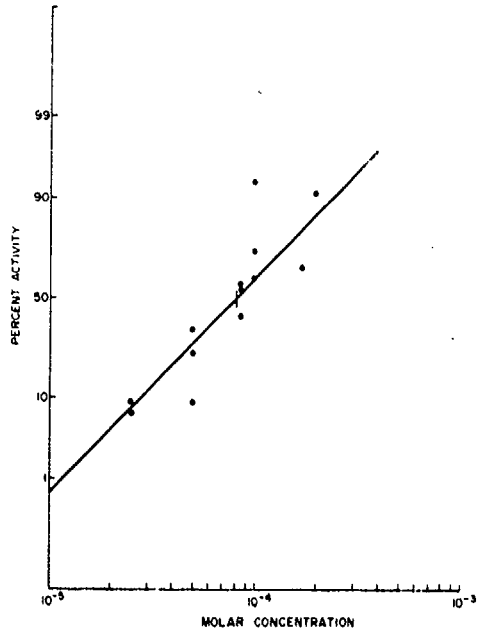

FIGURE 1. Frequency of elimination of the lac$^+$ marker from F lac containing *E. coli* 3876 as a function of the quinacrine concentration.

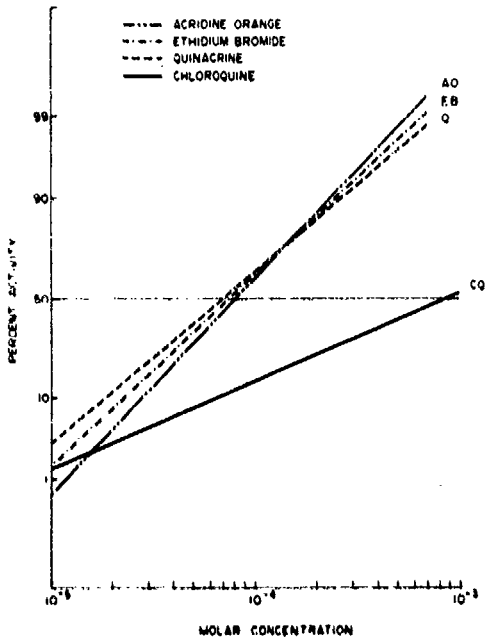

FIGURE 2. Dosage response lines as in FIGURE 1 for the lac$^+$ elimination by acridine orange: AO, ethidium bromide: EB, chloroquine: CQ, and quinacrine: Q.

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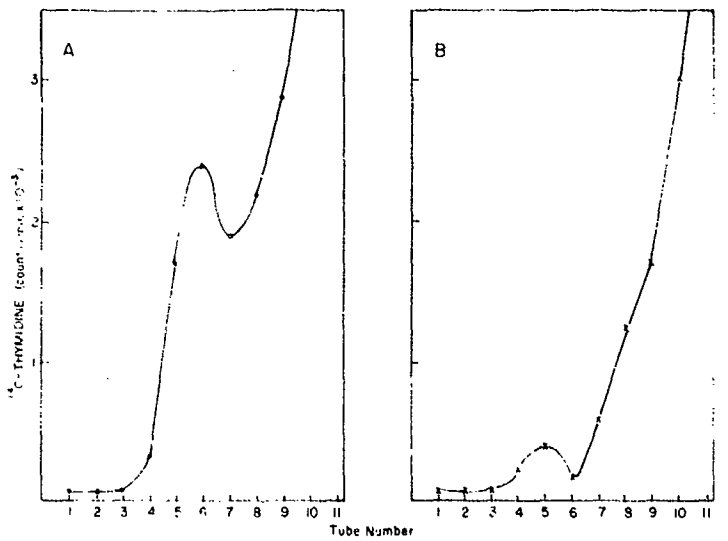

FIGURE 3. Ethidium bromide-buoyant density profiles of DNA. A: from F lac containing *E. coli*; B: from *E. coli* from which the lac⁺ marker had been eliminated by quinacrine.

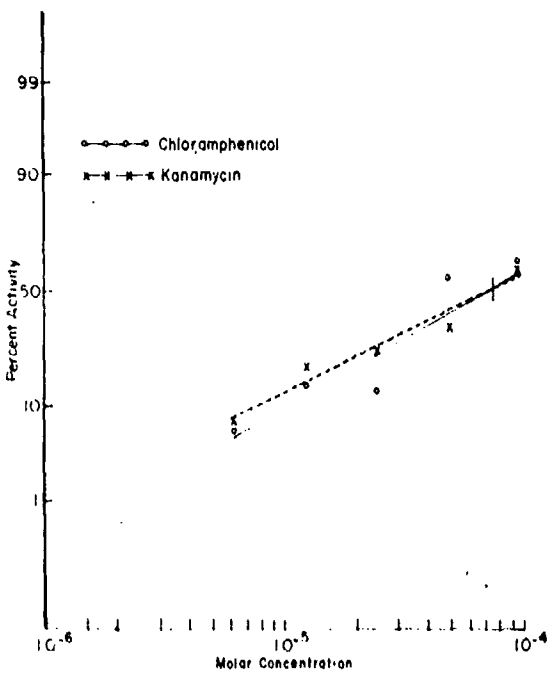

FIGURE 4. Frequency of curing kanamycin and chloramphenicol R factors in *E. coli* RS-2 as a function of the concentration of ethidium bromide.

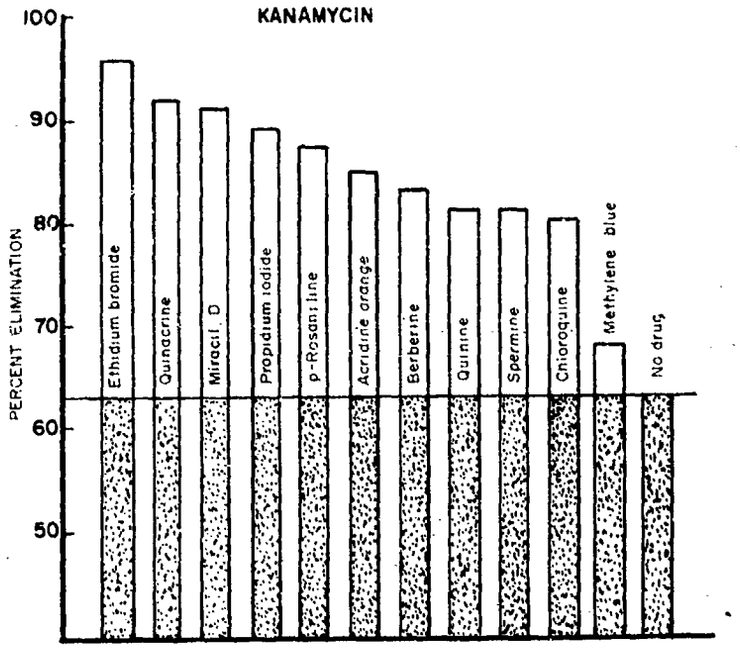

Figure 5. Elimination frequencies of the kanamycin resistance determinant in S. typhimurium.

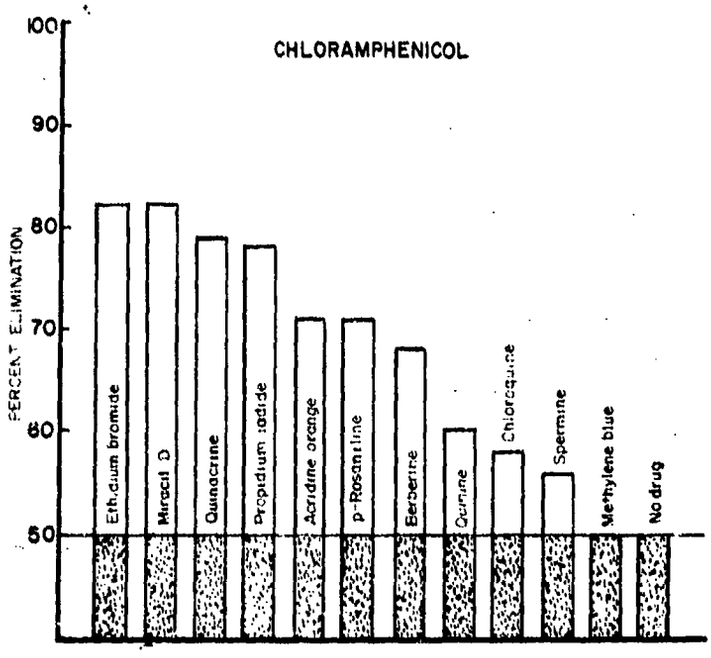

Figure 6. Elimination frequencies of the chloramphenicol resistance determinant in S. typhimurium.

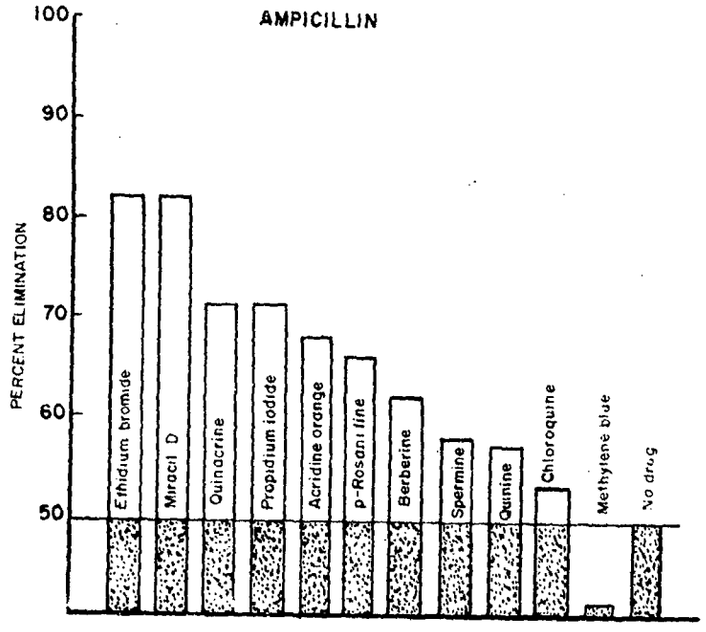

Figure 7. Elimination frequencies of the ampicillin resistance determinant in S. typhimurium.

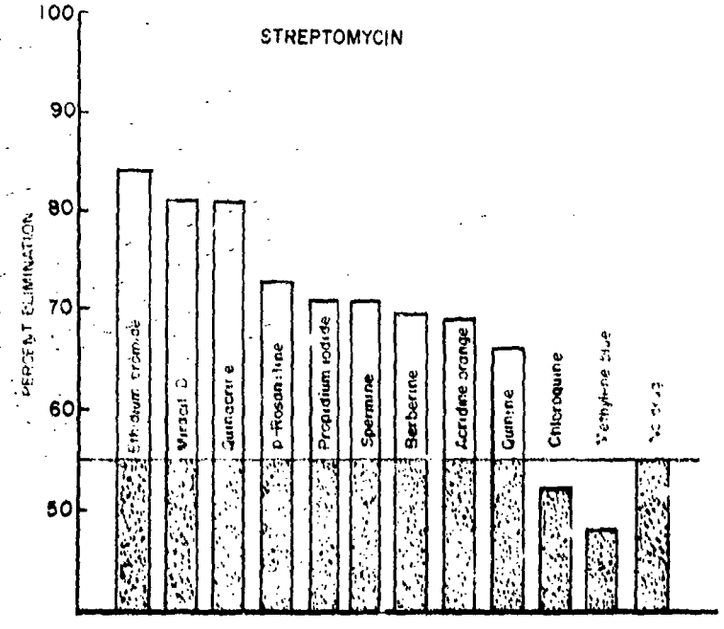

Figure 8. Elimination frequencies of the streptomycin resistance determinant in S. typhimurium.