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MECHANISMS OF STREPTOMYCIN AND TETRACYCLINE RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

by

© JUI-TENG TSENG

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Mechanism of Streptomycin and Tetracycline Resistance in Pseudomonas aeruginosa" submitted by Jui-teng Tseng in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A survey of Pseudomonas aeruginosa isolated from clinical specimens was made in an attempt to correlate the spectrum of their streptomycin and tetracycline resistance and the mechanism of resistance. The strains can be classified into three groups, according to their level of resistance to streptomycin: susceptible, low-level resistant, and high-level resistant strains. The mechanism of resistance of high-level resistant strains is either an R factor-mediated inactivation of streptomycin by phosphorylation or streptomycin-resistant ribosomes. However, such high-level resistant strains comprised less than 10% of the total strains isolated, the majority of the strains resistant to streptomycin were of low-level resistance. The latter are associated with a diminished uptake of streptomycin, and no evidence of streptomycin inactivation, resistant ribosomes or R factors could be detected. The spectrum of susceptibility of P. aeruginosa to tetracycline also covers a wide range. Susceptible strains (MIC less than 10 µg/ml) were rare (< 10%). The MIC for R^- strains was generally about 25 µg/ml and that of R^+ strains 75 to 200 µg/ml. Strains with an MIC higher than 75 µg/ml were also rare (< 10%). In both R^- and R^+ strains, significant tetracycline uptake did not occur until tetracycline concentration included in uptake studies approximated the MIC of that strain. In R^+ strains, a further reduction in tetracycline uptake resulting from pre-incubation with 1 µg/ml tetracycline was observed. No evidence of tetracycline inactivation or ribosomal resistance was

detected in R^- or R^+ strains. Thus, the majority of P. aeruginosa strains isolated from clinical specimens are of low-level resistance to either streptomycin or tetracycline due to defective permeability. Uptake studies of these antibiotics in the presence of second biological agents which affect the cell wall were also done. Antiserum and complement were used to modify the lipopolysaccharide lipoprotein, and carbenicillin was used to modify the peptidoglycan. These studies indicate that the cell membrane appears to be the ultimate barrier responsible for the differential susceptibility between the susceptible and low-level resistant strains to these antibiotics.

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LIST OF ABBREVIATIONS

DNA	- deoxyribonucleic acid
RNA	- ribonucleic acid
DNase	- deoxyribonuclease
ATP, GTP	- 5'triphosphate of adenosine and guanosine
EDTA	- ethylene diamine tetraacetate
Tris	- Tris (hydroxymethyl) aminomethane
OD	- optical density
MIC	- minimal inhibitory concentration
TS	- trypticase soy
CPM	- counts per minute
M	- Molar
mM	- milliMolar, millimole
l	- liter
ml	- milliliter
µl	- microliter
g	- gram
mg	- milligram
µg	- microgram
ng	- nanogram
cm	- centimeter
mm	- millimeter
nm	- nanometer
LPS	- lipopolysaccharide
v/v	- volume per volume
TCA	- trichloroacetic acid
RTF	- resistance transfer factor

INTRODUCTION AND LITERATURE REVIEW

It is a common observation that the frequency of occurrence of infection due to Pseudomonas aeruginosa is increasing. The reasons for the increase are not completely understood, but undoubtedly they bear some relationship to the widespread use of antibiotic therapy and the natural resistance of Pseudomonas aeruginosa to most of the widely used antibiotics. It is not unusual for strains of this organism isolated from infections to be resistant to most of the following antibiotics: penicillin, ampicillin, erythromycin, kanamycin, streptomycin, chloramphenicol, neomycin, or tetracycline. One of the possible approaches to this problem is to determine the nature and relative frequency of the mechanisms which account for antimicrobial resistance in that organism. The information obtained could be used to concentrate subsequent investigation on the most common mechanism so that the drug or the microorganism could be modified to reduce such resistance. Two examples of this approach are 3'.4'-dideoxykanamycin B (Umezawa et al. 1971) and the use of ethylenediaminetetraacetate to increase susceptibility to an antibiotic (Barrett and Asscher, 1972).

A number of theoretically possible changes in the functions of a bacterial cell have been suggested as possible mechanisms involved in drug resistance. Among these are decreased permeability to the drug, increased destruction of the antimicrobial inhibitor, increased synthesis of an essential metabolite or drug antagonist,

and changes in the properties of an enzyme or ribosomes resulting in reduced affinity for the drug.

The competitive relationship between para-aminobenzoic acid (PABA) and the sulfonamides was largely responsible for the revival of interest in chemotherapy, particularly the approach that makes use of structural analogues of essential metabolites. Early work in some strains of bacteria has shown that a high degree of resistance to sulfonamide is linked with an increased production of PABA (Landy et al., 1943, 1944). Although this correlation has been confirmed by a number of studies, there are many instances in which it has been impossible to demonstrate any increased production of PABA by sulfonamide-resistant organisms. More recent work on mechanisms involved in resistance to the sulfonamides has focused attention on the production of altered enzymes. Wolff and Hotchkiss (1963) found that extracts of sulfonamide-resistant strains of the Pneumococcus had sulfonamide-resistant capacities for the conversion of PABA to compounds with folic-like activity for Streptococcus faecalis. Pato and Brown (1963) have also been able to demonstrate in sulfathiazole-resistant mutants, enzyme systems for the formation of folic acid that differ from the parent strain only in that the conversion of PABA to dihydropteroic acid is less susceptible to inhibition by the sulfonamides. The altered enzymes of the resistant mutants differ structurally from the enzymes of the parent strain in that they do not combine as readily with sulfonamides. These workers also observed, however, an additional type of mutant believed to

differ in its permeability characteristics from the parent strain in such a way that sulfonamides cannot readily permeate the cells. These observations indicate that a single mechanism is not always satisfactory in explaining resistance to a single drug by all strains or species.

In all naturally occurring penicillin-resistant staphylococcal strains as well as penicillin-resistant strains of many other species, it has been shown that resistance to the drug is linked to the production of an enzyme, penicillinase, which inactivates penicillin (Pollock, 1964). The action of this enzyme is due to an opening of the beta-lactam ring of the penicillin molecule with the formation of the antibiotically inactive penicilloic acid. The action of penicillinase, however, does not give a complete answer to the problem of resistance. No penicillinase can be detected in several species of gram-negative bacteria completely resistant to penicillin. Also, artificially induced penicillin resistance in laboratory strains of staphylococci is not accompanied by penicillinase production. The acquisition of resistance by virtue of the enzymatic capacity for inactivation of antimicrobial drugs is highly unlikely to be the result of a single-step mutation or of even several mutations in an organism which is truly negative for this or related activity. The development of the drug-inactivation type of resistance would require the acquisition and eventual integration into the genome of a considerable segment of genetic material to code the amino acid sequence of the drug-destroying enzyme.

Penicillin resistance evoked by laboratory training has nothing to do with penicillinase, but is based on a mechanism of cell-wall formation which is unsusceptible to the inhibition by penicillin (Roger and Jeljaszewicz, 1961).

It has been possible to transfer the genetic information for penicillin β-lactamase production from positive to negative strains of Staphylococcus by transduction (Ritz and Baldwin, 1961). Evidence has been presented that the genetic determinant of penicillinase formation in Staphylococcus aureus is incorporated in an extrachromosomal element or "plasmid" (Novick, 1963); this conclusion has been supported by other workers (Harmon and Baldwin, 1964; Hashimoto et al., 1964). In the wild type of all of these penicillinase-positive staphylococcal strains thus far studied, the penicillinase gene expresses itself inducibly (Swallow and Sneath, 1962).

Impaired ability to take up a drug is a frequent accompaniment of drug resistance. Such impairment may be the result of decrease in the affinity or activity of the cellular receptors of the drug. On the other hand, it could be a consequence of the loss of a stereospecific mechanism necessary for the drug to penetrate an otherwise impermeable cell wall or membrane. Cooper and Rowley (1949) showed that penicillin-insensitive Staphylococcus took up less S^{35}-penicillin than sensitive organisms, regardless of whether they were exposed to the labelled drug in the resting or in the growing state. Eagle (1954) used isotopically labelled penicillin

for a study of the binding of penicillin in different organisms. A comparison of susceptible and such naturally insensitive strains which did not produce penicillinase shows a correlation with the binding of the penicillin. The more susceptible the strains, the more penicillin binding that could be demonstrated after exposure to the drug.

Schwartz et al. (1959) reported that mutant strains of E. coli selected for resistance to D-serine and to canavanine were unable to concentrate these analogues from the medium. The D-serine-resistant mutant is unable to concentrate glycine, and the canavanine-resistant mutant cannot concentrate arginine. Apparently these analogues gain entry into the cell via the permeases designed for the entry of natural amino acids. Evidence of this type, obtained under conditions in which utilization of the analogue is blocked, constitute the most satisfactory proof of altered permeability.

Actinomycin, which inhibits RNA synthesis in vitro, and in organisms, in vivo, does not do so in normal cells of E. coli. However, brief treatment of E. coli cells with ethylenediamine-tetraacetic acid renders them completely sensitive to the subsequent action of actinomycin (Leive, 1965). The cultures remain viable when treated with EDTA, and are not osmotically fragile. It was suggested that EDTA increases the permeability of gram-negative organisms to a variety of compounds, including some antibiotics. Since some cases of bacterial resistance are a consequence of the

development of a permeability barrier which prevents antibiotics from reaching its site of action, it is possible that conversion of E. coli to actinomycin sensitivity depends on a change of bacterial permeability produced by EDTA. Weiser et al., (1968) also showed that the disodium salt of EDTA enhanced the effect of penicillin G, ampicillin, tetracycline and chloramphenicol on a resistant strain of Pseudomonas aeruginosa. This effect was shown to be due to synergism of the EDTA and antibiotic combinations.

Experiments with cell-free polypeptide synthesis suggest that there is one site on a sensitive ribosome to which streptomycin binds in order to derange protein synthesis; any mutation which eliminates this site, or alters it in such a way that the bound drug can no longer exert its effect, would constitute a mutation to resistance (Speyer et al., 1962). Cell-free extracts from a streptomycin-resistant mycobacterium incorporated labelled amino acid in the presence of the antibiotic, whereas an extract from sensitive cells was inhibited (Erdos and Ullmann, 1959). In an extension of this approach, Flaks and his co-workers (1962) have shown that streptomycin interferes with the polyuridylate-stimulated incorporation of labelled phenylalanine into acid-insoluble polypeptide, thus offering evidence in support of the theory of Spotts and Stanier (1961) that the ribosomes of the sensitive and resistant strains have different affinities for streptomycin. In the streptomycin-resistant organism, a modification in the ribosomal site concerned with the binding of t-RNA to the messenger changes the structure of the site so that the

correct t-RNA is paired whether or not streptomycin is present. Still further modification might make the site require the presence of a streptomycin molecule in order to function properly. The latter case would constitute a mechanism for the classical streptomycin dependence (Davies et al., 1964). Subsequent experiments by Tanaka and Kaji (1968) revealed that the determining factor is a protein of the core particle of the 30S subunit of ribosomes, and the complete 30S subunits are required for the binding of dihydrostreptomycin.

Although bacterial mutants with streptomycin-resistant ribosomes have been encountered frequently, mutant strains with tetracycline-resistant ribosomes have been described rarely. Laskin and Chan (1964) studied the effects of tetracycline on cell-free systems derived from a clinical isolate of E. coli resistant to more than 100 µg/ml of tetracycline. Poly U-directed phenylalanine incorporation in these preparations was about as sensitive to the tetracycline as were the preparations from the sensitive strain. Okamoto and Mizuno (1964) also reported that the cell-free systems from two resistant strains were not resistant to tetracycline. One strain was developed by in vitro passage in chloramphenicol-containing medium and exhibited considerable cross-resistance to tetracycline, the second was resistant by virtue of carrying a multiple drug resistance factor. Izaki and Arima (1963) described the accumulation of a large amount of oxytetracycline by sensitive E. coli cells was dependent upon an energy-

yielding system. The cells of a multiple drug resistant strain of E. coli accumulated significantly less oxytetracycline, and the cells of a more highly resistant strain accumulated no significant amount of antibiotic. Similar results were subsequently described by Franklin and Godfrey (1965). Most of this evidence, therefore, favours the proposal that tetracycline resistance is due to an alteration in permeability. However, Craven et al. reported in 1969 a strain of E. coli which exhibited an unusually high rate of spontaneous mutation. When a tetracycline-resistant mutant (resistant to 250 µg/ml) was selected from this strain by laboratory training and examined for in vitro ribosomal resistance to tetracycline, the result showed a clear difference between the resistant mutant and a sensitive control. The resistant mutant was much less susceptible to the inhibition of ^{14}C-phenylalanine incorporation by tetracycline at concentrations of 10 to 20 µg/ml. than the sensitive strain.

The fact that drug resistance can be transferred by infection from one bacterial cell to another was discovered in Japan (Akiba et al., 1960). Since that time, extensive epidemiological studies were carried out in many countries (Smith and Armour,1966; Anderson, 1968). Contagious drug resistance is often multiple, rendering organisms resistant simultaneously to several unrelated antibacterial drugs of therapeutic importance. Resistance to more than ten drugs has been found to be specified by R factors; these include carbenicillin (Roe et al., 1971), ampicillin (Datta and

Richmond, 1966), streptomycin (Ozanne et al., 1969; Benveniste et al., 1970), tetracycline (Izaki et al., 1966; Franklin, 1967), chloramphenicol (Shaw, 1967; Susuki and Okamoto, 1967), kanamycin and neomycin (Ozanne et al., 1969; Kondo et al., 1968; Benveniste and Davies, 1971a), spectinomycin (Benveniste et al., 1970; Smith et al., 1970), sulfonamide (Watanabe, 1963), lividomycin (Kobayashi et al., 1971; Kondo et al., 1972), and gentamicin Martin et al., 1971; Benveniste and Davies, 1971b). A very wide range of gram negative bacilli can be infected with transmissible antibiotic resistance.

There is evidence that transmissible drug resistance factors consist of autonomously replicating, non-homogenous, extrachromosomal units of DNA that are both genetically and physically distinct (Falkow et al., 1966; Anderson, 1968). According to the model proposed by Watanabe and Fukasawa (1961), R-factors arise by linear linkage of a transfer unit which mediates transmission of the plasmid during bacterial conjugation, and drug resistance determinants (r-determinants) which carry genetic information specifying resistance to antimicrobial agents. Anderson and his collaborators (1965, 1968) have found that at least certain classes of R-factors include transfer units that can be transmitted alone, as well as in combination with resistance determinants. Furthermore, they have shown that ordinary non-transferable resistance determinants occurring in nature can be mobilized by RTF units which are then able to effect their passage into recipient bacteria. Once within a recipient cell, the linked

transfer and resistant components can separate and replicate independently of each other. Anderson's model predicts that, in certain conditions, DNA species representing transfer units and resistance determinants might be detectable as separate molecules, whereas in other circumstances only a single DNA molecular species containing the joined transfer factor and r-determinants may be present.

There are several different mechanisms by which R-factors determine resistance to antibiotics. Resistance to carbenicillin in R^+ strains is associated with the production of carbenicillinase (Roe, Jones, and Lowbury, 1971). Strains of bacteria which contain tetracycline resistance determinants appear to possess an inducible inhibitor of drug uptake (Izaki et al., 1966; Franklin, 1967; Sompolinski et al., 1970). The inhibitor could be induced by low sub-inhibitory concentrations of tetracycline or its derivative, β-apooxytetracycline, which has very little antibacterial activity. Most evidence for this mechanism comes from studies of the uptake of radioactive tetracycline in R^+ and R^- strains. There is no evidence for inactivation of the drug or alteration of its target site on the ribosome (Franklin, 1967). The mechanism of chloramphenicol resistance specified by R factors has been elucidated by Suzuki and Okamoto (1967) and by Shaw (1967), who found that a crude extract of an R^+ strain, in the presence of acetyl CoA, was capable of converting chloramphenicol to an inactive O-acetyl derivative. The product of acetylation is a mixture of 3-acetoxychloramphenicol and 1, 3-diacetoxychloramphenicol. Both products are completely inactive

as antibiotics and are produced by the action of chloramphenicol acetyltransferase. Streptomycin can be inactivated in R^+ strains in two different ways by two enzymes which transfer, respectively, the terminal phosphate (Ozanne et al., 1969) or the adenosine monophosphate residue (Umezawa et al., 1968) of ATP to the 3-hydroxyl group of L-glucosamine ring of streptomycin (Benveniste et al., 1970). The resulting phosphate ester or phosphodiester derivatives of streptomycin are inactive as antibiotics. For neomycin and kanamycin, N-acetylation and O-phosphorylation are the common inactivation mechanisms used by R^+ strains (Benveniste and Davies, 1971a; Kondo et al., 1968). Gentamicin resistance mediated by R factor is found to inactivate gentamicin by adenylation (Benveniste and Davies, 1971b). Enzymatic inactivation of gentamicin by N-acetylation has also been found in certain strains of Pseudomonas aeruginosa (Brzezinska et al., 1972) It has recently been shown that this resistance mechanism is probably determined by an R-factor, at least in some cases (Bryan et al., 1973).

Some of the mechanisms of drug resistance in P. aeruginosa have been described. The presence of R-factors was suggested by Smith and Armor (1966). Doi et al. reported in 1968 that there are streptomycin, kanamycin and neomycin inactivation enzymes in all the ten resistant strains of P. aeruginosa obtained from the Institute of Medical Science, University of Tokyo. Investigation by other workers (Mitsuhashi, 1971; Watanabe et al., 1971) revealed that the frequency of occurrence of R-factor in P. aeruginosa is very low. However, these workers depended upon the use of E. coli as a recipient.

The frequency of Pseudomonas R-factors as determined remained unclear, and the possibility that some Pseudomonas R-factors existed which transferred at low frequency or not at all to E. coli was not examined. R-factors carrying resistance to carbenicillin were detected in Britain (Sykes and Richmond, 1970; Fullbrook et al., 1970). Inactivation of lividomycin, paromomycin, neomycin kanamycin, and streptomycin by phosphorylation in P. aeruginosa was also described by Kobayashi et al. (1971, 1972). Besides the inactivation of streptomycin and the ribosomal resistance, an organism could also be resistant to streptomycin because of low permeability to the drug. In 1962, Hancock reported that both naturally resistant organisms (e.g. the Clostridia), and resistant strains of Staphylococcus aureus showed rates of uptake of the order of 1% of that in the most susceptible organism used, Bacillus megaterium. Beggs and Williams (1971) demonstrated that uptake of streptomycin by Mycobacterium tuberculosis is an active, energy-dependent process. Addition of NaCN at 5×10^{-4}M strongly inhibits the uptake. Perry (1969) also showed that in group H Streptococci only the streptomycin-susceptible strains accumulate significant amounts of ^{14}C-streptomycin. The resistant strains accumulate very little streptomycin. Although these workers gave some evidence that streptomycin-resistant strains of bacteria could be so because of low permeability to the drug, however, experiments by Anand and Davis (1960) showed that incubation of streptomycin with susceptible strains of E. coli for ten to fifteen minutes causes

a gradual leakage of intracellular acid-soluble nucleotides. Such an observation raises the possibility that streptomycin causes defective formation of protein and perhaps other membrane constituents and thus damage to the permeability barrier of the susceptible cells, which may then facilitate the leakage of streptomycin into the cells, resulting in high uptake of streptomycin by the susceptible organisms.

The objective of our research is to examine the nature and relative frequency of the mechanisms of resistance to common antimicrobial agents in hospital isolates of P. aeruginosa. This organism has been selected because it is a major agent of nosocomial infection and a prime reason for that is its broad antimicrobial resistance. An understanding of such mechanisms is a prerequisite for subsequent studies of methods to modify antimicrobial resistance and to the rational use of antimicrobial agents by the medical profession in order to prevent or forestall new antimicrobial resistance. Also essential is a knowledge of the nature and frequency of occurrence of infectious drug resistance in P. aeruginosa because of the rapidity with which such resistance can spread in wards, hospitals and large geographic regions and because of the potential selection of R-factor-containing strains. Streptomycin and tetracycline are useful models with which to undertake such a study because of the availability of knowledge on their mode of action and/or certain aspects of streptomycin inactivation. Mechanisms of resistance in P. aeruginosa have been shown to

include enzymatic phosphorylation (Doi et al., 1968) which was, in all the cases studied, found to be transferable (Bryan et al., 1972). Ribosomal resistance could presumably be another form of resistance which confers total indifference to streptomycin. However, the relative frequency of these in P. aeruginosa, and whether there are other resistance mechanisms was unknown. The mechanisms of tetracycline resistance in P. aeruginosa have never been investigated.

The study reported here was undertaken to assess the distribution of resistance mechanisms in strains of P. aeruginosa specifically selected from hospitalized patients to represent a natural population of therapeutic interest. Correlation of levels of streptomycin or tetracycline sensitivity to a mechanism and the frequency of such mechanisms has been carried out in order to indicate which functional or structural components of P. aeruginosa strains might be most gainfully modified by additional biological agents. In the event that a strain was found to be resistant to streptomycin or tetracycline because of low permeability, we also took our investigation one step further to determine which layer of the cellular envelope is the main barrier responsible for low uptake in resistant strains. The combined action of bacterial antiserum and complement has been shown to produce fairly specific lesions in the outer cell wall, especially lipopolysaccharide in E. coli (Feingold et al., 1968a, b). A complement and lysozyme-free system was selected to determine whether uptake of streptomycin or tetracycline can be modified by damage to lipopolysaccharide.

Carbenicillin was used in an uptake study of streptomycin or tetracycline to determine whether modification of the peptidoglycan layer can increase the accumulation of these antibiotics. We felt these studies would provide information for the rational use of antibiotics and a second agent in the treatment of infection by P. aeruginosa. Therefore, such a study would be useful with both academic and practical implications.

MATERIALS AND METHODS

1. Reagents: All chemicals were of reagent grade and were obtained from commercial suppliers.

2. Buffer:

   A. Buffer A: 50 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 7.5 mM Mg acetate.

   B. Buffer B: 10 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 10 mM Mg acetate, 6 mM mercaptoethanol.

   C. Buffer C: 50 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 7.5 mM Mg acetate, 6 mM mercaptoethanol.

   D. TMK solution: 0.06 M KCl, 10 mM Mg acetate, 0.06 M mercaptoethanol and 0.1 Tris-HCl pH 7.4.

   E. Borate buffer: 0.1 M (sodium) borate buffer pH 8.5.

2. Media: Trypticase soy (TS) broth and agar (TSA) (BBL). MacConkey agar (Difco). Brain Heart Infusion (BHI: Difco). The minimal medium used was that of Norris and Campbell (1949) modified to contain 500 mg of $MgSO_4 \cdot 7H_2O$/liter.

Low phosphate broth was prepared according to the method modified from that of Cheng et al. (1971). It has the following composition: 0.02M NH_4Cl, 0.02 M KCl, 0.02 M NaCl, 0.12 M Tris HCl pH 7.5, 0.5% glucose, 0.5% Bactopeptone (Difco), 0.0016 M $MgSO_4$.

4. Organisms:

P. aeruginosa strains were obtained as isolates from clinical specimens. E. coli CSH 2/NR3 (obtained from J. Davies, University of

Wisconsin) was used to produce dihydrostreptomycin phosphate. *E. coli* K12-58/E521 was used to produce streptomycin adenylate. Bacteriophage R17 and its propagating host *E. coli* AB 257 Hfr were supplied by W. Paranchych, University of Alberta, Edmonton. Mutant strains 280 met^-, 679 trp^- were isolated by the method of Ornston *et al.* (1969), 280 $cb^r su^r$ was isolated as a carbenicillin-resistant (50 µg/ml) colony. *E. coli* $K12F^-$:ATCC 14948 was used as recipient in intergeneric matings.

5. Strain characterization: All methods used to characterize *P. aeruginosa* were those of Cowan and Steel (1965) except acetamide hydrolysis (Bühlman, Vischer and Bruhin, 1961) and growth in triphenyl tetrazolium chloride (Wahba and Darrell, 1965). The DNA base ratios were determined by the method of Owen *et al.* (1969). Some of the strains were also typed by the methods of Govan and Gillies (1969).

6. Mating systems: Mating mixtures were prepared with equal volumes of logarithmic-phase donor and recipient strains grown in TS broth and adjusted to a cell density of 0.5 A_{550} unit (Beckman DBG Spectrophotometer) prior to mixing. Two or 4 ml amounts of that mixture were used in filter matings (Matney and Achenbach, 1962) with incubation of the filter on TS agar. Samples were removed from filters with a sterile loop to TS or minimal broth. Mating was interrupted by vigorous agitation, and the cell density was adjusted to 0.5 absorbance units at 550 nm (1.2×10^9 colony-forming units per ml) with the appropriate broth.

7. Selection methods: Selection was carried out by conventional auxotrophic method for 679 trp^- x 280 met^-, 679 trp^- x E. coli K-12F$^-$, and 280 met^- R931 (the recipient of 280 met^- x 931) x 280 Cb^rSu^r matings by plating resuspended samples of filter mating on minimal medium or minimum medium plus DL methione (100 µg/ml). The number of organisms plated was assessed from the initial absorbance (0.5 at 550 nm) and by duplicate plate counts.

Pyocine selection was used for the matings 931 x 280 met^-, 931 x E. coli-K12F$^-$, 1162 x 280 met^-, and 1162 x E. coli K12F$^-$.

Pyocines were produced by the method of Farmer and Herman (1969) and stored at 4°C. Donor organisms were screened for initial susceptibility to pyocines by applying 25 µl drops of undiluted pyocine to a lawn of the test organism on TS agar prepared exactly as for Kirby-Bauer (Bauer et al., 1966) disc antibiotic testing. The plates were incubated at 37°C for 18 and 42 hours, and if fewer than 20 colonies grew in the pyocine drop zone the pyocine was considered satisfactory for selection, provided it failed to inhibit the recipient strain.

For use in pyocine selection after mating, the pyocine was titrated with donor and recipient strains. Volumes of 0.1 ml containing 10^8 organisms were added to 1.9 ml volumes of serial twofold dilutions of the pyocine (in TS broth) and incubated in 50-ml Erlenmeyer flasks at 37°C for 20 minutes with vigorous shaking. 0.1 ml volumes (diluted in the case of the unsusceptible recipient strains 280 or E. coli) were applied to TS agar and incubated as above. The pyocine dilution producing less than 10% killing of strain 280 (E. coli K-12

F^- was totally unsusceptible) and allowing growth of fewer than 20 colonies of the donor after 42 hours of incubation was used for selection after mating.

Pyocine selection of the resuspended filter mating was carried out by adding 0.1 ml of that material to 1.9 ml of the appropriate pyocine dilution selected as above and incubated as for the pyocine titration. Appropriately diluted 0.1 ml volumes were added to TS agar or MacConkey agar (the latter for E. coli matings) containing streptomycin (16.5 µg/ml) and to TS or MacConkey agar for plate counts. E. coli platings were, in addition, incubated anaerobically. In some cases before final plating the E. coli mixture was concentrated 20-fold by centrifugation at 7,000 x g. Strain 280 was readily differentiated from the rare breakthrough of donor cells by an intense brown pigment and from E. coli by lactose fermentation of the latter on the MacConkey medium.

After selection by either method, about 10 colonies from each plate were picked and tested for antimicrobial susceptibility by the disc method and by determination of minimal inhibitory concentration (MIC). The MIC was also determined for donors. All Pseudomonas colonies showing R factor transfer were typed by the methods of Govan and Gillies (1969) to confirm their identity as strain 280.

8. Transduction: 2.5 ml suspensions of strain 931 or pyocine of 1363 prepared as described above were sterilized by filtration through Millipore filters (filters with pore size of 0.22 µm were used) mixed with 2.5 ml suspension of the recipient strain 280 and incubated at

37°C overnight (16 hours). Detection of any transfer was done by adding appropriately diluted 0.1 ml volumes to TS agar containing streptomycin (16.5 µg/ml).

9. Transformation: To rule out transformation, broth matings of strains 280 x 931 were incubated in the presence of 1, 10 and 50 µg of deoxyribonuclease I per ml (Worthington Biochemical Corp., 2000 units /mg) for 1, 4, and 20 hours.

10. MIC determination: A conventional broth dilution method was used with 2.5 ml of broth in each tube (19 x 125 mm). The inoculum size was 10^7 organisms, and the minimal inhibitory concentration was determined visually after 15 hours of incubation at 37°C.

11. ^{3}H-dihydrostreptomycin radiochemical purity: Thirty µl ^{3}H-dihydrostreptomycin solution was chromatographed on a Sephadex G-10 column (0.9 x 20 cm) and eluted with 0.1 M sodium phosphate buffer (pH 6.8). The radioactivity was determined on 0.5 ml of each 0.8 ml fraction in 10 ml of Bray's (1960) scintillation fluid (Beckman model LS-250 liquid scintillation counter).

12. Dihydrostreptomycin uptake study: A 1-ml amount of a slime-free overnight culture of a Pseudomonas strain in TS broth was inoculated into 20 ml of fresh broth in a 125 ml flask and incubated at 37°C in a water bath with vigorous shaking. At the mid-log phase of growth, the culture was diluted with TS broth to an absorbance of 0.21 at 550 nm, and 19 ml was transferred to a new 125 ml flask. One ml of an aqueous solution containing 5 x 10^6 disintegration per minute (D.P.M.)

of ^{3}H-dihydrostreptomycin (Amersham Searle, 2.1 mCi/mg) with various concentrations of cold dihydrostreptomycin was added. The mixture was incubated for 2 hours at 37°C in a waterbath with vigorous shaking. The total growth of bacteria was determined by measuring the absorbance at 550 nm. The culture was rapidly chilled in an ice-waterbath to stop further uptake. The bacteria were harvested by centrifugation at 21,000 g for 15 minutes in a refrigerated centrifuge. The pellet was washed three times by suspending it in 25 ml of cold TS broth, and the bacteria recovered as above. The pellet was then suspended in distilled water to a final volume of 2.3 ml and subjected to ultrasonic vibration with a Bronson Biosonik III (0.95 cm probe) for 1 minute. 0.3 ml of the sonicated suspension was dispersed in 5 ml of Bray's scintillation fluid, and counted in a Beckman Model LS-250 liquid scintillation counter. Two ml of the remaining sonicated suspension was centrifuged at 30,000 g for 30 minutes in a refrigerated centrifuge and the absorbance at 260 nm of the supernatant (S-30 supernatant) was also taken. The counts per minute were converted to disintegrations per minute by obtaining the counting efficiency from the internal standard, i.e. 25 µl of aqueous solution containing a known quantity of ^{3}H-dihydrostreptomycin was added to the same vial and re-counted, and the counting efficiency was then calculated. Non-specific background binding of dihydrostreptomycin was determined by incubating the culture containing 0.025% NaN_3 with an identical concentration of dihydrostreptomycin at 0 to 4°C for 2 hours, after which the bacteria were processed as given. The

actual uptake of dihydrostreptomycin was calculated by subtracting the non-specific background binding of the drug from the total uptake and is given as nanograms of streptomycin per A_{550} unit of growth per ml. The uptake per A_{260} unit of the S-30 supernatant was also calculated. However, under the ordinary growing conditions used in our study, there is a close correlation between absorbance at 260 nm of the S-30 supernatant and the absorbance at 550 nm of the growth culture, therefore only the uptake per A_{550} unit of growth per ml is given in the result in most cases.

13. Preparation of R17 RNA: R17 RNA was prepared by a method similar to that of Gesteland (1964) with some modification. Fifty ml of an overnight culture of E. coli AB 257 Hfr was inoculated into 8 liters of TS broth in a Magnaferm Fermentor (New Brunswick Scientific) and incubated with vigorous aeration (5 liter/min flow rate) until the absorbance of the culture was 0.3 at 550 nm. It was then infected with R17 phage at a multiplicity of about three plaque-forming units/bacterium. Aeration and incubating were continued for 3 hours to allow phage release. A titer of 1.6×10^{12} P.F.U./ml was obtained. The lysate was cooled, and 2643 gm of $(NH_4)_2SO_4$ was added slowly with continuous stirring (2.5 M final concentration) and allowed to stand overnight at 4°C. The resultant precipitate was sedimented by centrifugation for 25 minutes at 5500 g, suspended in 150 ml of standard saline citrate (SSC; 0.15 M NaCl, 0.015 M Na citrate) clarified by centrifugation at 1500 g for 20 minutes, and then precipitated with one-third volume of cold methanol and left overnight

at -20°C. The precipitate was collected by centrifugation at 5000 g for 25 minutes, suspended in 80 ml of SSC, and clarified by similar low speed centrifugation. The solution was then centrifuged for 1 hour in a Spinco model L2-65B ultracentrifuge at 100,000 g. The translucent pellet was suspended in 8 ml of SSC and clarified by centrifugation at 1500 g for 20 minutes. 160 mg of sodium dodecyl sulfate was added to the solution (2% concentration) and left at room temperature for 15 minutes. The solution was then chilled and subsequent operations were carried out at 4°C. After adding 80 mg of bentonite, RNA was purified by shaking with an equal volume of SSC-saturated distilled phenol for 10 minutes. The two layers were separated by centrifugation at 1500 g for 10 minutes, the aqueous layer was pipetted off, and the phenol layer was again extracted with 3 ml of SSC. The combined aqueous layers were shaken once more with an equal volume of phenol for 5 minutes and the aqueous solution recovered as above. The RNA was precipitated from the aqueous phase by adding 0.1 volume of 0.1 M EDTA and 2 volumes of cold ethanol, and leaving the suspension at -20°C for 48 hours. It was then sedimented by centrifugation at 3000 g for 20 minutes at 4°C. The pellet was suspended in 2 ml of Buffer A, and the absorbance at 260 nm and 280 nm were determined (A_{260} = 310, A_{280} = 180). The preparation had an RNA concentration of approximately 12.9 mg/ml ($E^{1\%\ RNA}_{1\ cm}$ = 240 at 260 nm, Ehrenstein, 1967).

14. Amino acid incorporation study in cell-free system. Polypeptide synthesis was carried out with RNA from phage R17 as messenger. S100 fractions and ribosomes from Pseudomonas strains were prepared by a

method described by Modolell and Davis (1968). The reaction mixture contained 50 mM tris (hydroxymethyl) aminomethane-hydrochloride pH 7.8, 60 mM NH_4Cl, 7.5 mM Mg acetate, 10 mM reduced glutathione, 1 mM adenosine triphosphate, 0.03 mM guanosine triphosphate, 5 mM creatine phosphate, 50 µg/ml creatine kinase, 0.008 mM ^{14}C-valine (Schwarz/Mann, 50 ci/µmole), 0.05 mM each of the 19 amino acids, 0.7 mg R17 RNA/ml, 1.0 mg of E. coli K12 transfer RNA/ml (Schwarz/Mann), 0.2 volume of S100 supernatant fluid, and 0.2 volume of ribosomal suspension (12 mg/ml). Streptomycin, if present, was used at 2 or 50 µg/ml. If tetracycline was used, the concentration was 20 µg/ml. The reaction mixtures were incubated at 34°C for 30 minutes, and measurement of the incorporation of radioactive amino acid into protein was done by a paper disc method modified from that of Mans and Novelli (1961). Fifty µl of reaction mixture was spread on an appropriate square of silica gel-impregnated paper (1.4 x 1.4 cm). Fifty µl of IN NaOH was added immediately to the wet paper to stop the reaction and hydrolyze the ^{14}C-Valine-t RNA which would otherwise cause a high background. After 45 minutes when the paper appeared nearly dry, it was washed three times by dropping it into 40 ml of ice-cold trichloroacetic acid solution (10%) and agitated gently for 15 minutes each time. The paper was next sequentially washed in 20 ml of ethanol-ether mixture (1:1, v/v) and in 20 ml of ether. The papers were dried and counted in 10 ml of toluene scintillation fluid (Beckman Scientific).

15. Streptomycin phosphotransferase and streptomycin adenylate synthetase. Strains were examined for streptomycin phosphorylation or adenylation by a method modified from that of Umezawa et al. (1967). Two ml of slime-free overnight culture of Pseudomonas strain in TS broth was inoculated into 80 ml of fresh broth containing streptomycin 0.5 µg/ml in a 500 ml flask and incubated at 37°C with vigorous agitation. When the culture reached the late log-phase of growth (A_{550} = 0.9), the bacteria were harvested by centrifugation at 21,000 x g for 15 minutes at 4°C. The pellet was washed once by suspending it in 20 ml of cold TMK solution and the bacteria recovered as above. It was then suspended in 3 ml of TMK solution and subjected to ultrasonic vibration for 1 minute at 4°C. The sonicated bacterial suspension was centrifuged at 30,000 g for 30 minutes in a refrigerated centrifuge and the S-30 supernatant was dialyzed against 500 volumes of TMK solution for 12 hours at 4°C. The dialyzed solution was used as crude cell extract for enzyme detection.

The method was further modified so that reaction mixtures consisted of the following: 1 ml of cell extract; 1 mg of adenosine triphosphate (disodium); 50 µg of dihydrostreptomycin; and 1 x 10^6 D.P.M. of ^{3}H-dihydrostreptomycin in a total volume of 1.05 ml. The mixtures were incubated at 37°C for 10 hours, a 50 µl amount of which was chromatographed on a Dowex 50 W column (0.9 x 4.2 cm) with the use of a stepwise gradient of 0.25 to 4M ammonium acetate, and the radioactivity was determined on 0.4 samples of each 4 ml fraction in 5 ml of Bray's scintillation fluid. The dihydrostreptomycin standard was chromatographed by use of a reaction mixture in which water replaced

the cell extract. De-phosphorylating or de-adenylating conditions were as follows: 100 µl of the above incubated reaction mixture was again incubated at 37°C for 8 hours with either 20 µg of snake venom phosphodiesterase (Crotalus adamanteus; Sigma Chemical Co., 0.29 units/mg) or E. coli alkaline phosphatase (Worthington Biochemical Corp., 26 units/mg) in 0.1 M borate buffer in a final volume of 0.4 ml. Dowex 50 W chromatography was then carried out as described above.

16. Bioassay and chromatographic study of the culture filtrate. Cultures containing dihydrostreptomycin were examined for the residual potency of the dihydrostreptomycin by a broth dilution technique using P. aeruginosa strain 280 as the test bacterium. Overnight cultures were centrifuged at 21,000 x g for 15 minutes at 4°C, and the supernatants were filtered through 0.2 µm filters (Sartorius). The amount of dihydrostreptomycin in the filtrate was confirmed by measuring the radioactivity of an aliquot of the filtrate and by use of the original specific activity of the streptomycin in the culture. Filtrates were compared for antimicrobial potency to dihydrostreptomycin standards of of similar concentration. A drop of filtrate was also chromatographed on Dowex 50 W as described previously.

17. Preparation of antisera to Pseudomonas strains. Ten ml of log-phase cultures of Pseudomonas strains in TS broth containing 1.2×10^9 organisms per ml ($A_{550} = 0.5$) was centrifuged at 21000 x g for 15 minutes at 4°C. The pellet was washed once by resuspension in 10 ml of normal saline and the bacteria recovered as above. It was then suspended in 1.5 ml of aqueous suspension containing 50% (v/v) of

incomplete Freund's adjuvant (Difco) and subjected to ultrasonic vibration for 30 seconds. The preparation was injected subcutaneously into rabbits and followed by two weekly subcutaneous injections of 1.2×10^{10} sonicated organisms prepared by the same method but suspended in distilled water only. After the third injection a booster dose of 4.8×10^{10} sonicated organisms was injected intramuscularly one week later. The rabbits were bled 5 days after the last injection. Such antisera usually had visible agglutination titer of 640 or 960 in TS broth.

18. Methods used in examining the action of antiserum and complement on the P. aeruginosa cell envelope.

(a) Plate counts after incubation with lysozyme. This experiment was initiated because of the generally accepted concept that the gram-negative bacteria would become susceptible to lysozyme if the permeability of the lipopolysaccharide-lipoprotein layer of the cell wall were modified by another agent to allow penetration of lysozyme molecules. In this experiment antiserum and guinea pig complement were absorbed with 2.5 mg per ml of bentonite twice for 10 minutes each at 4°C to remove the lysozyme. Such absorbed sera had no detectable lysozyme activity as determined by their inability to lyse a suspension of Micrococcus lysodeiticus in phosphate buffer (Shugar, 1952). The absorbed antiserum and complement were added to suspensions of Pseudomonas strains in TS broth adjusted to an absorbance of 0.2 at A_{550} nm. The final concentrations of antiserum and complement were 1/250 and 1/20 (v/v) respectively. If lysozyme was added (Sigma 18000 units/mg) it was

used at a final concentration of 25 µg/ml. The mixtures were incubated at 37°C for 30 minutes. Plate counts were subsequently done by serial dilutions in cold TS broth and plated on trypticase soy agar and trypticase soy agar containing 20% sucrose.

(b) Electron microscopy. Pseudomonas strains treated with antiserum and complement with or without lysozyme as described above were sedimented by centrifugation at 21,000 x g for 10 minutes at 4°C. The cells were fixed in suspension with osmium tetroxide following the technique of Kellenberger et al. (1958). After dehydration in a series of acetone concentrations ranging from 25% to 100%, the blocks were treated twice each for 30 minutes in propylene oxide. They were left in 1:1 mixture of propylene oxide: Epon unstoppered overnight and were embedded in fresh Epon the following morning (Luft, 1961). The Epon mixture used was that producing hard blocks, and contained equal parts of Epon 812 and methyl nadic anhydride with the appropriate amount of accelerator (2, 4, 6 tri(dimethylaminomethyl) phenol) added. Sections were stained with uranyl acetate and lead citrate and were examined with a Philips EM 300 electron microscope.

(c) Comparison of the levels of acid soluble labelled nucleotides in the intracellular pool and in the external medium. In order to assess whether complement and antiserum had any effect on the bacterial cell membrane, the levels of labelled acid-soluble nucleotides in the external medium and in the internal pool were compared as an indicator of membrane leakage. Five ml of fresh culture of strain 1885 in TS broth at an absorbance of 0.45 A_{550}nm was diluted into 20 ml

with fresh broth containing 2 x 10^7 dpm of ^{3}H-uracil (New England Nuclear 1 mci/4.28 mg) and incubated for 30 minutes at 37°C with vigorous shaking. By this time, about 85% of the labelled uracil in the external medium was taken up by the bacteria. The culture was rapidly chilled in an ice-water bath to stop further incorporation of components of the intracellular acid-soluble nucleotide pool into RNA or DNA. The bacteria were harvested by centrifugation at 21,000 x g for 15 minutes at 0 - 4°C. The pellet was washed by resuspending in 20 ml of cold TS broth and the bacteria recovered as above. The final pellet was suspended in 15 ml of cold TS broth containing 20% of sucrose. The suspension was divided into 3 portions of 4.7 ml each. To the first portion, 0.05 ml of antiserum and 0.25 ml of guinea pig complement were added. 0.3 ml of TS broth were added to the second portion as a negative leakage control and 0.3 ml of aqueous solution of polymyxin containing 20 µg of polymyxin were added to the third portion as a positive leakage control. These preparations were incubated at 37°C for 30 minutes and centrifuged at 21,000 x g for 15 minutes at 0 - 4°C. The supernatant and bacteria were processed separately as follows:

1. One ml of cold trichloroacetic acid (10%) and 0.1 ml of 0.5% (w/v) bovine serum albumin was added to 1 ml of supernatant. Centrifugation was carried out at 6000 x g for 10 minutes at 0 - 4°C and the pellet discarded. 0.9 ml of 1.2M tris (base) solution was added to neutralize the supernatant. One ml of the mixture was then dispersed in 10 ml of Bray's scintillation fluid for determination of radioactivity.

2. The bacteria were suspended in 2.1 ml of 5% trichloroacetic acid solution and left at room temperature for 30 minutes. The material was centrifuged at 6000 x g for 10 minutes at 0 - 4°C. 0.9 ml of 1.2 M tris (base) solution was added to the supernatant, and 1 ml of the neutralized mixture was dispersed in 10 ml of Bray's scintillation fluid.

19. Treatment of P. aeruginosa strain 1059 with high concentrations of magnesium ion. Cheng, Ingram and Costerton (1970) reported that Pseudomonas strain ATCC 9027 contains an inducible alkaline phosphatase which was released upon growth of the organism in medium containing high concentrations of magnesium ion, or upon washing the bacteria cells with a high concentration of magnesium chloride (> 0.1 M). Therefore, we explored the possibility of modifying the cell wall by increasing the concentration of magnesium in the medium. Overnight cultures of strain 1059 in low phosphate (LP) media were transferred to fresh low phosphate broth and incubated at 37°C with shaking. At mid-log phase, the culture was diluted to an absorbance of 0.21 at 550 nm with fresh LP broth. One ml of 2M $MgCl_2$ solution was added to 19 ml of the diluted culture and incubation was continued for 2 hours. The bacteria were harvested by centrifugation at 21,000 g for 15 minutes, re-suspended in 5 ml of LP broth, and subjected to ultrasonic vibration for 1 minute as described previously. The alkaline phosphatase assay was done on the supernatant and the sonicated bacterial suspension by a method modified from that of Garen and Levinthal (1960). 1.5 ml of

p-nitrophenylphosphate solution (1 mM) in 0.2 M sodium borate buffer (pH 8.5) was mixed with 1.5 ml of the crude extract and the increment of absorbance at 410 nm per minute (Δ A) was recorded for 5 minutes. The total units of alkaline phosphatase in the supernatants or associated with the bacteria were calculated by the following formula:

$$\text{Total units} = \frac{\Delta \text{ A/min} \times 100 \times \text{dilution} \times 3}{1.62 \times 10^4 \times 1.5}$$

Qualitative catalase tests were also done by adding 0.5 ml of 3% H_2O_2 solution into 0.5 ml of supernatant or sonicated bacterial suspension. These tests were performed in order to study whether leakage of intracellular proteins or enzymes occurred.

A control culture of strain 1059 in LP broth without incubation with $MgCl_2$ was also examined for any leakage of alkaline phosphatase or catalase by the same method given above.

20. Tetracycline uptake determination.

The procedure used was similar to that previously described for the study of dihydrostreptomycin uptake except that cultures were incubated with ^{3}H-tetracycline (3.2 mg/mc New England Nuclear) for only 1 hour at 37°C and subsequent washings in cold TS broth were only done twice. Uptake values for tetracycline have been corrected for non-specific binding of tetracycline and are given as ng/A550 nm/ml. If the strains were pre-incubated with a low sub-inhibitory concentration of tetracycline, the concentration used was 1 µg/ml and was pre-incubated for 3 hours prior to uptake determination.

21. Examination for inactivation of tetracycline.

P. aeruginosa strains 931 and 494 were exposed respectively to 30 µg/ml and 15 µg/ml tetracycline containing 1 x 10^7 d.p.m. of H^3-tetracycline under the following conditions. Bacteria were inoculated to 20 ml of TS broth in a 125 ml flask to an initial density of 0.1 A_{550} units. Tetracycline was then added and the mixture was incubated in a shaking water bath at 37°C for 3 hours. The final density was adjusted to 0.4 A_{550} units with TS broth. The cells were rapidly chilled in an ice water bath, centrifuged at 4°C at 21,000 g for 15 minutes and the pellet washed twice with 25 ml cold TS broth. The final pellet containing about 1 x 10^{10} cells was extracted with 2 ml of 0.1 N HCl: methanol (1:3) for 5 minutes at 25°C in the dark. The mixture was centrifuged at 2000 g (25°C) for 10 minutes and the pellet removed and re-extracted two additional times. The extraction process removed more than 95% of the radioactivity associated with the bacteria. The extract was concentrated by vacuum and chromatographed on silica gel-impregnated paper treated with 2% di-sodium EDTA using n-butanol: methanol: 1% citric acid (4:1:2). The paper was dried, cut into 1 cm fractions, suspended in 5 ml of Omnifluor (Beckman Scientific) and counted in a Beckman Model LS-250 scintillation counter. Control preparations of ^{3}H-tetracycline were similarly chromatographed. The Rf value for ^{3}H-tetracycline was 0.60.

Bioassays of filtrates of cultures treated with tetracycline for 3 hours were carried out as previously described for dihydrostreptomycin. Aliquots of the filtrate were also chromatographed as described above.

A 25 µl drop containing about 1 µg of tetracycline in water, extracted from strain 931, was applied to TSA inoculated with Staphylococcus aureus (NCTC-6571) exactly as for Kirby-Bauer antimicrobial testing. One µg of standard tetracycline exposed to a bacterial suspension as described, but at 4°C and otherwise identically extracted was applied separately to the same TSA as described. Plates were incubated at 37°C for 18 hours and the zones of inhibition determined (6mm).

22. Uptake of dihydrostreptomycin and tetracycline in the presence of second agents which modify the cellular envelope.

(a) Uptake of dihydrostreptomycin by strains 280 and 1059 in the presence of a sub-inhibitory concentration of carbenicillin: The minimal inhibitory concentrations for carbenicillin of strains 280 and 1059 were 5 µg and 25 µg respectively (determined as described previously). The uptake of dihydrostreptomycin by strains 280 and 1059 in TS broth was determined as described except that sub-inhibitory concentrations of carbenicillin (2.5 µg/ml for strain 280 and 12.5 µg/ml for strain 1059) were included in the media.

(b) Uptake of dihydrostreptomycin and tetracycline by strain 1885 in the presence of antiserum and complement: Determination of uptake in TS broth containing 20% sucrose with or without antiserum and complement (0.5% and 5% v/v respectively) was done by the method described previously. Since some agglutination occurred due to the presence of antiserum, uptake is given as nanograms of dihydrostreptomycin or tetracycline per A_{260} unit of the S-30 supernatant instead of per A_{550} unit of growth.

(c) Uptake of tetracycline by carbenicillin spheroplasts of strains 1059 and 280: Strain 280 (or 1059) was grown in TS broth at 37°C with vigorous shaking. At mid-log phase, the culture was centrifuged at 21000 x g for 15 minutes, and the bacteria were re-suspended in TS broth containing carbenicillin 2.5 µg/ml (or 20 µg/ml for strain 1059) and 20% sucrose. Incubation at 37°C was continued for one-and-a-half hours. At this time, more than 90% of the bacteria were found to be converted to spheroplasts by phase contrast microscopy. The culture was diluted with TS broth containing carbenicillin and sucrose to an absorbance of 0.21 at 550 nm. Determination of uptake of tetracycline was then done as previously described. Uptake was given as nanograms of tetracycline per A_{550} unit of growth per ml.

23. Method of studying the rate of release of intracellular tetracycline at 37°C and its correlation with the rate of incorporation of ^{14}C-leucine.

Strain 280 was allowed to accumulate ^{3}H-tetracycline at a tetracycline concentration of 10 µg/ml for one hour and the bacteria were harvested by centrifugation and re-suspended in cold TS broth as described previously. The re-suspended bacteria were then incubated in a 37°C waterbath with shaking for 10 minutes, 20 minutes, and 30 minutes respectively. The culture samples were rapidly chilled in an ice-water bath and the radioactivity associated with the bacteria was determined as given previously.

For studying the rate of incorporation of ^{14}C-leucine into bacteria which had been pre-treated with an inhibitory concentration of tetracycline, strain 280 was grown in minimal broth. At mid-log

phase of growth, the culture was adjusted to a density of 0.20 A_{550} absorbance with fresh minimal media. 0.1 ml of aqueous solution containing tetracycline 200 µg was added to 20 ml of culture (final concentration of tetracycline was 10 µg/ml) and incubation at 37°C was continued for one hour. The bacteria were sedimented by centrifugation at 0 - 4°C and re-suspended in 20 ml of cold minimal broth containing leucine 20 µg/ml. Twenty µl of ^{14}C-leucine (50 µci/µm, 10 µci in 0.5 ml Schwarz/Mann) was mixed with 10 ml of the re-suspended culture and the mixtures were incubated in a 37°C waterbath with shaking. At zero time and every 5 minutes, 1 ml of sample was removed and mixed immediately with 2 ml of 10% TCA solution, and 0.1% aqueous solution of bovine serum albumin. The latter was added as a carrier. The preparation was then heated in a 90°C waterbath for 15 minutes to hydrolyse any ^{14}C-leucine-tRNA which might cause a high background. The pellet was harvested and recovered by centrifugation at 5000 x g for 10 minutes at 4°C after each of two washes with 3 ml of cold 10% TCA solution. The final pellet was suspended in 0.5 ml of 0.05 M sodium phosphate buffer and then dispersed in 5 ml Bray's solution and counted in a Beckman Model LS-250 liquid scintillation counter.

24. Uptake and incorporation of ^{14}C-proline by R^+ (R factor-containing) and R^- cells in the presence of tetracycline with or without pre-incubation of the cells in a sub-inhibitory concentration of tetracycline.

Strains of P. aeruginosa were inoculated into minimal media with or without 1 µg/ml tetracycline and incubated at 37°C for 3 hours. The

cultures were then diluted with minimal media to a density of 0.2 absorbance at 550 nm. The growth was stopped by placing the flasks in an ice-water bath. To 9.8 ml of each culture was added 0.1 ml of proline (1 mg/ml) and 0.1 ml of a tetracycline solution so that the final concentration of tetracycline in the mixture would be slightly lower than the MIC of that strain. 10λ of ^{14}C-proline (Amersham/ Searle 285 mCi/mM 50 µci in 1 ml) was added. Immediately a 2 ml "0 time" sample was removed for the background count. The flasks were re-incubated in the 37°C shaker water bath for 30 minutes, 2 ml samples were then removed and to each was added 4 ml of cold 10% TCA and 0.1 ml of 1% BSA. The mixtures were heated in a 90°C waterbath for 15 minutes. The radioactivity associated with the acid-insoluble pellet was then determined as described previously.

25. Methods used in detecting binding proteins for dihydrostreptomycin and tetracycline.

In order to investigate whether there is any binding protein involved in the transport of streptomycin or tetracycline, an equilibrium dialysis was done using lysozyme-EDTA supernatant prepared by the following method: strains of P. aeruginosa were inoculated into 25 ml of TS broth with or without tetracycline 1 µg/ml and incubated in a shaker waterbath at 37°C until the culture reached a density of 0.5 absorbance units at 550 nm. The bacteria were harvested from 20 ml of culture by centrifugation at 21,000 x g for 15 minutes in a refrigerated centrifuge, and the pellet was washed once with 20 ml of normal saline and then recovered by similar centrifugation. The bacteria were suspended in 5 ml of aqueous solution containing potassium EDTA 10 mM

(pH 7) lysozyme 500 µg/ml and NaCl 3%. The mixtures were incubated at 37°C for 30 minutes. The supernatant was collected by centrifugation at 21,000 x g for 15 minutes at 4°C and the pellet discarded. For equilibrium dialysis, 1 ml of the supernatant was put into one side of a 1 ml dialyzing cell (Chemical Rubber Co.). The other side of the cell was filled with 1 ml of aqueous solution containing k-EDTA 10 mM, lysozyme 500 µg/ml, NaCl 3% and 1 x 10^6 d.p.m. of undiluted ^{3}H-dihydrostreptomycin or ^{3}H-tetracycline. The cells were placed on a shaker and left at 4°C. At 16, 24, and 40 hours, 50 λ of sample were removed from each cell, dispersed in 5 ml Bray's solution and counted in the liquid scintillation counter.

RESULTS

I. Strain Characterization

All of the Pseudomonas strains were confirmed as P. aeruginosa by the procedures given in Table I. Strains 931 and 280 were further confirmed by DNA base ratios (68%) and by the presence of one to three polar flagella on electron microscopy. They were typable by the pyocine typing method of Govan and Gillies (1969). The latter method is dependent on the ability of Pseudomonas strains to produce pyocine that gives a characteristic pattern of inhibition of a set of indicator strains. Table II is a list of pyocine typing results for some of our representative strains.

II. Transfer of drug resistance

1. Pyocine selection method: When this experimental work was initiated, no satisfactory counter-selection method applicable to large numbers of potential R factor donors existed for P. aeruginosa. For that reason, three pyocine-producing strains (371, 1363, and 280) were examined for production of selective pyocine which would effectively inhibit the donor organisms in P. aeruginosa mating systems. The pyocine of strain 1363 proved the most efficacious, inhibiting strains 931 and 1162 but not 679. No pyocine examined inhibited strain 679 to a satisfactory degree. Further examination of the pyocine of strain 1363 by the screening procedure on other strains of P. aeruginosa demonstrated inhibition of 19 out of 24 strains, suggesting the possibility that this pyocine would be useful for selective purposes on a broad scale.

TABLE I

CHARACTERIZATION OF P. AERUGINOSA STRAINS

Method	Strains			
	679	1162	931	280
Oxidase	+	+	+	+
O - F test	O+ F-	O+ F-	O+ F-	O+ F-
Acetamide hydrolysis	+	+	+	+
Pigment				
King A Medium	+	+	+	+
King B Medium	+	+	+	+
Motility	+	+	+	+
Gluconate oxidation	+	+	+	+/-
Serial growth at 42°C(a)	+	+	+	+
Arginine dihydrolase	+	+	+	+
Growth in triphenyltetrazolium chloride	+	+	+	+
DNA base ratio	-	-	68%	68%
Flagella	-	-	Polar 1 - 3	Polar 1 - 3

(a) = 3 consecutive transfers

TABLE II

PYOCINE TYPING OF SOME OF OUR REPRESENTATIVE

STRAINS OF PSEUDOMONAS AERUGINOSA

Strains	Type	Sub-type
280	1	c
915	3	
1059	17	
2379	9	
1731	10	
1162	1	c
679	1	b
1363	22	
371	6	
1330	1	c
1515	1	b
1885	22	
931	2	
487	5	
494	16	

The effectiveness of the pyocine of strain 1363 in selectively inhibiting strain 931 following the mating 931 x 280 met$^-$ was determined by replicate plating (Lederberg and Lederberg, 1952). TS-agar streptomycin (streptomycin 16.5 µg/ml) plates containing colonies considered to be recipients of drug resistance were replicated to minimal agar which supported the growth of strain 931 but not 280 met$^-$. No 931 colonies could be detected on any plate examined (10^{-1}, 10^{-2}, 10^{-3} dilutions). Thus the pyocine selection method was judged satisfactory for use provided the procedure as outlined in the Methods was strictly adhered to. The use of 280 met$^-$ as a recipient and replication onto minimal agar plates allowed an accurate assessment of donor breakthrough following pyocine selection. Strain 1162 (1162 x 280 met$^-$) frequently exhibited breakthrough but by use of the above replication technique drug resistance transfer could be detected.

2. Transfer of drug resistance by mating: Strains 931, 1162 and 679 trp$^-$ were mated with 280 met$^-$ by the filter method. In each of these cases the recipient was confirmed as given in the Methods and by auxotrophy for methionine.

Strain 931 transferred resistance to streptomycin and to tetracycline; strains 1162 and 679 tryp$^-$ transferred resistance to streptomycin and sulfonamide. The MIC of donor and hybrid strains are shown in Table III. The high level of streptomycin resistance is noteworthy. The hybrid strains obtained generally demonstrated a slightly lower resistance to streptomycin than the donor, though

TABLE III

MIC, DISC ZONE TO SULFONAMIDE AND PRESENCE OF STREPTOMYCIN PHOSPHOTRANSFERASE OF STRAINS BEFORE AND AFTER DRUG RESISTANCE TRANSFER.

Strain	MIC[a] (µg/ml) Tetracycline	MIC[a] (µg/ml) Streptomycin	Disc Zone to sulfonamide (mm)	Streptomycin phospho transferase
Recipients				
P. aeruginosa 280	10	5	35	-
E. coli K12 F^-	----	2.5	30-35	-
Donors				
P. aeruginosa 931	150	1500	35	+
P. aeruginosa 679	----	>2000	< 10	+
P. aeruginosa 1162	----	>2000	< 10	+
Hybrids[b]				
P. aeruginosa 280 R931	75	400	35	+
P. aeruginosa 280 R679	----	1600	< 10	+
P. aeruginosa 280 R1162	----	2000	< 10	+
P. aeruginosa 280 cb^r su^r R931	75	400	< 10	+
E. coli K12 F^- R679	----	40	< 10	+
E. coli K12 F^- R1162	----	400	< 10	+

[a] - inoculum was 10^7 organisms in 2.5 ml TS broth, grown for 15 hr.

[b] - hybrids are designated by the number of the recipient strain followed by R (resistance transfer) and the number of the donor strain.

(hybrid = R^+ recipient)

of a level considerably higher than similar resistance in E. coli (Harwood and Smith, 1969). The sulfonamide resistance was only examined by the disc sensitivity method (Table III). The zone diameter for that drug was < 10 mm for the donor and for the recipients after transfer. The resistance to sulfonamide and streptomycin was transferred to E. coli R1162 and E. coli R679. The sensitivity to penicillin, ampicillin, cephaloridine, gentamicin, kanamycin, tetracycline, chloramphenicol, carbenicillin and polymyxin (disc) was not changed.

3. Frequency of drug resistance transfer: The frequency obtained after 20-hr matings on filters are shown in Table IV for both transfer to E. coli and to P. aeruginosa 280. Strain 931 in six repetitions did not transfer drug resistance to E. coli whereas 679 trp^- or 1162 transferred at very low frequencies. The order of difference between the Pseudomonas and E. coli recipient is at least 10^3 to 10^5. The survival of E. coli was unaffected by pyocine treatment or auxotrophic selection methods. An extremely marked difference in the ability of E. coli K12 F^- and strain 280 to act as a recipient was thus demonstrated. The MIC's produced for streptomycin resistance in E. coli hybrids were also very much reduced relative to those seen in the donors, particularly in the case of strain 679 and E. coli R679 (Table III).

4. Hybrid transfer of drug resistance: Matings of the hybrid strain 280 met^- R931 with 280 cb^r su^r (a mutant strain of 280 resistant to 50 µg/ml carbenicillin and with a disc zone of < 10 mm for

TABLE IV

TRANSFER FREQUENCY OF DOUBLE DRUG RESISTANCE
TO *E. COLI* AND *PSEUDOMONAS* STRAINS[a]

Donor	Recipient (Frequency of double drug resistance/recipient cell)	
	E. coli K12 F^-	P. aeruginosa 280 met^- or 280 cb^r su^r [b]
P. aeruginosa 931	$< 5 \times 10^{-8}$	1×10^{-2} to 1×10^{-3}
P. aeruginosa 679	2 to 6×10^{-8}	1×10^{-3}
P. aeruginosa 1162	5×10^{-7}	1×10^{-4}
P. aeruginosa 280 met^- R931[b]	-	1×10^{-4}

a - based on 20 hr filter matings.

b - 280 cb^r su^r was used as the recipient only in the mating P. aeruginosa 280 met^- R931 x 280 cb^r su^r.

sulfonamides) clearly showed that the hybrid had acquired the ability to act as a donor. As shown in Table III, 280 cb^r su^r also acquired streptomycin phosphotransferase in the above mating.

5. Transformation and transduction: The slight possibility that transduction or transformation accounted for the transfer of drug resistance was excluded by the results in Table V.

III. Spectrum and mechanism of P. aeruginosa streptomycin resistance

1. Distribution of streptomycin sensitivity in P. aeruginosa strains:

The frequency of different sensitivity levels to streptomycin in 200 strains of P. aeruginosa is shown in Table VI. Each strain was selected from individual hospitalized patients situated in different wards. Of these strains 49% would be reported as resistant using Kirby-Bauer criteria (1966) and an additional 39.5% as intermediate. Strains were classified as susceptible, low level- and high level-resistant. Studies of resistance mechanism were carried out on one or more representative strains from each group.

2. ^{3}H-dihydrostreptomycin radiochemical purity: Figure 1 illustrates the elution profiles of ^{3}H-dihydrostreptomycin from the Sephadex G 10 chromatography by a procedure given in the Methods. A single sharp peak was obtained.

3. Uptake of dihydrostreptomycin: The kinetics of uptake of ^{3}H-dihydrostreptomycin with time in P. aeruginosa strains (Fig.2) conformed to that described previously in other bacteria (Anand and Davis, 1960; Beggs and Williams, 1971). For the susceptible strain

TABLE V

BROTH MATINGS OF *P. AERUGINOSA* 931 x 280 CONTAINING DNase I AND OF *P. AERUGINOSA* 931 FILTRATE OR 1363 PYOCINE FILTRATE x *P. AERUGINOSA* 280

Conditions[a]	Doubly Resistant Colonies/10^5 Recipient Cells
(1) Control *P. aeruginosa* 931 x 280	630
(2) Control + 1 µg/ml DNase I	540
Control + 10 µg/ml DNase I	640
Control + 50 µg/ml DNase I	910
(3) Filtrate of 931 + Strain 280	0
(4) Filtrate of Pyocine 1363 + Strain 280	0

a - Mating conditions are specified in the Methods. The duration of mating was 20 hr.

TABLE VI

DISTRIBUTION OF STREPTOMYCIN RESISTANCE LEVELS IN *P. AERUGINOSA*

Class	MIC (µg/ml)	No. of strains (total 200)	Per cent
(1) Susceptible	10 or less	23	11.5
(2) Low-level resistant	10 - 50	79	39.5
	50 - 200	80	40
(3) High-level resistant	> 200	18	9

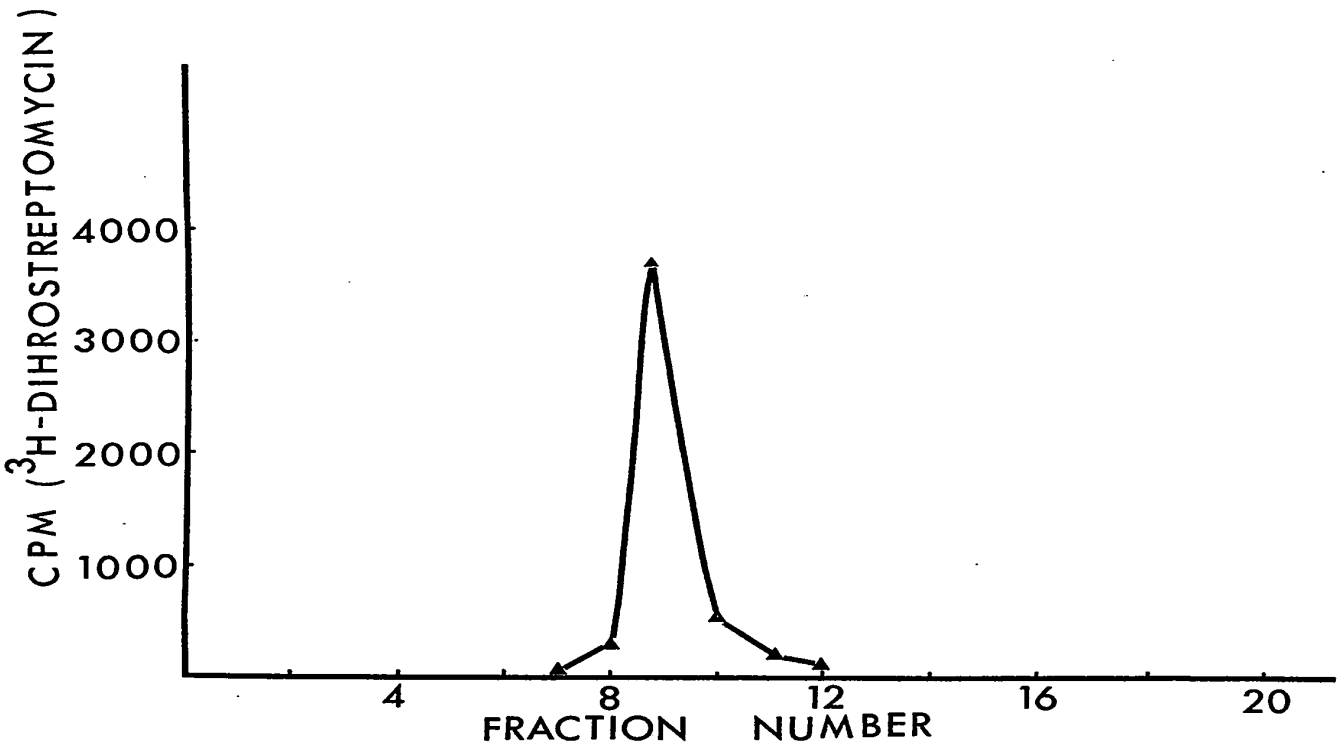

CPM (^{3}H-DIHROSTREPTOMYCIN)
4000
3000
2000
1000
4
8
12
16
20
FRACTION NUMBER

FIG. 1. G-10 sephadex gel filtration of ^{3}H-dihydrostreptomycin.

^{3}H-dihydrostreptomycin diluted in 0.1 M sodium phosphate buffer was applied to a G-10 sephadex column. The column specifications and operation are given in the Materials and Methods.

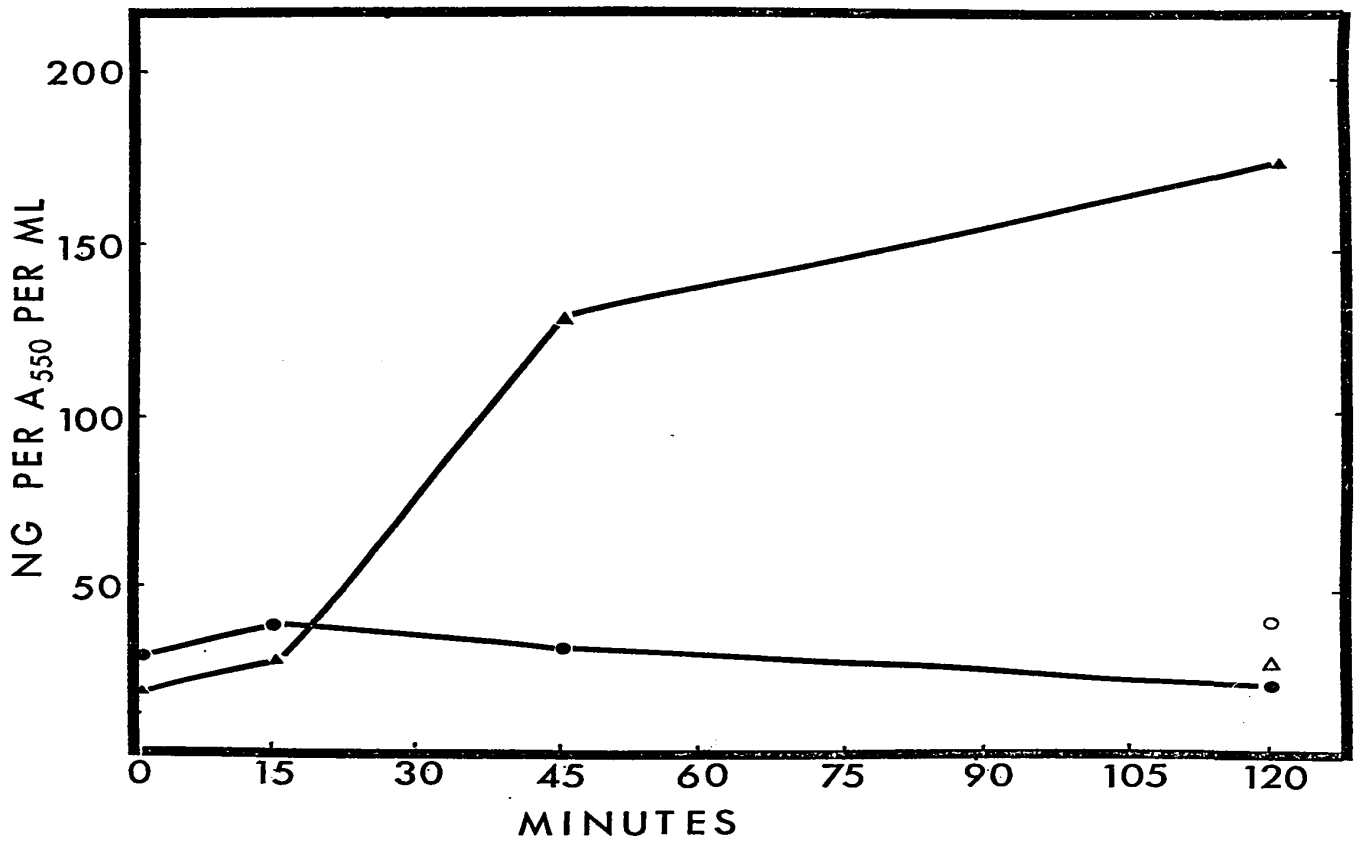

200
150
100
50
NG PER A_{550} PER ML
0
15
30
45
60
75
90
105
120
MINUTES

FIG. 2. Kinetics of uptake of dihydrostreptomycin by strains 280 and 1330 in TS broth at a dihydrostreptomycin concentration of 5 µg/ml.

Strain 280	▲
Strain 1330	●
Strain 280 with NaN_3	Δ
Strain 1330 with NaN_3	o

280 (MIC, 5 µg/ml) the uptake was biphasic at 5 µg of streptomycin/ml. The initial flat portion of the curve or primary uptake was very rapidly achieved, being essentially complete by the time the first sample was taken (0 minute sample). The second phase of uptake occurred with strain 280, but was not seen with the low level resistant strain 1330 at 5 µg of streptomycin /ml. In the presence of 0.025% sodium azide, the uptake of streptomycin by either strain 280 or 1330 was approximately equal to the primary uptake in strain 280. Thus, the primary uptake is most likely non-specific binding of the strongly cationic streptomycin to the bacterial cells.

Table VII presents streptomycin uptake data in TS broth at different streptomycin concentrations in representative strains of P. aeruginosa. As noted in the Methods the values have been corrected for primary uptake and represent the true uptake values. It can be observed that except for strains 931 and 915 there is an extremely close correlation between MIC and actual uptake of streptomycin. The uptake values obtained for the susceptible strains 280 and 1731 are not achieved in low level resistant strains 2379, 1059, 1330, and 1885 until the streptomycin concentration approximates the MIC for the resistant strains. The pattern exemplified by strain 1885 is typical. Low levels of streptomycin incorporation occur below the MIC but a sudden increase occurs at about the MIC.

Strain 280 GSK^r is a mutant of strain 280 obtained by culturing strain 280 in TS broth with increasing concentrations of gentamicin. The MIC to gentamicin in this mutant is about 25 µg/ml. Similar to

TABLE VII

ACTUAL UPTAKE OF DIHYDROSTREPTOMYCIN BY STRAINS OF *P. AERUGINOSA* AT VARIOUS DIHYDROSTREPTOMYCIN CONCENTRATIONS

Strain	MIC (µg/ml)	Actual uptake (ng/A550/ml)							
		Dihydrostreptomycin concentrations (µg/ml)							
		5	10	25	50	100	150	200	400
280	5	151.4[a]							
1731	5	124.8							
2379	10	36	169						
1059	20	4.8	78.1	587					
1885	50	< 1.0	6.7	18	312				
1330	125	< 1.0				6.1	673	859	
280GSK^r	400	< 1.0						< 1.0	303
931	1500	13.1				269			
915	20,000	2.8				74.4	229	454	

a = values underlined represent streptomycin uptake at or about the MIC.

the observation of Weinstein et al. (1971) the mutant shows cross resistance to kanamycin and streptomycin, has a prolonged generation time and is auxotrophic. It is clearly a strain which would be selected against in nature and thus does not represent a natural mode of resistance. However, as Table VII clearly demonstrates, this strain has an MIC of 400 µg/ml for streptomycin and fails to incorporate streptomycin below that MIC, thus supporting the possibility of a relationship between permeability and MIC.

In 1970 Turnock reported the isolation of a mutant of E. coli with increased sensitivity to streptomycin. Incubation of this mutant strain with streptomycin produced inhibition of protein synthesis and loss of viability with almost no lag period (the bactericidal effect of streptomycin is attributed to its essential irreversible binding to ribosome); while in the ordinary strains addition of inhibitory concentration of streptomycin to a culture required a lag of several minutes before any cell death or inhibition of protein synthesis could be detected (Dubin, Hancock and Davis, 1963; Turnock, 1970). Such results implied the idea that access of streptomycin to its site of action in a normal cell is restricted, and that this restriction, which is not effective in the mutant ultrasensitive to streptomycin, probably involves a permeability barrier. We have explored the possibility of similar behavior for one of our Pseudomonas strains most sensitive to streptomycin. Strain 280 at a density of 0.5 absorbance at 550 nm in TS broth was incubated with dihydrostreptomycin at a final concentration of

100 µg/ml (20-fold higher than MIC) for 1 hour at 0 - 4°C (a procedure equivalent to the method used to assess the background binding.) The bacteria were subsequently harvested by centrifugation at 21000 g for 10 minutes in a refrigerated centrifuge. The plate counts of the re-suspended bacteria (1.12×10^9 organisms/ml) was similar to that of the standard control (1.2×10^9 organisms/ml). It was thus concluded that little or no streptomycin would penetrate into bacterial cells at 0 - 4°C, and that the method used to assess the background binding is essentially correct.

4. Streptomycin phosphotransferase and streptomycin adenylate synthetase:

In order to assess the frequency of inactivating enzymes as a cause of streptomycin resistance, ^{3}H-dihydrostreptomycin was incubated with cell-free extracts of various strains under the conditions given in the Methods. After incubation with the cell extract of strain 280 met$^-$ the ^{3}H-dihydrostreptomycin is eluted by 3 to 4 M ammonium acetate, essentially identical to that of the ^{3}H-dihydrostreptomycin standard. After exposure to the cell extract of *E. coli* CSH2 with R factor NR 3 (known to produce streptomycin phosphotransferase) or of *P. aeruginosa*, 280 met$^-$ R931, the ^{3}H-dihydrostreptomycin is eluted by 0.75 to 1.0 M ammonium acetate. The hybrid, thus, has acquired streptomycin phosphotransferase. Fig. 3 illustrates the elution profile of ^{3}H-dihydrostreptomycin after incubation with various cell extracts of these strains.

To confirm that the product was dihydrostreptomycin phosphate, the cell extract of strain 931, after incubation with ^{3}H-dihydrostreptomycin,

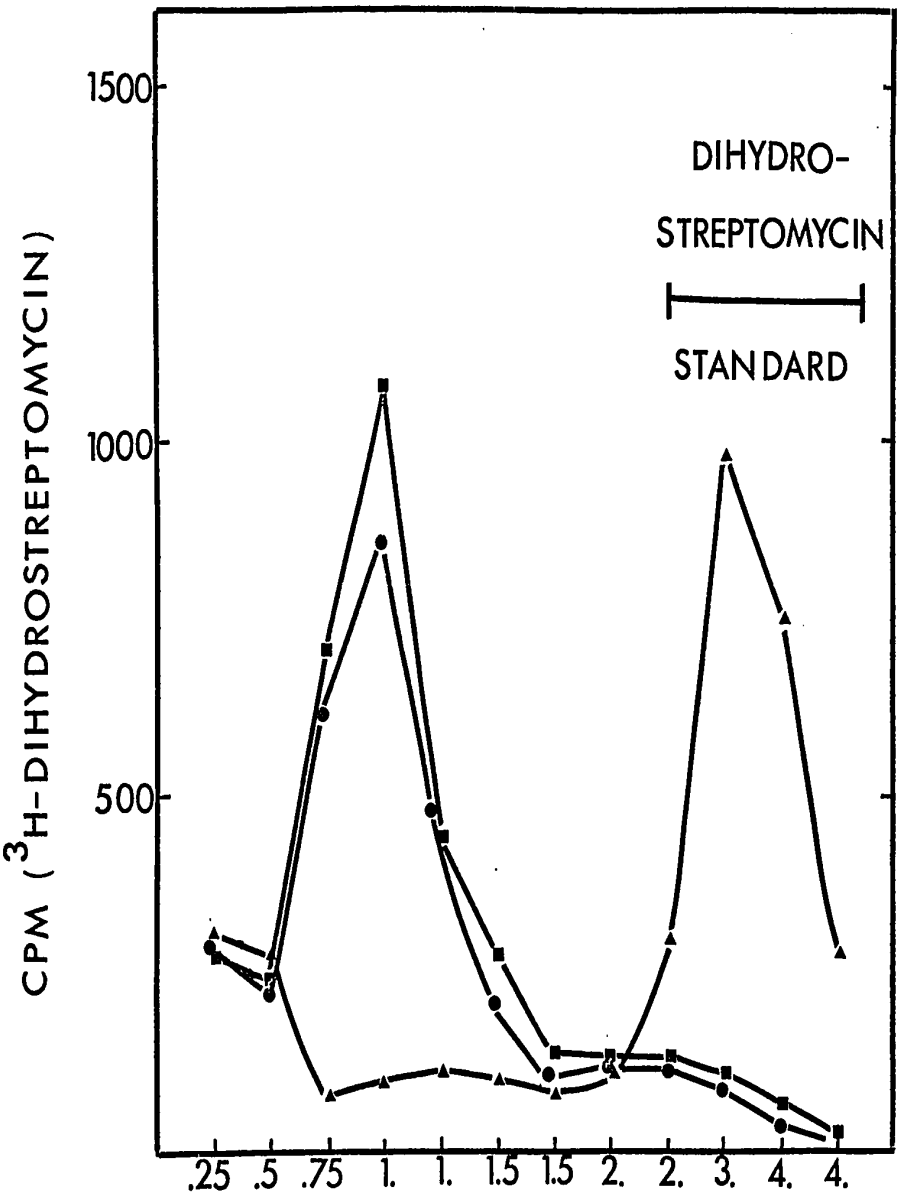

1500
1000
500
CPM (^{3}H-DIHYDROSTREPTOMYCIN)
DIHYDRO-
STREPTOMYCIN
STANDARD
.25 .5 .75 1. 1. 1.5 1.5 2. 2. 3. 4. 4.
MOLARITY OF AMMONIUM ACETATE

FIG. 3. Effect of cell extracts on phosphorylation of dihydrostreptomycin.

Cell extract of a strain producing streptomycin phosphotransferase (E. coli CSH-2 with R factor NR_3 ●) P. aeruginosa strain 280 (▲) or P. aeruginosa 280 R931 (■) were incubated with ^{3}H-dihydrostreptomycin, and then applied and eluted from Dowex 50W as described in Materials and Methods. Identical treatment of a reaction mixture containing cell-free extract determined the elution position of a ^{3}H-dihydrostreptomycin standard.

was chromatographed without further treatment, or after treatment, with either snake venom phosphodiesterase or E. coli alkaline phosphatase as given in the Methods. The results in Fig. 4 demonstrate that the product was susceptible to E. coli alkaline phosphatase. It was thus concluded that strain 931 produces streptomycin phosphate. Strains 1162, 679 trp^-, 280 R1162, 280 R679 trp^-, E. coli K12F^-, E. coli hybrids of 679 trp^- and 1162, and 280 Cb^r Su^r R931 (obtained by mating 280 met^- R931 x 280 Cb^r Su^r) were also examined for the production of streptomycin phosphotransferase or streptomycin adenylate synthetase. The results given in Table III show that the donors 1162 and 679 produce streptomycin phosphotransferase and that this property was acquired by the recipient 280 met^-. Fig. 5 illustrates that E. coli K12 F^- acquired streptomycin phosphotransferase after filter matings with strains 679 and 1162. The sequential hybrid 280 Cb^r Su^r R931 also contained streptomycin phosphotransferase. Fig. 6 illustrates the elution profile of dihydrostreptomycin adenylate prepared by incubation of ^{3}H-dihydrostreptomycin with the cell extract of E. coli K12-58/E521, a strain known to adenylate streptomycin. It was also confirmed to be sensitive to snake venom phosphodiesterase and insensitive to E. coli alkaline phosphatase.

Table VIII shows that low-level resistant strains had no detectable phosphorylating enzymes for streptomycin. Adenylating enzymes were not detected in any of 21 strains examined (including low-level resistant strains). Strains possessing phosphorylating activity demonstrated high levels of resistance to streptomycin, and the

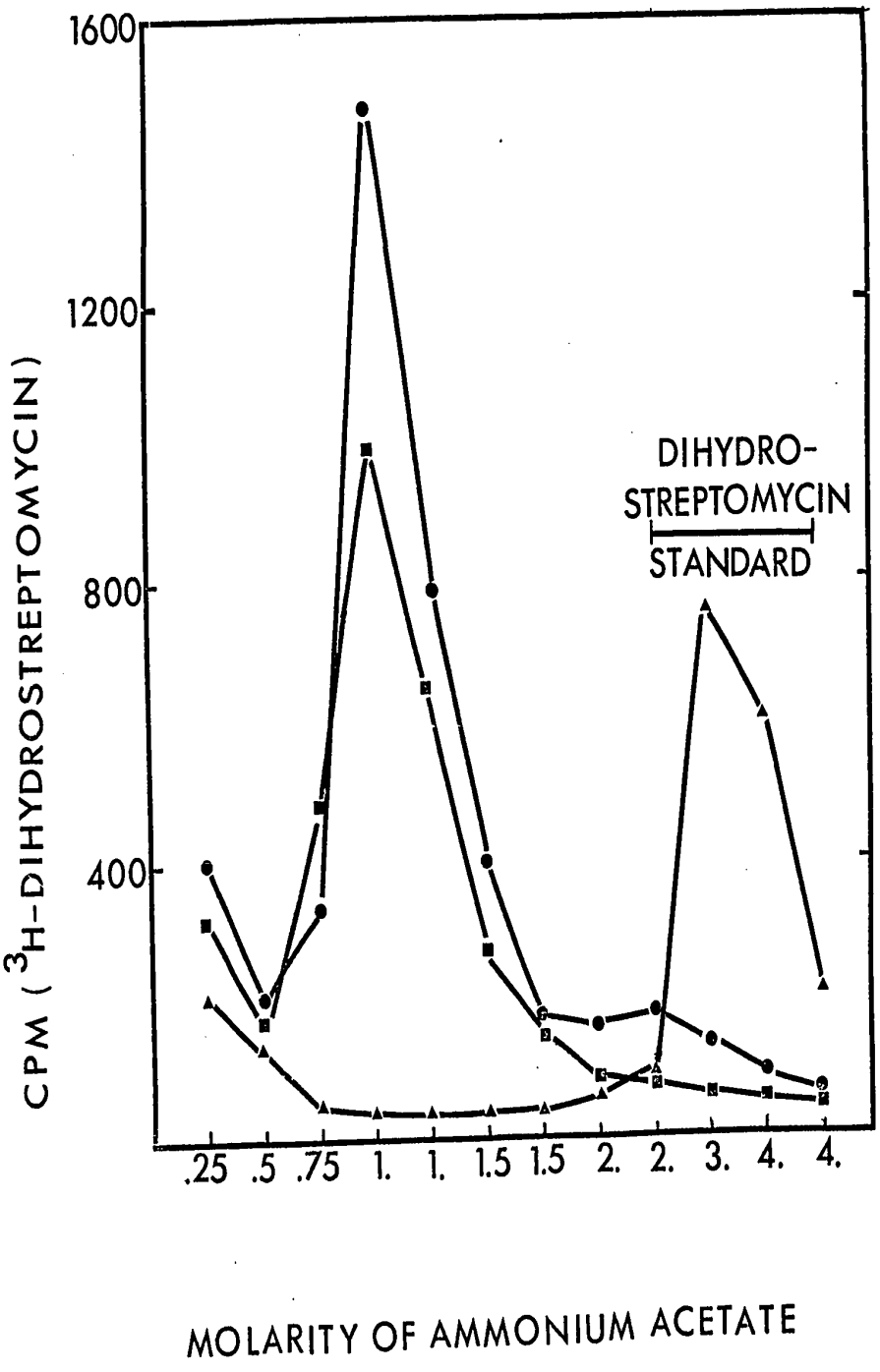

1600
1200
800
400
CPM (^{3}H-DIHYDROSTREPTOMYCIN)
DIHYDRO-
STREPTOMYCIN
STANDARD
.25 .5 .75 1. 1. 1.5 1.5 2. 2. 3. 4. 4.
MOLARITY OF AMMONIUM ACETATE

FIG. 4: Product of ^{3}H-dihydrostreptomycin obtained by incubation with cell extract of P. aeruginosa 931 (●) and the effect on that product obtained from incubation with snake venom phosphodiesterase (■) or E. coli alkaline phosphatase (▲).

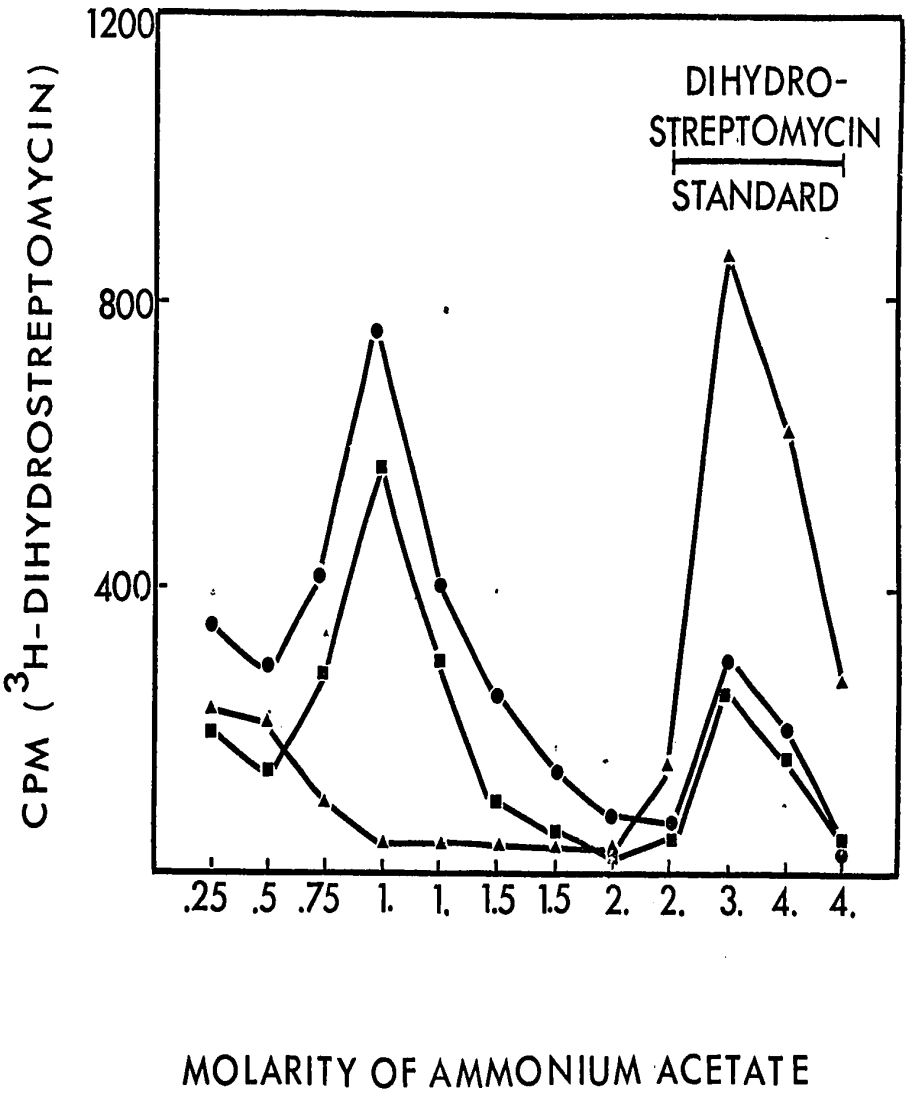

1200
800
400
CPM (^{3}H-DIHYDROSTREPTOMYCIN)
DIHYDRO-
STREPTOMYCIN
STANDARD
.25 .5 .75 1. 1. 1.5 1.5 2. 2. 3. 4. 4.
MOLARITY OF AMMONIUM ACETATE

FIG. 5: Presence or absence of dihydrostreptomycin phosphorylating activity in E. coli K12F⁻ ATCC 14948 (▲), E. coli K12F⁻ R1162 (●) and E. coli K12F⁻ R679 (■) strains.

Cell extracts of those strains were incubated with ^{3}H-dihydrostreptomycin, and then applied and eluted from Dowex 50W as described in Materials and Methods.

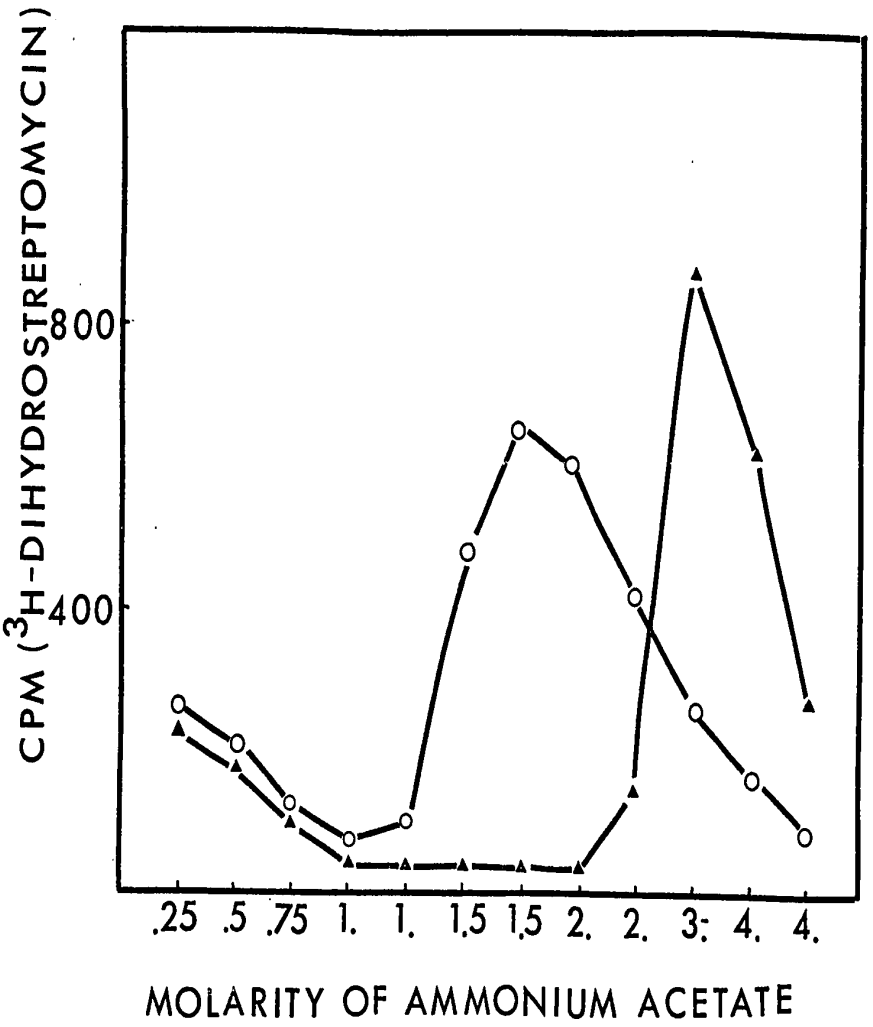

CPM (^{3}H-DIHYDROSTREPTOMYCIN)
800
400
.25 .5 .75 1. 1. 1.5 1.5 2. 2. 3. 4. 4.
MOLARITY OF AMMONIUM ACETATE

FIG. 6: Elution profile of the product of ^{3}H-dihydrostreptomycin incubated with cell extract of E. coli K12-58/E521 (o), and the effect on that product produced by incubation with snake venom phosphodiesterase (▲).

TABLE VIII

STREPTOMYCIN PHOSPHOTRANSFERASE OR STREPTOMYCIN ADENYLATE SYNTHETASE IN P. AERUGINOSA STRAINS

Strain	MIC (µg/ml)	Streptomycin Phosphoro-transferase	Streptomycin Adenylate Synthetase	Trans-fer-ability[a]
Sensitive				
280	5	-	-	-
1731	5	-	-	-
2379	10	-	-	-
Low-level resistant				
1059	25	-	-	-
1330	125	-	-	-
1885	50	-	-	-
High-level resistant				
931	1500	+	-	+
1162	> 2000	+	-	+
679	> 2000	+	-	+
280R931[b]	500	+	-	+
915	20000	-	-	-

[a] transferability to the recipient P. aeruginosa strain 280 by conjugation.

[b] hybrid of strains 931 x 280 mating.

phosphorylating activity was transferable with one or more other resistance markers to a second P. aeruginosa strain. We detected seven strains of P. aeruginosa producing streptomycin phosphate in this work. In each case, the phosphorylating activity has been transferable to a second P. aeruginosa strain. Strain 931 possesses phosphorylating activity. When studied for uptake it accumulated an amount of streptomycin, at 100 µg of streptomycin/ml (well below its MIC), about twice that seen in susceptible strains at their MIC. Thus, accumulation of large quantities of streptomycin is not dependent upon first reaching the MIC for a strain (Table VII).

Culture filtrates of representative low-level resistant strains were examined by bioassay for inactivation of extracellular streptomycin such as might occur if streptomycin were modified to a form which was rapidly excreted from the cell. If the latter were true the data might mimic defective streptomycin permeation. However, no detectable inactivation occurred. In addition when low-level resistant strains were examined for adenylation or phosphorylation of streptomycin the labelled dihydrostreptomycin always eluted in a position identical to that of a dihydrostreptomycin standard.

5. Ribosomal resistance to streptomycin: It is well established that resistance to streptomycin can be mediated by a specific ribosomal mutation (Davies, 1964). Table IX illustrates that low-level resistant strains and strains possessing streptomycin-inactivating enzyme do not exhibit ribosomal resistance, which is as would be expected. Strain 915 did demonstrate ribosomal resistance. The frequency of strains

TABLE IX

INHIBITION OF AMINO ACID INCORPORATION BY STREPTOMYCIN IN CELL FREE SYSTEMS OBTAINED FROM P. AERUGINOSA STRAINS

Experiment No.	Cell free system S100	Cell free system Ribosomes	Incorporation of ^{14}C-valine (cpm)[b] Control	Incorporation of ^{14}C-valine (cpm)[b] With streptomycin 50 µg/ml
1	1731[a]	1731	1784	189
	1731	915	1167	887
2	1059	1059	1241	161
	1059	931	1016	134
3				With streptomycin 2 µg/ml
	1731	1731	1431	458
	1731	1330	1096	228

a the number of the bacterial strain used as a source of the ribosome or S100 fractions which were prepared as described in the Methods.

b background cpm were 100 to 125.

such as 915 was found to be about 5% of all P. aeruginosa strains in this series.

6. Summary: From the above findings and for the reasons expanded upon in the Discussion, we concluded that the large majority of strains of P. aeruginosa isolated from clinical specimens are of low resistance to streptomycin due to low permeability.

IV. Modification of the lipopolysaccharide-phospholipoprotein components of the cell envelope.

1. Action of antiserum and complement on Pseudomonas strain 1885

This experiment was initiated in order to produce a selective LPS lesion for the possible modification of uptake of antibiotics in P. aeruginosa. Strain 1885 at a density of 0.2 A_{550} absorbance was treated with homologous antiserum with or without lysozyme by a procedure given in the Materials and Methods. Table X illustrates the effect on viability determined by plate counts after such treatment. Lysozyme-free antiserum and complement showed a considerable bactericidal effect on strain 1885 when subsequent plate counts were done using ordinary TSA plates. The bactericidal effect, however, was abolished by the inclusion of 20% sucrose in the TSA plates. Lysozyme alone or antiserum (complement inactivated) alone did not have any effect on bacteria as would be expected. However, if lysozyme, antiserum and complement were used together, the bactericidal effect persisted even if 20% sucrose was included in the medium. Such observations would suggest the following sequence of events during the serum bactericidal reaction. The antiserum and complement act on the

TABLE X

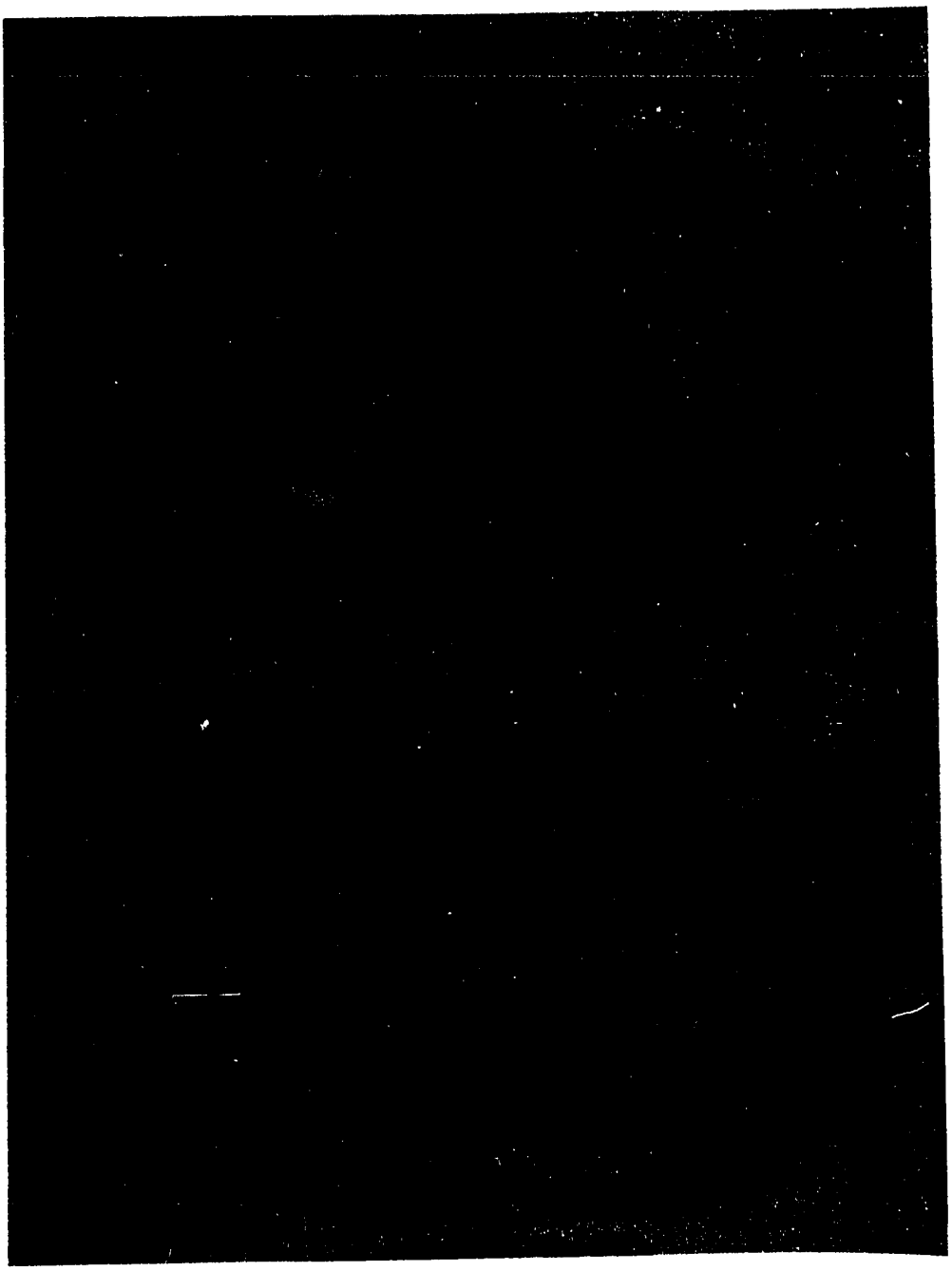
VIABILITY OF STRAIN 1885 TREATED WITH ANTISERUM AND COMPLEMENT WITH OR WITHOUT LYSOZYME

	TSA Plate (Colony counts/ml)	Sucrose TSA Plate (Colony counts/ml)
Control	5.2×10^8	4.9×10^8
Treated with antiserum (complement inactivated[a]) alone	4.8×10^8	5.0×10^8
Treated with lysozyme (25 µg/ml) alone	5.4×10^8	5.2×10^8
Treated with lysozyme-free complement and antiserum	0.8×10^8	4.5×10^8
Treated with complement antiserum and lysozyme (25 µg/ml)	0.6×10^8	0.7×10^8

[a] Antiserum was incubated in 56°C waterbath for 30 minutes to inactivate the complement.

lipopolysaccharide-phospholipid complex of the cell wall, resulting in loss of wall material from the cell and an impairment of the supportive function of this structure. The peptidoglycan polymer remains intact, and the cells remain viable if osmotic support (sucrose) is present. However, the cells become suceptible to lysozyme, and lose viability even with 20% sucrose in the TSA plates if complement, serum and lysozyme are used together. Fig. 7 shows the electron micrograph appearance of the bacterial cells treated with lysozyme alone. The bacterial cell walls show the normal wavy, three electron dense layers. Fig. 8 is the appearance of the bacterial cells treated with antiserum and complement. The three electron dense layers are still present, but formation of "membrane" material (blebs) is noted. Fig. 9 shows the appearance of cells treated with serum, complement and lysozyme. The cell wall assumes a layered structure and is seen as an electron-lucent layer sandwiched between two electron-dense double-track layers. The electron-lucent layer thus appears to be the peptidoglycan polymer which is degraded by the action of lysozyme. The cells are converted from their rod shape into spheroplasts. Leakage of intracellular material is also apparent and numerous spherules or blebs are present.

Table XI illustrates that treatment of strain 1885 with antiserum and complement as given in the Methods does not cause any additional leakage of acid-soluble nucleotides from the bacteria as compared to the control. In this study, polymyxin is used as a positive control. Strain 1885, after such polymyxin treatment,



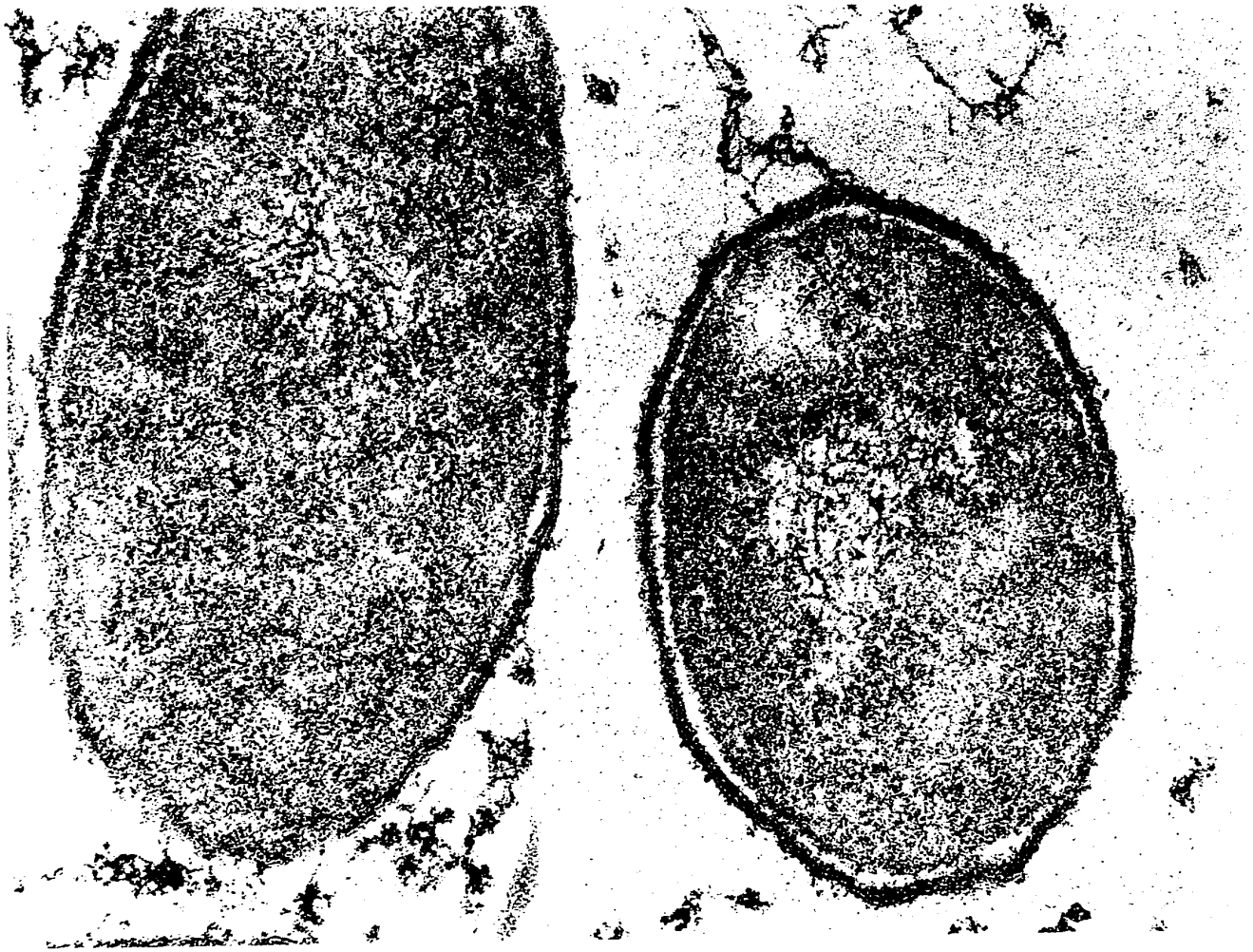


FIG. 7: Electronmicrograph of strain 1885 treated with lysozyme alone.

The normal three electron dense layers of the cell wall are present.

Magnification 140,000 x

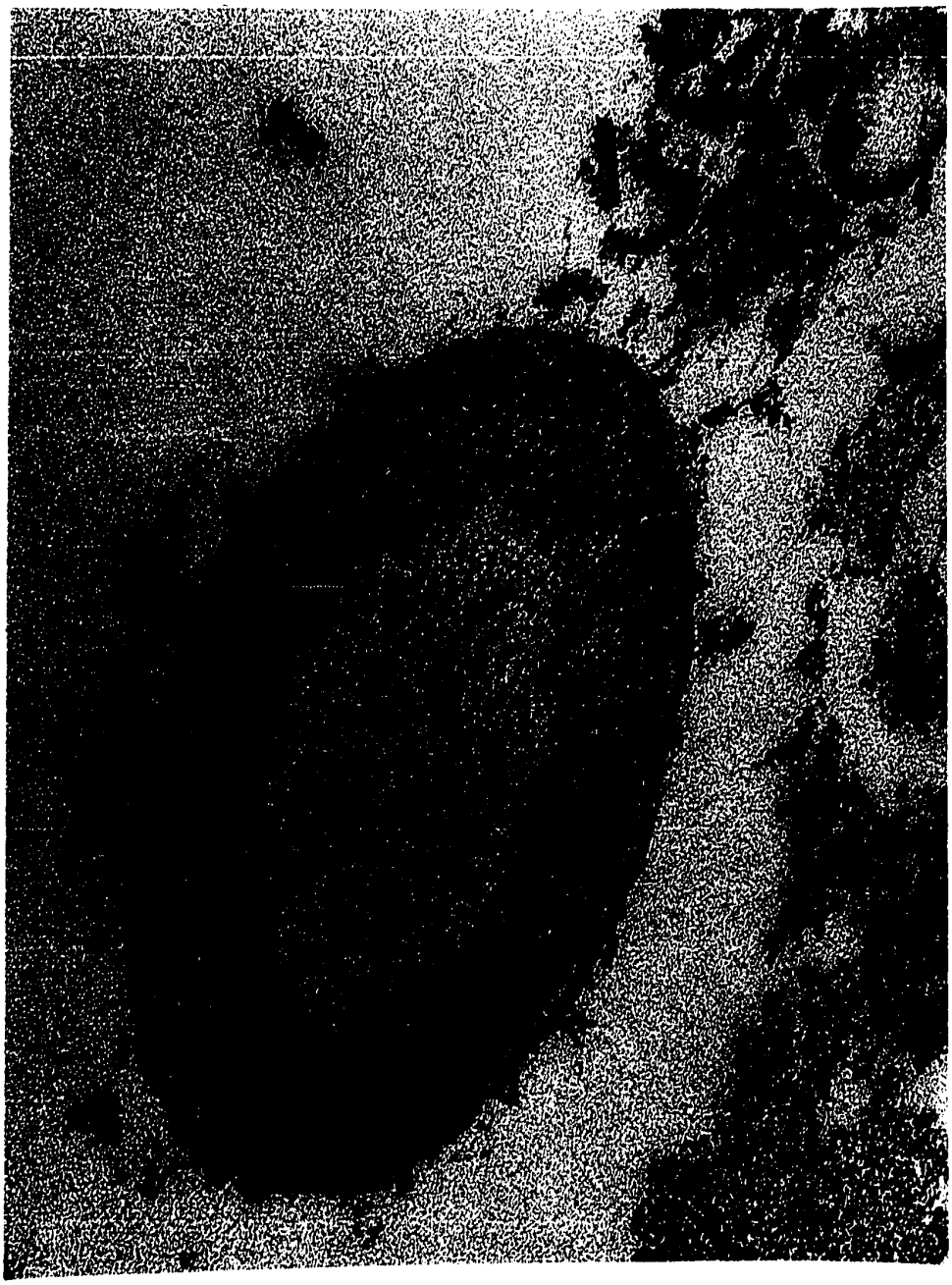


FIG. 8: Electronmicrograph of bacterial cells treated with lysozyme-free antiserum and complement.
Three electron dense layers of the cell wall are still present. The cells remain rod shaped, some blebs are present.
Magnification 140,000 x

FIG. 9: Electronmicrograph of bacterial cell treated with serum, complement and lysozyme.

These cells assume a spherical shape. An electron-lucent layer sandwiched by two electron-dense layers is clearly visible. Numerous blebs or vesicles are present, and cell contents have been lost by most cells. Magnification 74,000

TABLE XI

LEVELS OF TOTAL LABELLED ACID-SOLUBLE NUCLEOTIDES IN THE EXTERNAL MEDIUM AND IN THE INTERNAL POOL OF STRAIN 1885

	Count per min[a]	
	Total count from external medium	Total count from internal pool
Control	2340	9900
Treated with antiserum and complement	2430	10218
Treated with polymyxin (4 µg/ml)	6150	9084

[a] Background counts per min were 20 to 25.

leaked catalase from the cells into the medium as examined by a qualitative procedure given in the Methods. From this data, we assume that antiserum and complement have a negligible effect on the barrier property of the cell membrane of strain 1885.

2. Effect of $MgCl_2$ on strain 1059 in low phosphate media:

Cheng et al. in 1970 reported that growth of Pseudomonas strain ATCC 9027 in medium containing an $MgCl_2$ concentration greater than 0.1 M caused the release of the periplasmic enzyme alkaline phosphatase; this indicated that high concentrations of $MgCl_2$ might modify the LPS-lipoprotein complex. However, the results of our study showed that growth of strain 1059 in low phosphate medium released the alkaline phosphatase almost completely into the medium, whether 0.1 M of $MgCl_2$ was present or not, as illustrated in Table XII. Catalase was not released from the cells.

V. Effect of different media on streptomycin uptake.

Although we could not conclude whether magnesium chloride had any effect on the barrier property of lipopolysaccharide of P. aeruginosa from the release of alkaline phosphatase, we studied the effect of $MgCl_2$ on ^3H-dihydrostreptomycin uptake in low phosphate medium. As shown in Table XIII, low phosphate medium caused a reduced MIC coupled with an increased streptomycin uptake as compared to TS broth. The inclusion of 0.1 M $MgCl_2$ in low phosphate medium reversed this effect, causing strain 1059 to exhibit a rise in MIC and a corresponding decline in streptomycin uptake.

TABLE XII

ALKALINE PHOSPHATASE AND CATALASE ACTIVITY OF STRAIN 1059 GROWN IN LOW PHOSPHATE MEDIUM WITH OR WITHOUT 0.1 $MgCl_2$

		Alkaline phosphatase (total units)	Catalase
Treated with 0.1 M $MgCl_2$	External medium	0.123	- [a]
	Associated with bacteria	0.037	+
Control	External medium	0.123	-
	Associated with bacteria	0.007	+

[a] Catalase test was done qualitatively by mixing 0.5 ml and 3% H_2O_2 with 0.5 ml of test solution and looking for the bubbling. Negative sign indicates no catalse activity.

TABLE XIII

ACTUAL UPTAKE OF DIHYDROSTREPTOMYCIN BY P. AERUGINOSA STRAINS IN VARIOUS GROWTH MEDIA

Strain	Media	MIC (µg/ml)	Actual uptake at 10 µg/ml dihydrostreptomycin (ng/A550/ml)
1885	T.S.	50	6.7
	Low phosphate	25	89.5
1059	T.S.	20	78.1
	Low phosphate	12.5	167.8
	Low phosphate + 0.1 M $MgCl_2$	37.5	19.5

VI. Mechanism of P. aeruginosa tetracycline resistance.

1. Tetracycline uptake studies: Tetracycline uptake has been shown to be an energy-requiring system in E. coli (Franklin, 1967) and Staphylococcus aureus (Sompolinski et al., 1970). This was confirmed to be true also in P. aeruginosa from the results shown in Fig. 10. Thus, in the absence of 0.025% NaN_3, there is an increase with time in the amount of ^3H-tetracycline associated with strain 280; in the presence of 0.025% NaN_3 at 4°C the increase in tetracycline accumulation with time by strain 280 is eliminated. Thus the cell-associated radioactivity obtained with 0.025% NaN_3 at 4°C under our experimental conditions represents background levels which appear to be non-specific binding of tetracycline to the bacterium. Fig. 10 also illustrates the tetracycline uptake pattern at 10 µg/ml tetracycline of a representative susceptible and resistant strain. It may be observed that the amount of tetracycline associated with strain 494 increases only slightly with time and that the total amount of tetracycline associated with that strain is approximately equal to that of strain 280 examined at 4°C in the presence of 0.025% NaN_3.

The tetracycline uptake of several strains of P. aeruginosa at various concentrations of tetracycline is seen in Table XIV. Several points are apparent from that table. In general the concentrations of tetracycline used in uptake studies must approximate the MIC of a R^- (R factor negative) resistant strain before tetracycline uptake is equivalent to that of susceptible strains 280 and 2379 at 10 µg/ml tetracycline. When the concentration of tetracycline used

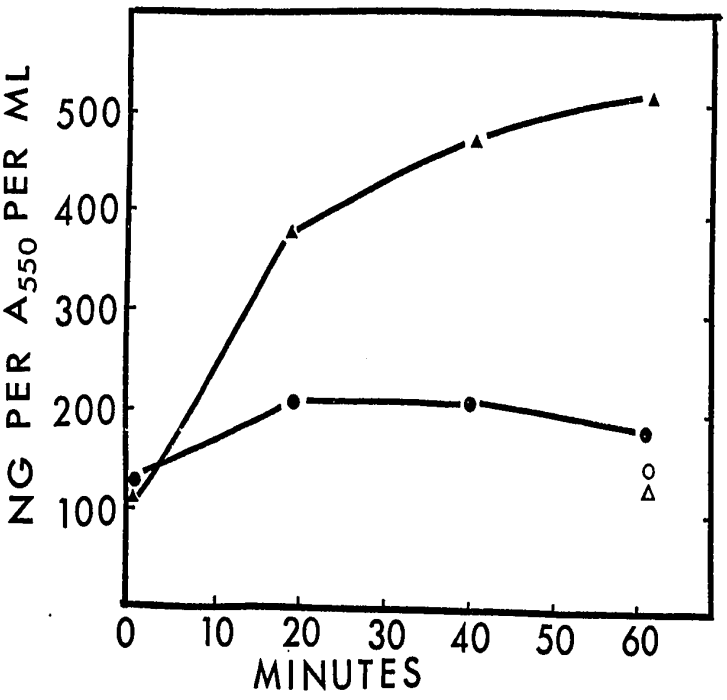

NG PER A_{550} PER ML
500
400
300
200
100
0
10
20
30
40
50
60
MINUTES

FIG. 10: Kinetics of uptake of tetracycline by strains 280 and 494 in TS broth at a tetracycline concentration of 10 µg/ml.

Strain 280 ▲—▲, strain 494 ●—●
Strain 280 with NaN_3 at 0 - 4°C Δ
Strain 494 with NaN_3 at 0 - 4°C o

TABLE XIV

UPTAKE OF TETRACYCLINE BY STRAINS OF P. AERUGINOSA
AT VARIOUS TETRACYCLINE CONCENTRATIONS

Strain	MIC (µg/ml)	Tetracycline Concentration(µg/ml)						
		10	17.5	20	25	50	100	200
R^-								
280	10	495.2[a]						
2379	10	573.2						
1731	22.5	71.8		392.8				
1885	25	60.9	163.4		846.4			
1885 pre-treated[b]					725.5			
494	25	71.9			715.5	5074.6		
1059	20	201.1			863.7			
R^+								
280R931	75	135.2			240.2	587.6		
280R931 pre-treated						337.1		
931	150	27.8					3870.9	
931 pre-treated						47.2	420.0	3207

[a] uptake value expressed in nanograms per A_{550}/ml.

[b] pre-treated strains were exposed to 1 µg/ml tetracycline for 3 hr at 37°C prior to uptake determination.

in uptake studies exceeds a strain's MIC, the tetracycline uptake increases very rapidly as is seen with strain 494. A second point is the marked reduction in tetracycline uptake produced by acquisition of Pseudomonas R factor, 931. Thus the hybrid strain 280R931, obtained by mating strains 931 and 280, displays about one quarter the uptake shown by strain 280 at 10 µg/ml tetracycline.

Data from Table XIV also establish that pre-incubation of resistant R^+ (R factor-containing) strains with sub-inhibitory tetracycline concentrations reduces tetracycline uptake. The most marked example is strain 931 but reduction also occurs with 280R931. In these R^+ strains the correlation of MIC and tetracycline uptake is best shown by the uptake values obtained when strains are pre-incubated with low sub-inhibitory tetracycline concentrations. A negligible effect of pre-incubation is seen with R^- strain 1885 in which the tetracycline resistance is presumed to be chromosomal.

2. Inhibition of amino acid incorporation by tetracycline: Table XV shows that there is no evidence to suggest that ribosomes are insensitive to tetracycline in any strain examined. The presence of the R factor, R931, does not influence ribosomal susceptibility to tetracycline.

3. Inactivation of tetracycline: Several procedures described in the Methods were used to examine for inactivation of tetracycline by resistant strains. Bioassays of cell-free supernatants after 3-hr exposure at 37°C of strains 931 and 494 to 30 µg/ml and 15 µg/ml tetracycline respectively demonstrated that no detectable reduction

TABLE XV

INHIBITION OF AMINO ACID INCORPORATION BY TETRACYCLINE IN CELL-FREE SYSTEMS OBTAINED FROM STRAINS OF P. AERUGINOSA

Expt. No.	Cell-free system		Incorporation of ^{14}C-valine (counts/min)[a]	
	S100	Ribosomes	Control	With tetracycline (20 µg/ml)
1	1731[b]	1731	940	263
	1731	1330[c]	1096	191
	1731	931	860	110
2	931	1731	1046	272
	931	931	1368	174
3	280	280	1752	226
	280	1330	830	162

[a] Background counts per minute were 100 to 125.

[b] The number of the bacterial strain used as a source of the ribosomes or S100 fractions which were prepared as described in Materials and Methods.

[c] Strain 1330 is a R^- tetracycline-resistant strain.

in the bio-activity of tetracycline occurred. Tetracycline was extracted from resistant bacteria which had been exposed to tetracycline, and subjected to silica gel-impregnated paper chromatography. The Rf value obtained for extracted tetracycline from strain 494 and the R-factor-carrying strain 931 was 0.6. That value was identical to that obtained with a tetracycline standard or with tetracycline from filtrates of 931 or 494 subjected to the same chromatography protocol. As shown in Fig. 11, a single sharp peak was obtained with the tetracycline extracted from strain 931. Extracted tetracycline from strain 931 also retains similar biological activity to a tetracycline standard when examined by plate diffusion as described in Materials and Methods. Thus there is no evidence of any altered form of tetracycline. In addition, from this data it seems unlikely that the explanation for difference in permeability between susceptible and resistant strains could be accounted for by the modification of tetracycline to a more rapidly excretable form.

4. Summary: The tetracycline uptake data show that there is a close correlation between a strain's MIC and its uptake value. The ribosomes from resistant and susceptible strains are equally sensitive to tetracycline in the inhibition of amino acid incorporation in the cell-free system studied. Incubation of labelled tetracycline with resistant strains failed to detect any tetracycline with reduced biological activity or altered chromatographic characteristics. From these findings, we have thus concluded that resistance to tetracycline in P. aeruginosa is due to defective permeation of the drug.

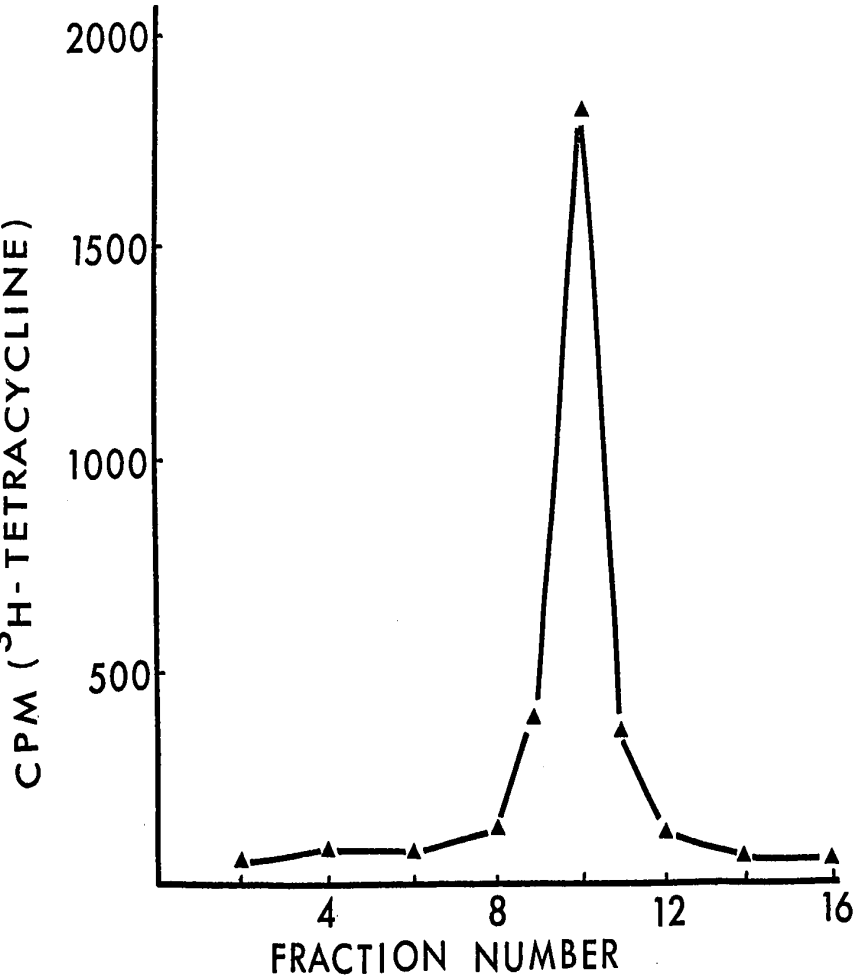

2000
1500
1000
500
CPM (^{3}H-TETRACYCLINE)
4
8
12
16
FRACTION NUMBER

FIG. 11: Silica gel-impregnated paper chromatography of ^{3}H-tetracycline from filtrate of strain 931 treated with tetracycline for 3 hours.

A drop of filtrate was applied to a strip of silica gel-impregnated paper treated with 2% Di-sodium EDTA and chromatographed overnight in the dark at room temperature using N-Butanol:Methanol:10% citric acid (4:1:2). The paper was dried, cut into 1 cm fractions, and suspended in toluene scintillation fluid.

VII. Modification of dihydrostreptomycin or tetracycline uptake by second agents

1. Uptake of dihydrostreptomycin by strains 280 and 1059 in the presence of carbenicillin: In order to determine whether the peptidoglycan polymer acts as an important barrier to streptomycin penetration in the low-level streptomycin-resistant strain, uptake of dihydrostreptomycin at a sub-inhibitory concentration of carbenicillin (half of the MIC) was studied in one such strain and compared with a susceptible strain. Table XVI illustrates the results of such a study. Low-level resistant strain 1059 exhibited an increase of 1.6-fold in uptake of dihydrostreptomycin in the presence of carbenicillin. A similar degree of increment in uptake was also noted in the susceptible strain 280 with carbenicillin. The difference in streptomycin sensitivity, thus, appears not to be due to a differential permeability barrier of the peptidoglycan of the two strains.

2. Uptake of dihydrostreptomycin and tetracycline in the presence of antiserum and complement: Antiserum to strain 1885 and complement was shown to alter the permeability of the lipopolysaccharide-lipoprotein layer of the cell wall by rendering the organisms sensitive to the action of lysozyme. Therefore an uptake study of dihydrostreptomycin and tetracycline was done in the presence of antiserum and complement in order to determine whether lipopolysaccharide-lipoprotein acts as an important penetration barrier for these antibiotics in the low-level resistant strains. The results are illustrated in Table XVII. No significant change in uptake of tetracycline occurred, but a slight increase in uptake of dihydrostreptomycin was observed.

TABLE XVI

ACTUAL UPTAKE OF DIHYDROSTREPTOMYCIN BY STRAINS OF *P. AERUGINOSA* WITH OR WITHOUT SUB-INHIBITORY CONCENTRATIONS OF CARBENICILLIN

Strain	MIC (µg/ml)	Conditions	Dihydrostreptomycin Concentration (µg/ml) 2.5	10
1059	20	Control		78.1[a]
		With carbenicillin		120.9
280	5	Control	29.6	
		With carbenicillin	49.5	

[a] Actual uptake expressed in nanograms per A_{550} unit per ml.

TABLE XVII

UPTAKE OF DIHYDROSTREPTOMYCIN AND TETRACYCLINE BY STRAIN 1885 IN TS BROTH CONTAINING 20% SUCROSE WITH OR WITHOUT ANTISERUM AND COMPLEMENT

	Dihydro-streptomycin 10 µg/ml	Tetracycline 10 µg/ml
Control	2.0[a]	22.0
With antiserum and complement	2.4	23.0

a = actual uptake expressed in nanograms per A_{260} unit per ml of the S-30 supernatant of bacteria.

3. Uptake of tetracycline by carbenicillin spheroplasts:

A tetracycline uptake study was done using carbenicillin spheroplasts of a low-level resistant strain (1059) and a susceptible strain (280) by the procedure given in Methods. Table XVIII illustrates the results of this study. A three- to four-fold increment in uptake was observed in carbenicillin spherosplasts from both susceptible and low-level resistant strains. Since spheroplasts from the resistant strain do not have a relatively higher increment of uptake than spheroplasts from the susceptible strain, the difference in tetracycline sensitivity between the strains cannot be explained by a differential permeability barrier of the strains' cell walls to tetracycline.

VIII. Tetracycline and incorporation of labelled amino acids in bacteria

1. Release of intracellular tetracycline at 37°C and its correlation with the rate of incorporation of ^{14}C-leucine:

Tetracycline accumulated by bacteria is released from the cells rather rapidly at 37°C, as illustrated in Fig. 12. In this experiment cells were centrifuged free of tetracycline after 60 minutes of uptake and incubated at 37°C. The tetracycline remaining with the cells was determined as given in the Methods. In strain 280 when the uptake was carried out at 10 µg/ml tetracycline, the cells lost 70% of tetracycline in 10 minutes; 85% in 20 minutes, and 95% in 30 minutes. Another control study has shown that less than 2% of the labelled tetracycline accumulated by strain 280 is lost upon subsequent storage at 0 - 4°C in TS broth for 30 minutes. The rate

TABLE XVIII

UPTAKE OF TETRACYCLINE BY CARBENICILLIN SPHEROPLASTS OF STRAINS 280 AND 1059

Strain		Tetracycline concentration 4 µg/ml	10 µg/ml
280	Control	50.6[a]	
	Carbenicillin spheroplasts	215.6	
1059	Control		102.7
	Carbenicillin spheroplasts		321.9

[a] = actual uptake expressed in nanograms per A_{550} unit/ml.

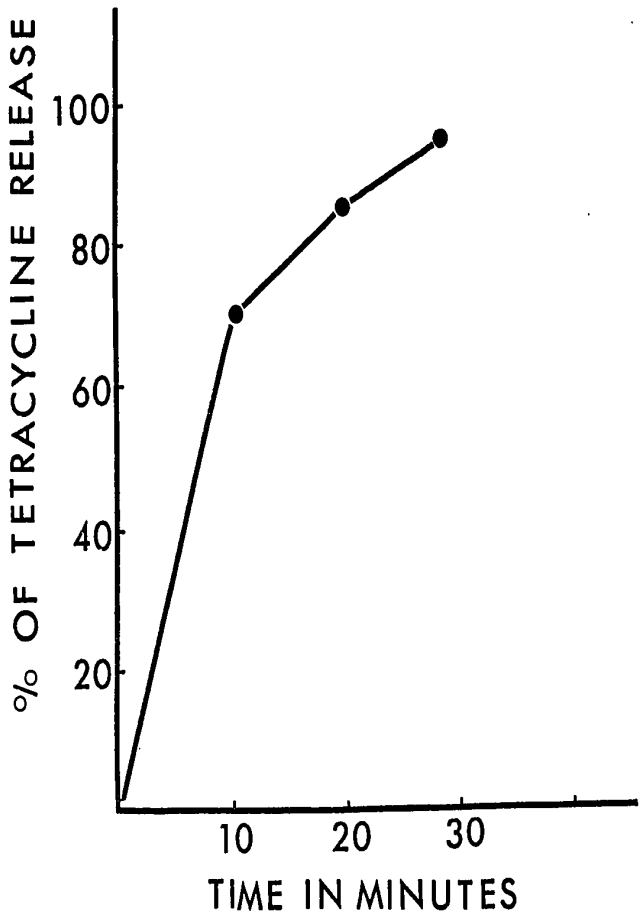

% OF TETRACYCLINE RELEASE
100
80
60
40
20
10
20
30
TIME IN MINUTES

FIG. 12: Release of tetracycline from bacterial cells.

Strain 280 was allowed to accumulate tetracycline at concentration 10 µg/ml for one hour at 37°C and then centrifuged free of tetracycline. Cells were re-suspended in TSB at 37°C and ^{3}H-tetracycline remaining with the cells determined as given in the Methods.

of incorporation of ^{14}C-leucine into strain 280 which was incubated with 10 µg/ml tetracycline for 1 hour and then washed free of tetracycline was determined. As seen in Fig. 13, a short lag period of 15 to 20 minutes was present, after which incorporation of ^{14}C-leucine proceeded at the normal rate with the same slope as that of a control. A good correlation between the release of tetracycline from bacteria and the re-starting of cellular protein synthesis was thus demonstrated.

2. Uptake and incorporation of ^{14}C-proline by strains of *P. aeruginosa* in the presence of tetracycline with or without pre-incubation with 1 µg/ml tetracycline:

It has been found that strains of *E. coli* bearing transferable resistance factors, when pre-incubated with a sub-inhibitory concentration of tetracycline, showed a marked increase in resistance to the drug (Franklin, 1967). Therefore, we tried also to assess the degree of resistance or susceptibility to tetracycline by the ability of the organism to continue protein synthesis *in vivo*, measuring the uptake and incorporation of ^{14}C-proline into the cellular acid insoluble material.

In this study, two R^+ strains (931 and 280 R931) and two R^- strains (1885 and 280 T^r - the latter was isolated as a mutant colony of 280 from a TSA plate containing 20 µg/ml tetracycline) were used. As shown in Table XIX, the R^+ strains became more resistant with pre-incubation and showed a twofold increase in proline incorporation. In R^- strains, no significant change in proline incorporation occurred with pre-incubation. In previous tetracycline uptake studies, it was

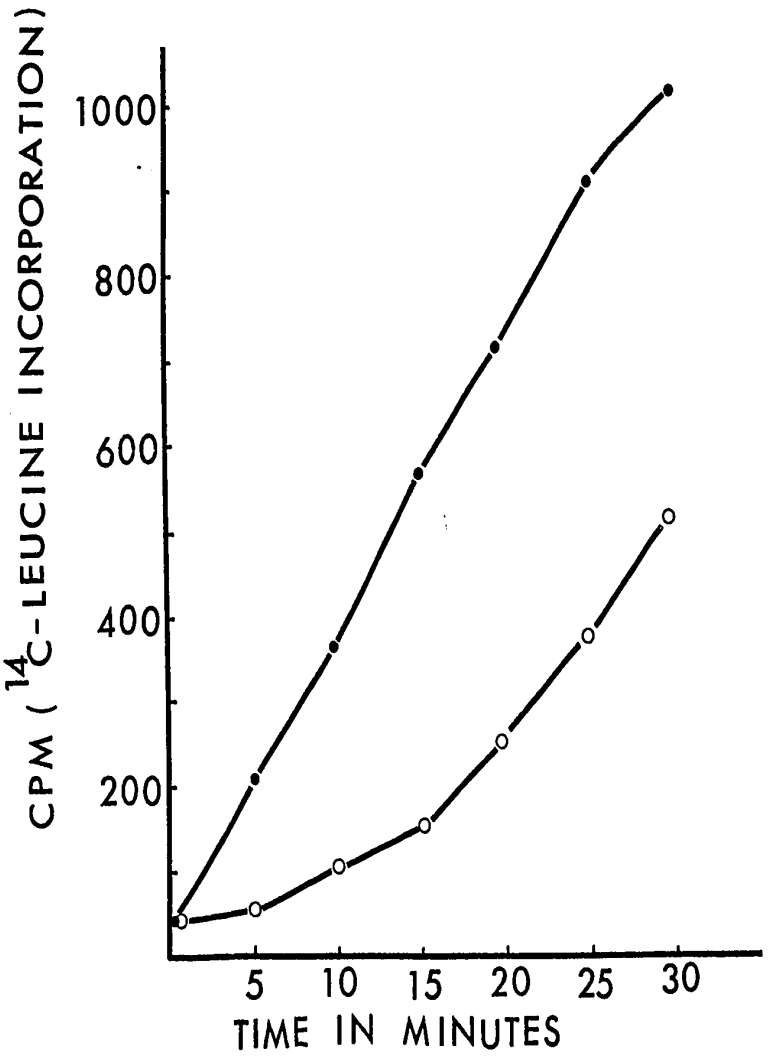

CPM (^{14}C-LEUCINE INCORPORATION)
1000
800
600
400
200
5
10
15
20
25
30
TIME IN MINUTES

FIG. 13: Rate of incorporation of ^{14}C-leucine into cellular acid-insoluble material of strain 280 with or without pre-treatment of bacteria with 10 µg/ml tetracycline, as described in Materials and Methods.

Control ●——●

Bacteria pre-treated with tetracycline o——o

TABLE XIX

INCORPORATION OF ^{14}C-PROLINE INTO CELLULAR ACID INSOLUBLE MATERIAL IN THE PRESENCE OF TETRACYCLINE

Strain	MIC (µg/ml)	Tetracycline concentration used (µg/ml)	^{14}C-proline incorporation (CPM) in 30 minutes		0 time
			With pre-incubation	Without pre-incubation	
931	150	100	2241.5	1198.1	51.9
280R931	75	50	3451.4	1662.8	
1885	25	20	1119.1	1080.8	
280TR	30	20	1233.5	1323.6	

demonstrated that only R^+ strains exhibited a marked reduction in tetracycline uptake with pre-incubation. Thus, there is a good correlation between these two studies.

IX. Periplasmic binding protein

In 1968 Penrose et al. reported the isolation and purification of a leucine-binding protein which appears to be involved in the transport of this and related amino acids (isoleucine and valine). The transport of several other substrates in Gram negative bacteria has also been shown to involve periplasmic binding proteins. These include sulfate (Pardee, 1966), galactose (Anraku, 1968), arginine (Wilson and Holden, 1969) and others. Therefore, a preliminary attempt to investigate whether periplasmic binding proteins were involved in the uptake of streptomycin and tetracycline was done. Centrifuged and washed bacterial cells were treated with EDTA and lysozyme containing 3% NaCl as described in Methods. In this study 3% NaCl was used to replace sucrose as an osmotic support in order to minimize non-specific ionic interaction of the strongly cationic streptomycin and proteins in the subsequent equilibrium dialysis study. After the lysozyme and EDTA treatment, the supernatant contained 300 µg/ml of released protein from strain 280 and 400 µg/ml of released protein from strain 1885, as determined by the method of Lowry et al. (1951). The bacterial cells were observed by phase contrast microscopy to be converted to spherical shape after such treatment, indicating that elimination of cell wall (and periplasmic content) from bacteria had occurred. As illustrated in Tables XX, XXI. and XXII,

TABLE XX

INVESTIGATION FOR THE PRESENCE OF PERIPLASMIC STREPTOMYCIN BINDING PROTEIN BY EQUILIBRIUM DIALYSIS

Strain		CPM (^{3}H-dihydrostreptomycin)		
		16 hrs	24 hrs	40 hrs
1885	Control	1241	1123	1145
	Supernatant[a]	1061	1187	1191
280	Control	1238	1157	1201
	Supernatant[a]	1124	1171	1159

[a] Supernatants were prepared from bacteria treated with EDTA and lysozyme as described in Materials and Methods.

TABLE XXI

INVESTIGATION FOR PERIPLASMIC TETRACYCLINE BINDING PROTEIN BY EQUILIBRIUM DIALYSIS

Strain		CPM (^{3}H-tetracycline)		
		16 hrs	24 hrs	40 hrs
1885	Control	1113	1114	1100
	Supernatant[a]	1074	1102	1122
280	Control	1140	1072	1076
	Supernatant[a]	1095	1077	1082

[a] Supernatants were prepared from bacteria treated with EDTA and lysozyme as described in Materials and Methods.

TABLE XXII

INVESTIGATION FOR PERIPLASMIC TETRACYCLINE BINDING PROTEIN BY EQUILIBRIUM DIALYSIS IN STRAIN 280R931

Growing Conditions		CPM (^{3}H-tetracycline)		
		16 hr	24 hr	40 hr
With tetracycline 1 µg/ml	Control	1048	1013	1034
	Supernatant[a]	1014	1052	1039
Without tetracline	Control	1055	1012	1025
	Supernatant[a]	1025	1030	1076

[a] Supernatants were prepared from bacteria treated with EDTA and lysozyme as described in Materials and Methods.

neither streptomycin nor tetracycline periplasmic binding proteins were detected in any strains studied by this method. It is unlikely, however, that the method used would be sensitive enough to detect binding proteins if they were produced in low quantities or if their affinity for the antibiotics were low.

DISCUSSION

Although streptomycin and tetracycline are not extensively used therapeutically for P. aeruginosa infections, they do serve as useful models to explore distribution of resistance levels and mechanisms. At least some of the information may also prove applicable to the study of other antibiotics.

The spectrum of susceptibility of P. aeruginosa to streptomycin covers a wide range. It extends from those strains resistant to high concentration (> 1000 µg/ml) to fully susceptible strains. The results of these studies indicate that the basis of the resistance of mutants selected in the laboratory is rarely similar to that of clinical isolates and point out the importance of studying resistant strains isolated from nature. High-level resistance accounted for less than 10% of our series and was the result of ribosomal resistance or inactivation of streptomycin by phosphorylation. Of the high-level resistant strains, about one in five was due to drug inactivation. Strains which acquired resistance by conjugation are all of high level resistance, and the mechanism of resistance is conferred by an R factor (Bryan et al., 1973) which controls the genetic information for producing streptomycin phosphotransferase. However, low-level resistance was very much more frequent (about 80%) and ranged from MIC values of 10 to 200 µg/ml; for about half of these strains more than 50 µg/ml of streptomycin was required to materially inhibit growth. For many of these strains, thus, the MIC equalled or exceeded peak concentrations achieved in serum after conventional

doses of streptomycin (Garrod and O'Grady, 1971), and these strains would be reported as resistant by the Kirby-Bauer disc antimicrobial susceptibility method. It is also possible that a similar range of antimicrobial susceptibility exists for other antimicrobial agents used against P. aeruginosa and could account for therapeutic failures or delayed cure. For example our laboratory has detected many more strains of P. aeruginosa which are resistant to about 6 to 15 µg/ml gentamicin than to 100 µg/ml or greater. Resistance in the latter category has been ascribed to ribosomal cause (Tanaka, 1970) or to gentamicin inactivation (Mitsuhashi et al., 1971), but no exaplanation of the former has been advanced.

The differences in sensitivity to streptomycin of strains of low-level resistance seems clearly to be dependent on permeability to the drug. At least five lines of evidence support that conclusion. Firstly, there is, as pointed out in the results, a close association between the MIC of a strain and uptake of streptomycin. Secondly, the relationship between MIC and streptomycin uptake is further supported by the observation that growth of two different strains in low phosphate medium resulted in a decrease in MIC and a corresponding increase in streptomycin incorporation of an equivalent streptomycin concentration. Similarly the presence of 0.1 M $MgCl_2$ in the growth medium resulted in an increase of the MIC for strain 1059 and decreased streptomycin incorporation. The composition of culture medium has been shown to influence measurement of bacterial susceptibility to aminoglycoside antibiotics (Henry and Hobby, 1949;

Medeiros et al., 1971; Youmans and Fisher, 1949). For example, Gilbert et al. (1971) reported the MIC to gentamicin is increased by increasing the concentration of magnesium (or calcium) in the growth medium. The data presented here suggest these cations may modify permeability to the drug.

Thirdly, the major objection to the proposal that diminished permeation is responsible for the low-level resistance is that membrane leakage might be secondary to a primary event which occurs at the MIC (possibly inhibition of protein synthesis). If this were true, it should not be possible to divorce uptake level and MIC to any significant extent. This supposition would likely be so whatever the primary effect, as membrane leakage seems sufficient to cause bacterial death. The incorporation data obtained with strains 931 and 915 seem to exclude that possibility. In those strains substantial uptake occurs at a streptomycin concentration considerably below the strains' MIC (see Table VII) demonstrating that uptake can be independent of the MIC. Strain 931 apparently owes its resistance to a streptomycin-inactivating enzyme and that of 915 primarily results from resistant ribosomes.

Fourthly, there is no other detectable mechanism to account for the MIC of low-level resistant strains, as there is no evidence of any known form of streptomycin inactivation nor, as would be expected, of ribosomal resistance. In addition, several attempts have also been made to transfer the low-level resistance to a susceptible strain, and have never succeeded. Therefore, no evidence

of R factors could be detected in the low-level resistant strains.

It is possible for other processes to mimic defective permeation such as bacterial modification of a drug to a rapidly excretable form or to a form which cannot enter the cells. These seem unlikely in view of the preceding points and of the failure to detect any streptomycin which possessed reduced biological activity or altered chromatography characteristics except in those strains possessing phosphorylation enzymes. It also seems unlikely that defective ribosomal binding of streptomycin accounts for low-level resistance. The ribosomes obtained from representative low-level resistant strains (1059, 1330) and from a susceptible strain (1731) are equally susceptible to 2 or 50 µg/ml streptomycin in the inhibition of amino acid incorporation. In addition, previous investigations have shown that ribosomal mutations result in a single step to high level streptomycin resistance (Davies, 1964) rather than to the low level resistance observed here.

We have also used the transfer of drug resistance directly between two strains of P. aeruginosa as a method to assess the frequency of occurrence of R factors. The demonstration of such transfer was feasible only because of the unique characteristics of the recipient strain 280. It was sensitive to seven antibiotics (carbenicillin, gentamicin, kanamycin, streptomycin, sulfonamide, tetracycline and polymyxin); resistant to most pyocines and produced a brown pigment uncommon among P. aeruginosa strains. Unfortunately, it was resistant to cephaloridine, ampicillin, and moderately resistant to chloramphenicol, but in spite of these problems it is a useful

strain to utilize as a recipient for transfer of the resistance to other drugs. The remarkable difference demonstrated by the Pseudomonas (strain 280) in recipient potential relative to the E. coli strain is of considerable significance. The utilization of E. coli as a recipient in survey techniques for transferable drug resistance is, in our opinion, unwise in view of the possibility and even likelihood that many examples of transferable drug resistance would not be detected. A positive result under these conditions would be acceptable but it would be difficult to justify a negative result. The reason for the difference in transfer frequency (of drug resistance) has not been established. Examination of our donor, recipient and mating mixtures of Pseudomonas by electron microscopy has failed to demonstrate any obvious mating pili or other unusual morphological features. It is, however, not surprising that severe mating incompatibility exists between Pseudomonas and E. coli because of the significant differences between these members of distinct genera.

Pyocine selection was judged to be a useful technique. In the case of strain 931 the results were excellent but for 1162 breakthrough was troublesome. However, in combination with an auxotrophic recipient it also proved to be a useful method. Pyocine selection would appear in some cases to be as good or better than the auxotrophic method in, at least, the case of strain 931. Its major promise was in its use as a selecting device for surveying large numbers of strains for transferable drug resistance. However, with the availability of rifampicin-resistant mutants, it is probable that

pyocine selection will not be extensively used in the future.

The streptomycin resistance mediated by transferable drug resistance in Pseudomonas is very much greater than most similar examples in E. coli. However, when introduced into E. coli, the Pseudomonas streptomycin phosphotransferase is associated with much lower streptomycin resistance (400 µg/ml vs 3200 µg/ml for 1162; 40 µg/ml vs 3200 µg/ml for 679) than in the donor.

Although R factors have been found in some strains of P. aeruginosa, they are not the major reason for the resistance of P. aeruginosa to streptomycin. Such a situation contrasts with that of E. coli where the resistance to streptomycin is usually due to R factors which code for the production of either streptomycin adenylate synthetase (Umezawa et al., 1968) or streptomycin phosphotransferase (Ozanne et al., 1969). We have not found any streptomycin-resistant P. aeruginosa strains which produce streptomycin adenylate synthetase. The reason why occurrence of R factors is uncommon in Pseudomonas is not known, but it may bear some relationship to the low-level resistance of most naturally occurring strains of P. aeruginosa to streptomycin. Thus no selective pressure has been imposed on P. aeruginosa for R factor-containing strains.

Our studies demonstrate that in a population of P. aeruginosa selected from hospitalized patients the large majority of strains reported as resistant are so owing to defective permeability to streptomycin. This study supports the contention that mechanisms of resistance should be examined in naturally-occurring resistant strains

and not in strains rendered resistant in the laboratory (Smith, 1970). In addition, resistance due to defective permeability is high enough to be therapeutically troublesome but perhaps low enough to be overcome by modification of permeation in the bacterium by a second biologically active agent.

The spectrum of susceptibility of P. aeruginosa to tetracycline covers a range of MIC values from 10 to 100 µg/ml. Strains bearing transferable resistance markers for tetracycline are usually resistant to high concentrations (> 100 µg/ml). However, such strains comprise less than 10% of the total strains tested. Most of our strains isolated from clinical specimens show tetracycline susceptibility values between 10 µg/ml and 30 µg/ml. Strains which have an MIC of more than 10 mg/ml are considered clinically resistant to tetracycline.

The results of our studies indicate that there is a close correlation between the MIC of a strain and its uptake of tetracycline. The ribosomes obtained from representative strains are all equally sensitive to tetracycline in the inhibition of amino acid incorporation. Incubation of labelled tetracycline with resistant strains failed to detect any tetracycline which possessed reduced biological activity or altered chromatography characteristics. A susceptible strain, which acquired a transferable resistance marker (28OR931) also shows decreased permeability to the drug. When strains of P. aeruginosa bearing a transferable factor for resistance to the tetracycline are grown in a low sub-inhibitory concentration of tetracycline, there is a striking increase in resistance as shown in the ^{14}C-proline

incorporation study (Table IX). This observation was associated with a marked fall in the accumulation of tetracycline by the cells. Since such adaptation phenomena occurred with very low concentrations of the drug and short pre-incubation times, the possibility that more resistant cells were being selected from a heterogeneous population of cells could be excluded. Thus it seems reasonable to assume that resistance to tetracycline in P. aeruginosa is due to diminished permeability to the drug. Similar observations have been reported for E. coli (Franklin, 1967) and Staphylococcus aureus (Inoue et al., 1970; Sompolinski et al., 1970).

Despite the apparent unique origin of R931 (Bryan et al., 1973) the mechanisms of resistance to streptomycin and tetracycline are the same as those of R factor mediated resistance in E. coli (Franklin, 1967; Okamoto and Suzuki, 1965). It seems possible that r determinants may be derived from a common source but that a distinct conjugation system has evolved in P. aeruginosa. It is also possible that there exists a limited number of methods by which R factor mediated resistance can be expressed. It would be of interest to examine a variety of characteristics of the various streptomycin phosphotransferases to determine their degree of relatedness and thus the probability of common origin. In the case of R factor or episomal tetracycline resistance, there is evidence reported here and elsewhere (Franklin, 1967; Sompolinski et al., 1970) that an inducible inhibition of tetracycline uptake occurs. Avtalion et al. (1971) have reported serological evidence for such an inhibitor. Further work to examine

serological cross-reactivity of the inhibitors from different sources would be of interest for the above reason.

Our studies of the resistance mechanisms of P. aeruginosa to streptomycin and tetracycline demonstrate that the large majority of strains selected from hospitalized patients are of low level resistance to these antibiotics due to defective permeability. R factor-mediated resistance to these antibiotics in P. aeruginosa is uncommon, and thus not a major therapeutic problem in using these antibiotics. Due to the significance of low permeability as a cause of resistance, we tried to determine whether the various layers of cell envelope could account for the difference in the permeability of susceptible and resistant Pseudomonas strains. We successfully modified the permeability of the outer cell wall (lipopolysaccharide lipoprotein) with antiserum and complement. From the results in Table XVII, such modification appeared fairly specific for the non-peptidoglycan portion of the cell wall, rendering the organism susceptible to the action of lysozyme, but we did not obtain any appreciable increment of uptake of these antibiotics in the low-level resistant strains by this method. A half to one-fold increase in streptomycin uptake was observed with maximum sub-inhibitory concentrations of carbenicillin in both susceptible and low-level resistant strains. An increment of three- to four-fold tetracycline uptake was also obtained with carbenicillin spheroplasts in both susceptible and low-level resistant strains. Peptidoglycan layer, therefore, appears to be a more important barrier than the lipopolysaccharide for the uptake of these two

antibiotics. It is of interest that penicillin (or carbenicillin) has been frequently used in combination with an aminoglycoside antibiotic for the treatment of infection and a more satisfactory effect is usually obtained than using one antibiotic alone. Although bacterial uptake of tetracycline may be increased by penicillin or carbenicillin, combinations of tetracycline and penicillin should not be used clinically because tetracycline is a bacteriostatic agent. Since uptake of streptomycin and tetracycline in both susceptible and low-level resistant strains is affected to a similar extent with carbenicillin, the cell membrane thus appears to be the ultimate barrier responsible for the difference in uptake between the susceptible and low-level resistant strains. No periplasmic binding protein responsible for transport of these antibiotics was detected in any of the representative strains tested. The impairment of the low-level resistant strains in uptake of these antibiotics could be the result of low affinity or activity of the membrane transport carriers to these drugs or it could be a consequence of the loss of a stereospecific mechanism necessary for the drugs to gain access to the transport carriers and to penetrate the membrane.

Streptomycin and tetracycline are not natural nutritional ingredients for the bacteria, therefore we would not expect the bacteria to have a specific transport system for the active accumulation of streptomycin or tetracycline. Apparently, these antibiotics gain entry into the cell via the transport system designed for the entry of some natural substrates. Mutant strains of E. coli selected for

resistance to D-serine or to canavanine are unable to concentrate glycine or arginine from the medium (Schwartz et al., 1959), thus are unable to grow in minimal media with glycine or arginine as the only carbon source. Similar studies could be done for streptomycin or tetracycline by isolating the resistant mutants from a susceptible strain which could not accumulate these antibiotics. Such mutants would be expected to have lost the transport system for some natural substrate. A knowledge of the transport system which the organism uses to concentrate these antibiotics would be helpful in the future to study what chemical modification of those antibiotics might be most gainful to render the organism more susceptible to those drugs.

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