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# PERMEABILITY ALTERATIONS ASSOCIATED WITH CHLORAMPHENICOL RESISTANCE IN *MICROCOCCUS PYOGENES* VAR. *AUREUS*

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APPROVED BY

DISSERTATION COMMITTEE

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# CHAPTER I

## INTRODUCTION

The increasing use of antibiotics in recent years has stimulated much interest in the mechanisms by which microorganisms are resistant or become resistant to these substances. Resistant strains are often serious problems in therapeutics. Thus, studies of the features of resistance have great practicality and potential application. Since these studies are often carried out at fundamental levels of cellular function, further development of knowledge of the physiology of microorganisms has occurred concurrently.

Although the resistance to antibiotic substances is only poorly understood, evidence has accumulated that several mechanisms may be operative.

### The Synthesis of an Enzyme With the Ability to Destroy or Inactivate the Drug

A striking example of such an enzyme is penicillinase, first described by Abraham and Chain (1940). While this enzyme is produced by many penicillin-resistant strains of several species, many highly resistant strains do not produce it. Other mechanisms must occur. For example, Eagle ($1954_a$) has noted that there are probably several mechan-

isms of resistance to penicillin in addition to the production of penicillinase. First, certain species do not liberate penicillinase but degrade penicillin after it enters the cells, e.g., (Escherichia coli, Proteus morgani, and Shigella paradysenteriae). Second, among the many species which do not degrade penicillin, resistant strains regularly bind less penicillin. Cell-free extracts of these strains bind penicillin in relation to the sensitivity of the intact cells. Finally, resistant variants may combine with penicillin to the same degree as the parent sensitive cells without being killed as a result of that combination.

Other mechanisms are implied by the penicillin-dependent strains of Micrococcus described by Barber (1953), and the cell-bound intracellular penicillinase in Bacillus cereus (Pollock, 1956).

The alterations by which cells become insensitive to antibiotic substances may be quite different depending upon the bacterial species and the antibiotics concerned. It is likely that these mechanisms are not exclusive in all instances and that a single strain may become resistant by one means at one encounter with an antibiotic and by another at a subsequent encounter with the same antibiotic. For example, Chandler, et al., (1951) isolated a penicillinase producing strain of Micrococcus pyogenes var. aureus which was resistant to 200 ug penicillin per ml. Resistance decreased to 0.156 ug per ml after 13 daily transfers in the presence of chlortetracycline and the ability to produce penicillinase was lost. Re-exposure to penicillin led to a resistance to 25 ug penicillin per ml, but by some other mechanism since no penicillinase was produced.

Szybalski (1953) reported that of the penicillin-resistant mutants of M. pyogenes var. aureus obtained by in vitro selection, approximately 1 in 10,000 produced penicillinase, whereas more than 99 per cent of those isolated from patients produced the enzyme. Wallmark (1954) found that every one of 327 resistant strains isolated from patients produced penicillinase, but no penicillinase producers could be obtained by serial propagation in increasing concentrations of penicillin. However, Roy and Lankford (1954) found 7 penicillinase producing strains in 313 strains made resistant by exposure to penicillin in vitro.

Enzymatic degradation of chloramphenicol has been noted by Smith and Worrel (1949, 1950). The enzyme, produced by E. coli, reduced the active nitro group of chloramphenicol to an inactive amine. Merkel and Steers (1953) reported that "chloramphenicol reductase" activity of E. coli increased with increasing resistance. However, these authors also noted that serial transfer of the organism in the absence of chloramphenicol resulted in complete loss of "chloramphenicol reductase" activity but only partial loss in resistance. Therefore, it was concluded that this enzymatic activity could not account completely for resistance. A similar apparently adaptive system has been described by Molho-Lacroix and Molho (1952) in the same species. A soluble enzyme which could reduce chloramphenicol has also been reported by Egata and Sato (1951). More recently Chabbert and Debruge (1956) reported that chloramphenicol-resistant strains of staphylococci produced a substance which diffused through agar and inhibited the action of chloramphenicol upon sensitive strains. It is not known whether or not this substance is an enzyme.

Bergman, et al., (1954) noted the inhibition of streptomycin activity by culture filtrates of Pseudomonas aeruginosa. This heat stable material was without effect in mice infected with pneumococci and treated with streptomycin.

## Altered Metabolism

Streptomycin inhibits a variety of metabolic reactions including the "oxalacetate-pyruvate reaction" (Umbreit, 1949; Oginsky, Smith, and Umbreit, 1949). In a recent review Umbreit (1955) has concluded that only the "oxalacetate-pyruvate" inhibition has any possible bearing upon the mode of action of streptomycin.

This reaction appears to be unique in that it does not resemble the known reactions of oxalacetate or pyruvate, but involves the formation of a compound identified as 2-phospho-4-hydroxy-4-carboxy adipic acid (Umbreit, 1953). Formation of this compound by E. coli is inhibited by streptomycin at levels comparable to those required to inhibit growth. What role this intermediate may play in metabolism is not yet known. Presumably, streptomycin resistant microorganisms can dispense with it by utilizing some alternate pathway. Such a phenomenon is postulated by Wyss and Schaiberger (1953) and by Smith, Oginsky and Umbreit (1949), who found that streptomycin-resistant and -dependent variants of E. coli could not effect the oxalacetate-pyruvate condensation in detectable amounts.

A strain of M. pyogenes var. aureus which was resistant to streptomycin acquired the ability to grow in the presence of 100 ug streptomycin per ml in a medium deficient in phenylalanine and aspartic

acid (Sevag and Rosanoff, 1952). The parent strain would not grow in this medium if 10 ug streptomycin per ml were present, but would grow in a complete medium containing 10 ug SM per ml. The resistant strain must have acquired another pathway of metabolism independent of the utilization of phenylalanine and aspartic acid.

Resistance due to increased synthesis of a metabolite which antagonizes an inhibitor is exemplified by the antagonism of sulfonamides by para-aminobenzoic acid (PAB). Mirick (1942) isolated a sulfonamide antagonist from culture filtrates of a resistant strain of pneumococci which produced 10 times more of the inhibitor than the sensitive parent strain; this inhibitor was identified as PAB. Landy, et al., (1943) found that micrococci resistant to sulfathiazole produced 70 times more PAB than the parent strain.

## Decreased Affinity of an Enzyme for an Inhibitor or the Production of an Enzyme Refractory to an Inhibitor

Davis and Maas (1952) have described the occurrence of enzymes with decreased affinity for the inhibitor, para-nitrobenzoic acid (PNB). This compound is a competitive analog to both para-aminobenzoic acid (PAB) and para-hydroxybenzoic acid (POB), which are similar in structure but have different metabolic functions. Resistant strains of E. coli were obtained in the presence of each of these metabolites. There was no cross resistance between the two resistant strains. It was concluded that decreased permeability or destruction of the antimetabolite could not be the basis of resistance. Furthermore, the persistance of a PAB requirement in a sulfonamide resistant derivative of a PAB auxotroph

excluded any altered metabolic pathway as an explanation of resistance. Finally, Davis and Maas demonstrated that resistant strains had an increase in competitive ratio of analog to metabolite which was proportional to their degree of resistance. In some strains there was not only a decreased affinity of the PAB utilizing enzyme for the analog, but also some decreased affinity for PAB.

Yaniv and Davis (1953) reported another example of an enzyme with an altered affinity for a specific inhibitor. A sulfonamide-resistant, secondary mutant was derived from an E. coli mutant which required PAB. With an excess of PAB, the two strains grew equally well, but at growth limiting concentrations of the vitamin. The resistant mutant yielded slower and lighter growth than its parent. The resistant strain, therefore, appeared to have an enzyme with decreased affinity for PAB.

An enzyme from E. coli strain E-26 which mediates the reduction of chloramphenicol and other aromatic nitro compounds to the corresponding arylamines was described by Saz and Marmur (1953) and Saz and Slie (1954). This enzyme was inhibited by aureomycin in concentrations of 1 to 100 ug per ml. When a strain of E. coli was rendered resistant to aureomycin by serial passage of the parent in increasing concentrations of the drug (Saz, et al., 1956), the nitro-reductase isolated from the resistant strain was not inactivated by aureomycin. The aureomycin-sensitive and -resistant enzymes were compared by Saz and Martinez (1956) and found to differ in chemical and physical properties. Thus it was concluded by Saz, et al., (1956) that resistance to aureomycin

reflects the synthesis of an altered enzyme which is insensitive to aureomycin.

Sevag and Gots (1948), obtained cell-free dehydrogenases from pneumococci which were resistant to various drugs including atabrine, propamide and acriflavin. They explained resistance in these instances by the fact that the enzymes were resistant to the drugs, but it has not been established that inhibition of these dehydrogenases is the mechanism by which the drug interferes with growth.

## Altered Permeability

Altered permeability as an explanation for drug resistance has been suggested (Davis and Maas, 1952; Gale, 1952) and Nachmanson (1954) has indicated that the success of many pharmacologic drugs may be due to a selective permeability of various cells of the body. Racker (1954) points out that a "disunity of permeability" to various metabolites and intermediates is known to exist among microorganisms and animal cells, and that studies on chemotherapeutic agents should always consider this possibility.

Recorded instances exist in which a decrease in permeability has been found to occur concomitantly with resistance. Maass and Johnson (1949) were unable to demonstrate an uptake of isotopically labelled penicillin by Saccharomyces cerevisiae and concluded that impermeability was a possible explanation for the resistance of this organism to penicillin. However, Eagle ($1954_b$), working with HeLa and L strain tumor cells in tissue culture, believed that the relative non-toxicity of penicillin for mammalian cells was not due to the failure of the antibiotic to

penetrate the cells, but was probably related to a low combining affinity of cellular components with the intracellular penicillin.

The oxalacetate-pyruvate reaction, which is sensitive to streptomycin has been demonstrated in animal cells. However, intact cells were not affected by streptomycin (Umbreit and Tonhazy, 1949), and it was concluded that permeability barriers exist in the cell wall and also at the surface of the mitochondria which prevent the entry of streptomycin.

Nicol (1954) has found that large amounts of analogs (A-methopterin or Aminopterin) were required to inhibit enzymatic formation of folinic acid from pteroylglutamic acid by resistant strains of Streptococcus faecalis. Much smaller amounts inhibited this enzyme in cell-free extracts of resistant organisms or in intact sensitive organisms.

There is at least one instance in which it has been demonstrated that resistance is not due to altered permeability. Saz, et al., (1956) have reported preliminary experiments using aureomycin labelled with $Cl^{36}$ in which the uptake of this material by sensitive and resistant E. coli was of the same order of magnitude. However, it was observed also that the nitro-reductase activity of cell-free extracts of the resistant strain was not affected by aureomycin.

It is not the purpose of this paper to discuss whether alterations which result in resistance occur through genetic mutation or adaptive processes. There is ample evidence to indicate that both may be operative since there is not always a single satisfactory explanation for the different phenomena of resistance. For detailed discussions

of this question, the reader is referred to reviews by Yudkin (1953), Braun (1953), Abraham (1953), Cavalli-Sforza and Lederberg (1953), Bryson and Szybalski (1955), Bryson and Demerec (1955), Gale and Davies (1953), Sevag, Reid and Reynolds (1955), Eagle and Saz (1955), and Schnitzer and Grunberg (1957).

From very early observations, the belief has been that bacterial cells possess mechanisms by which the passage of certain materials into and out of cells could be controlled. This subject has been reviewed by Knaysi (1951), Mitchell (1949), Weibull (1956) and Mitchell and Moyle ($1956_a$).

Although the permeability mechanisms of bacterial cells are not well understood, the subject is becoming increasingly important in the current literature. The use of isotopically labelled materials has great potential value in the study of these phenomena; however, the techniques involved are fraught with difficulty. Because of this, and also because different authors have used different organisms and experimental systems, a considerable amount of controversy has arisen and the problem is far from being solved.

Roberts, et al., (1955) and Cowie and Roberts (1955) made extensive studies of E. coli and concluded that this species has no osmotic barrier of any consequence. They visualize the bacterial cell as a sponge and the cell membrane as a surrounding hairnet which is unable to exclude the entrance or emergence of small molecules. These investigators have used isotopic techniques, for the most part, combined with methods for determining intracellular "water space" available for solutes (Cowie and Roberts, 1955). They found that E. coli is freely permeable to phosphate,

sulphate, glucose-1-phosphate, fructose 1:6-diphosphate, and the ions of potassium, rubidium, cesium and sodium. The ability of some species to concentrate certain ions and other materials (Gale, 1947; Taylor, 1947; Rothstein, 1955; Mitchell and Moyle, 1956$_a$) was explained by some type of selective intracellular binding and not by differential permeability. Cystine and glutamic acid were found to enter and leave the cells freely; passive diffusion was indicated as the mode of transport. These findings were considered in light of the fact that certain metabolic intermediates do not diffuse out of cells. It was suggested that, with certain compounds, some type of intracellular alteration occurs which makes such compounds non-diffusible. For example, acetate probably exists in the form of acetyl-coenzyme A and succinate as succinyl-coenzyme A (Lipmann, 1953). Also certain amino acids may remain within the cells because they are combined into peptides which are not diffusible. It was admitted by these investigators that permeability differences among species are likely and, indeed, they do occur. This is not in agreement with the conclusions of others which are discussed below.

Using several different methods Mitchell and Moyle (1956$_b$) measured permeability in E. coli, Micrococcus pyogenes var. aureus, Micrococcus lysodeikticus, Sarcina lutea and Bacillus megaterium. They reported that the permeability of the plasma membrane to a variety of sugars was too low to be measured; glutamic acid and glutamine did not penetrate the osmotic barrier to a significant extent. The cells were practically impermeable to a number of electrolytes including sodium chloride, potassium chloride, ammonium chloride, magnesium chloride and potassium bromide. These results constitute evidence in favor of the

existence of a plasma membrane of low permeability. However, M. pyogenes var. aureus was permeable to several thiocyanide salts and E. coli was not. All species were permeable to erythrol, pentaerythrol, D-ribose and glycerol. Mitchell and Moyle concluded that passive permeability exists for some substances.

Work with protoplasts of M. lysodeikticus and S. lutea yielded similar results (Mitchell and Moyle, $1956_c$) and also fragility studies have demonstrated that protoplasts of M. pyogenes are impermeability to sucrose and sodium chloride (Mitchell and Moyle, 1957).

Mitchell and Moyle (1953) investigated phosphorous uptake using $P^{32}$ labelled phosphate groups, and found that an exchange takes place across the cell surface of M. pyogenes var. aureus. Phosphorus uptake was studied in E. coli by Roberts and Roberts (1950) and was attributed to free diffusion, whereas Mitchell and Moyle prefer to explain the phenomenon on an active transport basis, theorizing carrier groups as ferry boats carrying an equal number of passengers in either direction. Mitchell and Moyle based their conclusion on the observation that the plasma membrane was impermeable to inorganic phosphate. Furthermore, since the plasma membrane contains the bulk of the cytochrome in M. pyogenes var. aureus (similar to B. megaterium (Weibull, 1953)), acid phosphatase, succinic dehydrogenase and lactic dehydrogenase (Mitchell, $1954_{a,b}$), Mitchell and Moyle view the plasma membrane "not only as a static barrier to the free diffusion of small molecular weight solutes, but also as a mosaic of specific small carriers which may allow the free movement of certain solutes with a specificity equal to that of enzyme-substrate combinations --- some of the carriers being enzymes themselves."

Obviously, there is disagreement in the data and conclusions of Mitchell and Moyle and of Roberts, et al. It should be noted, however, that most of the experiments cited involved different organisms and different techniques. Gram positive micrococci as studied by Mitchell and Moyle may exhibit vastly different membrane phenomena than the gram negative E. coli studied by Roberts and coworkers. This is supported by the fact that gram positive organisms do not lyse readily in hypertonic solutions, but may shrink, while gram negative organisms are more sensitive and lyse readily in non-isotonic environments (Knaysi, 1951).

Weibull ($1955_a$) found the osmotic barrier of B. megaterium to be impermeable to phosphate when only endogenous respiration was taking place, but a net transfer of phosphate could be detected when glucose was metabolized. He located this permeability barrier at the protoplasmic surface. Protoplasts prepared from B. megaterium were observed to lyse under adverse osmotic conditions, whereas whole cells were uneffected (Weibull, $1955_b$). This further indicates that this species has a selectively permeable osmotic barrier and confirms its location at the protoplasmic surface. Furthermore, Weibull (1956) examined the "ghosts" which remain after lysis of the protoplasts and is of the opinion that the "ghosts" are actually intact plasma membranes.

Rothstein (1956), in a review of investigations on yeasts, concluded that the cell surface of yeast cells contains enzymes, permeability barriers, cation binding sites and active transport mechanisms: the cell surface reactions involved in the absorption of sugars were shown to possess many of the properties of enzyme reactions.

Large quantities of potassium are taken up by yeast cells during

active metabolism; only slow diffusion outward occurs in the absence of substrate (Rothstein, 1955). Sodium is also concentrated in actively metabolizing cells, but in the presence of potassium, sodium is retained by a factor of only about 1 to 20 (Conway, 1954).

An active transport system for glucose in yeast is indicated by Rothstein (1954). Two such systems have been observed, one operating only under aerobic conditions. The kinetics and temperature effects of these reactions are characteristic of enzymes.

Using specific decarboxylases for amino acid assay, Gale ($1945_{a,b}$) investigated the assimilation of amino acids by bacteria. It was discovered that certain amino acids were accumulated in a free state within cells of Streptococcus faecalis (Gale, 1947) and could be released for analysis by subjecting cell suspensions to 100 C for 10-15 minutes. Using these techniques, Taylor (1947) carried out an investigation of free glutamic acid and lysine content of a wide variety of organisms. The organisms containing free amino acids were all gram positive, and staphylococci appeared to have the highest internal concentrations of glutamic acid.

Mathieson and Catcheside (1955) demonstrated the same phenomenon with the mold Neurospora, and observed that some amino acids interfered with the uptake of others. For example, arginine interfered with the structurally related compound, histidine. Cohen and Rickenberg (1955) found extensive intracellular concentration of valine in non-growing E. coli. With an energy source such as succinate, the intracellular concentration of valine often was increased 100 times that of the environment.

According to Gale (1947) lysine enters the cells by free diffusion, since an appreciable amount was taken up at 0 C and smaller amounts were taken up in the presence of an energy source. Inhibition of lysine entry in E. coli by glucose has also been reported by Mandelstam (1956). Gale further observed that the equilibrium concentration of lysine within cells was directly proportional to the concentration in the medium, an indication that active transport mechanisms are absent. The entry of lysine into M. pyogenes var. aureus and S. faecalis was uneffected by the presence of common metabolic inhibitors such as cyanide, iodoacetate, fluoride, azide, or 2,4-dinitrophenol. Lysine does not diffuse out of micrococci except in the presence of an energy source.

Gale found subsequently (1954) that investigations with radioactive lysine gave slightly different results. An increased uptake by M. pyogenes var. aureus could be observed in the presence of glucose, and this was decreased appreciably by 2,4-dinitrophenol. However, the rate and amount of lysine taken into cells was significant in the absence of glucose.

Glutamic acid is not assimilated by S. faecalis or M. pyogenes var. aureus except in the presence of an energy source such as glucose (Gale, 1947; Taylor, 1947); only insignificant amounts were accumulated at 0 C in the presence of an energy source. The rate and amount of uptake were independent of the concentration of glutamic acid in the medium except at very low concentrations. Accumulation was inhibited by 2,4-dinitrophenol in concentrations less than that required to inhibit glycolysis (Gale, 1951). Glutamic acid diffuses out of cells very slowly in water or saline and not at all if an energy source is present.

Clifton (1946) has shown that oxidative assimilation of carbon substrates is inhibited by sodium azide and 2,4-dinitrophenol. These materials appear to act in mammalian and yeast cells by uncoupling the generation of energy-rich phosphate bonds from oxidative processes (Loomis and Lipmann, 1948). According to Gale (1951), it is possible that the accumulation of free glutamic acid is dependent upon a coupled phosphorylation, and that the inhibitory action of these compounds is due to their uncoupling action.

Gale (1953) concluded that either glutamic acid is converted into a derivative which can penetrate the cell barrier and can be re-converted into glutamic acid, or that a carrier substance exists, which, in the presence of glucose, combines with glutamic acid and transports it across the barrier. This type of hypothesis was first offered by Osterhaut (1952) to explain the accumulation of materials within plant cells and Mitchell ($1954_{a,b}$) postulated a similar carrier system for phosphate in Micrococcus. Gourley (1952) suggested that adenosine triphosphate (ATP) may be the intermediate which carries phosphate across the membranes of erythrocytes, because isotopic inorganic phosphate was found to enter the internal ATP fraction faster than the internal inorganic phosphate fraction.

Evidence has been accumulated which points toward enzymes as the basis of many active transport mechanisms. Davis (1956), reporting on the work of Green, described experiments using Aerobacter aerogenes and 1,5 labelled radioactive citrate. It was found that glucose was utilized preferentially over citrate, and that an adaptive period elapsed prior to utilization of citrate. Since A. aerogenes is capable of utilizing

citrate as a carbon source, enzymes for its metabolism should be constitutive and present at all times. Thus the adaptation period was believed to involve the development of some system for rendering exogenous citrate accessible to the enzymes which ordinarily act upon endogenous citrate. Furthermore, the conditions necessary for this induction to citrate utilization were those necessary for net protein synthesis. Halvorson and Spiegelman,(1952) have shown that the formation of inducible enzymes requires net protein synthesis. In experiments in which a mixture of glucose and radioactive citrate served as carbon source Davis also demonstrated that the citrate was not utilized until the glucose was exhausted; also, under conditions where citrate was the sole carbon source, the utilization of citrate ceased upon the addition of glucose. Since the enzymes were present for citrate utilization, the interference by glucose was thought to involve the citrate transport system. Analyses of the supernates of heavy suspensions of cells revealed that citrate-grown cells removed citrate from the medium at a much higher rate than comparable glucose-grown cells.

Monod (1956) and Rickenberg, et al., (1956) discovered that the induction of beta-galactosidase in E. coli is preceeded by an inducible mechanism for the intracellular concentration of beta galactose. They called this the "y" system and postulated that such a system may exist for each type of carbohydrate metabolized by a given organism.

Another inducible permeability mechanism has been described recently by Pardee (1957). Strains of E. coli, not previously exposed to melibiose did not permit entry of this sugar. After exposure to melibiose, galactose, and other galactosides, one strain could concentrate

any of these sugars at an internal concentration of at least 20 times that of the medium. The sugars could be recovered unchanged from the bacteria. It was concluded that the accumulation mechanism was an inducible enzyme-like system probably similar to the "y" system of Monod and Rickenberg.

It can be seen from these observations that no theories of simple diffusion, pores, lipid layers or emulsions could be a suitable and complete explanation for the differential permeability of living cells. There are mechanisms of active accumulation and certain of these appear to be enzymatic and are probably inducible. It is conceivable that a permeability alteration could be responsible for resistance to deliterious substances. Indeed, many unknown undesirable or toxic materials may be screened out by this means.

Certain characteristics (e.g., differences in salt tolerance) of chloramphenicol-sensitive and -resistant strains of M. pyogenes var. aureus suggested the possibility of differences in permeability features. The present investigation was undertaken in order to determine whether or not permeability differences exist between sensitive and resistant strains, and their relationship to chloramphenicol resistance.

# CHAPTER II

## MATERIALS AND METHODS

The organisms used throughout were a strain of Micrococcus pyogenes var. aureus which is sensitive to chloramphenicol (Strain S) and a resistant mutant (variant) (Strain R). Strain S was obtained from the culture collection of the Department of Microbiology, University of Oklahoma Medical Center. Strain R was obtained by Ramsey and Padron (1954) by serial subcultivation of Strain S in media containing increasing concentrations of the antibiotic (Demerec, 1945). Resistance of Strain R was approximately 100 times that of Strain S. Strain R is non-pigmented and, in general, is slower to develop in culture than Strain S, but eventually produces luxuriant growth even in the presence of 500-800 ug chloramphenicol per ml.

Working stock cultures of Strain S were maintained on Difco AC agar. Strain R was maintained on AC agar containing 500 ug chloramphenicol per ml. These were transferred to fresh medium at monthly intervals.

### Salt Experiments

The basal medium containing Difco casamino acids, salts, vitamins and glucose (described by Ramsey and Padron, 1954) was prepared in four times the desired final concentration. This allowed for the addi-

tion of various test materials from concentrated stock solutions and/or water to obtain a final volume of 5 ml per tube. Glucose was autoclaved separately and added aseptically to each tube of the sterile cooled medium. This eliminates any error due to caramelization and precludes spurious results from uncontrollable stimulatory products of the heat degradation of glucose (Ramsey and Lankford, 1956). Riboflavin was omitted from this medium because of its toxicity for the test organisms (Wilson, 1957; Ramsey and Wilson, 1957).

Solutions of chloramphenicol, NaCl, KCl, $NH_4Cl$, $MgCl_2$ and $FeCl_2$ were prepared in water and added to each tube in the proper amounts to achieve the desired final concentration.

The resulting tubed medium was clear and colorless, and therefore suitable for the determination of growth by nephelometry using a Bausch and Lomb "Spectronic 20" photoelectric colorimeter with a wavelength setting of 525 mμ.

Inocula were harvested by centrifugation of 20-24 hour AC broth cultures, washed twice in the basal salt solution, resuspended and diluted to the desired concentration. All tubes of the test series were inoculated with one drop of the suspension as delivered by a 25 gauge hypodermic needle held with the bevel horizontal. All incubations were at 37 C.

Salt tolerance was enhanced by serial transfers in increasing concentrations of the desired salt in the basal medium until no further growth occurred.

## Amino Acid Experiments

Inoculum cells were grown for 18-22 hours in a medium developed and modified by Gale ($1945_a$, 1947). The medium contains salts, vitamins, glucose and "marmite" and is designed to produce cells which are deficient in amino acids. In the experiments described herein the medium was further modified by substituting yeast extract (Difco) for "marmite" (a yeast autolysate) and omitting riboflavin (Wilson, 1957). One ml of broth culture was used to inoculate Roux bottles containing 100 ml of the same medium supplemented with 2 per cent agar. After 18-22 hours the growth was harvested in 10 ml of the sterile salt mixture used for the diffusion experiments (Gale, 1947), washed once, recentrifuged, and resuspended in 1 ml sterile water to make a heavy suspension. For some experiments the cells were "starved" in the wash suspension for 2-4 hours. For others the wash suspensions were aerated for 2-4 hours by bubbling air passed through sterile cotton.

Diffusion experiments were carried out in tubes containing 5 ml of the sterile diffusion salt mixture to which amino acids and other test materials were added.

The amino acids used were DL lysine-1-$C^{14}$ (specific activity 0.83 mc/mM) and DL glutamic acid-2-$C^{14}$ (specific activity 0.47 mc/mM obtained from the California Foundation for Biochemical Research, Los Angeles, California. When desired, a sterile glucose solution was added to give a final concentration of 0.5 per cent. Final concentration of the amino acids was 100 ug per ml.

Sterile solutions thus prepared were dispensed into screw-

capped tubes and were allowed to equilibrate in a water bath at 37 C or in a salt-ice bath at 0 to 2 C. Two-tenths ml of the thick cell suspension was added to each tube and to tared cups for the determination of dry weight. One ml aliquots were removed at various times and centrifuged immediately for 6 minutes at 16,000 rpm in a refrigerated centrifuge. The supernatants were retained for analysis and the cells were washed immediately in 1 ml ice-cold water, and resuspended in 2 ml water. One ml of this suspension was saved for analysis as "whole cells." One ml was placed in boiling water for 10-15 minutes to release the "free amino acid pool" (Gale, 1947). After boiling, the suspensions were centrifuged and the supernatants, containing the released amino acids, were decanted and the sediments washed once with water and resuspended in 1 ml water for analysis as "bound amino acids."

The radio activity of the various components was used to estimate the quantity of amino acids present. Aliquots of 0.15 ml were placed upon aluminum planchets and spread to cover a circle of 2.? cm in diameter. The radioactivity was measured with a 1.9 mg/sq cm end window Geiger counter in a lead shield. Results are expressed as counts per minute per mg cells (dry weight).

## Chloramphenicol Absorption

Organisms were grown in Roux bottles on AC agar containing 5 and 500 ug chloramphenicol per ml for Strain S and Strain R respectively. Due to the disparity in growth rate of the cultures in the presence of chloramphenicol, Strain S was harvested at 36 hours and Strain R at 72 hours. The growth was harvested, washed and concentrated into a thick

suspension as described above. Aliquots of the suspension were added to tubes containing varying concentrations of chloramphenicol in the diffusion salt mixture or the basal medium previously described. After incubation, the supernatants, obtained by centrifugation, were assayed for chloramphenicol using a strain of Escherichia coli obtained from the culture collection of the Department of Microbiology, University of Oklahoma Medical Center. Assays were made using the basal medium containing aliquots of the chloramphenicol test solutions. Growth was determined by nephelometry as described above and was compared with standard curves obtained from growth in known concentrations of chloramphenicol.

# CHAPTER III

## RESULTS

Wilson (unpublished) originally observed that adjustment of a 30 per cent solution of yeast extract to pH 3.0 followed by neutralization resulted in a loss of activity as measured by chloramphenicol antagonism. It seemed possible that the loss of activity could be a reflection of the increased salinity of the medium brought about by the manipulations necessary for pH adjustment. Accordingly, experiments designed to test this possibility were performed in which the effect of varying salt concentrations on a chloramphenicol-sensitive strain (Strain S) and its resistant counterpart (Strain R) were determined.

### Effect of Increasing Concentrations of Salts upon Strains S and R

The organisms were inoculated into the basal medium containing increasing concentrations of sodium chloride, potassium chloride, ammonium chloride, magnesium chloride, ferric chloride, and glucose. It may be seen that potassium chloride and sodium chloride were more inhibitory toward Strain R than they were toward Strain S (Figure 1). In certain instances this difference was quite marked. For example, 0.8 M potassium chloride was completely inhibitory for Strain R whereas a 1.8 M concentration was required to inhibit Strain S. Ammonium chloride was slightly less inhibitory than potassium chloride or sodium chloride

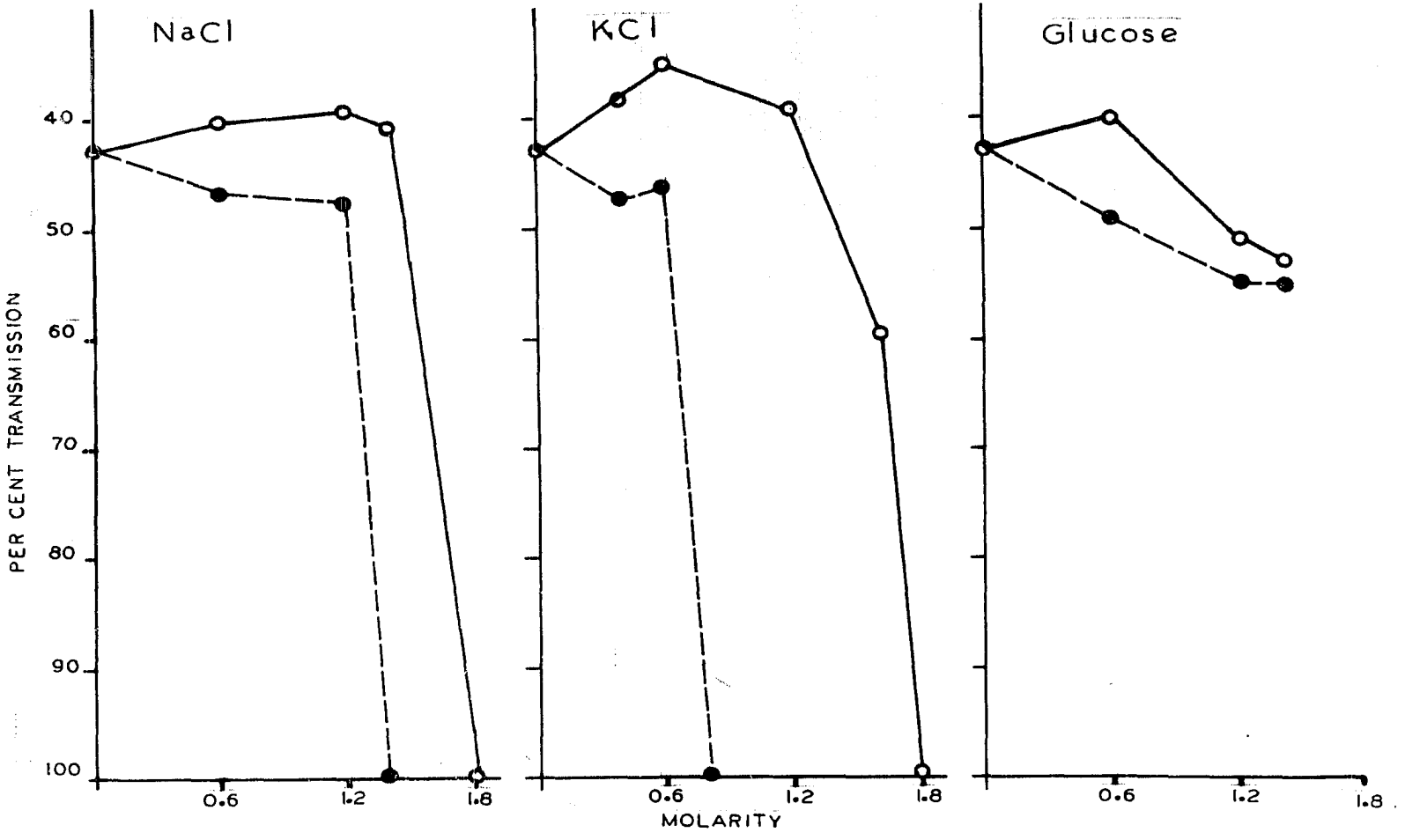



Figure 1. The Tolerance to Sodium Chloride, Potassium Chloride, and Glucose of Micrococcus pyogenes var. aureus, Strain S (solid lines) and Strain R (broken lines).

whereas magnesium chloride was more inhibitory. However, precipitation of the latter upon autoclaving made growth measurements by nephelometry impossible. Ferric chloride was toxic to both strains in the lowest concentration tested (0.2 N). By subculture it was determined that 0.6 N magnesium chloride demonstrated primary toxicity toward Strain R, while 1.0 N was required to render Strain S non-viable.

The difference in tolerance to glucose is insignificant; however it should be noted that the osmotic pressure of glucose at the highest concentration tested is only about one-half that of the salts. Comparisons of osmotic pressures are difficult to make since satisfactory methods for the determination of the osmotic pressure of high salt concentrations are not available.

## Effect of Increased Salt Tolerance upon Resistance to Chloramphenicol

Since Strain R demonstrated a decrease in salt tolerance it was of interest to determine whether an inverse relationship existed between resistance to salt and to chloramphenicol. In order to examine this possibility the salt tolerance of Strain R was increased by serial transfer in the basal medium containing increasing concentrations of either potassium chloride or sodium chloride. In sodium chloride, tolerance was increased to 2.0 M and in potassium chloride to 2.2 M. This required nine transfers and represented the maximum which could be obtained. Increased tolerance to one of these salts conferred increased tolerance to the other.

Figures 2 and 3 show that an increased salt tolerance resulted

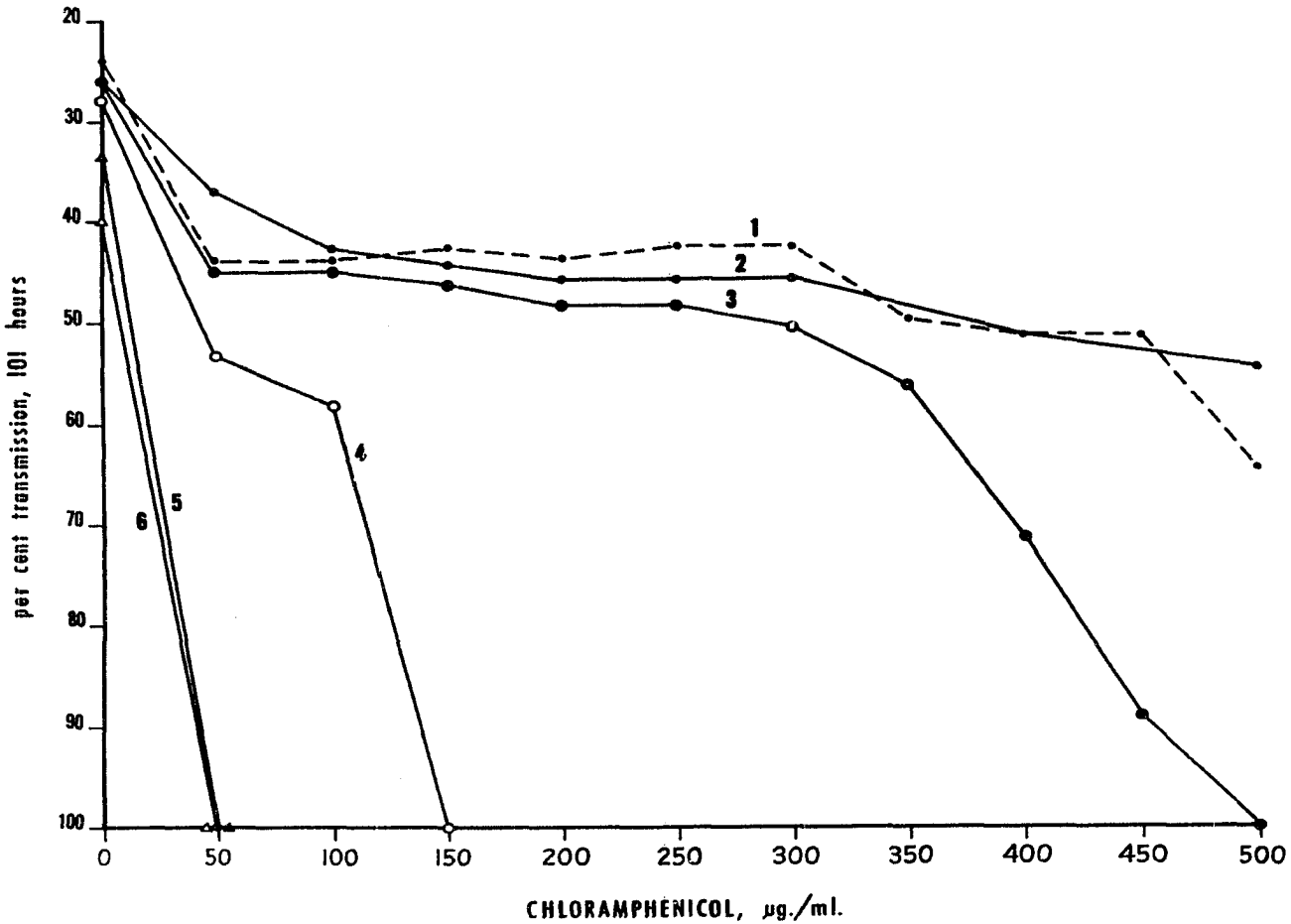



Figure 2. The Response of Strain R to Chloramphenicol Before and After Enhancement of Tolerance to Sodium Chloride. 1. Strain R after nine transfers in the basal medium (control). 2. Strain R (stock strain). 3. Strain R after nine transfers in increasing concentrations of sodium chloride. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M and 2.0 M added sodium chloride, respectively.

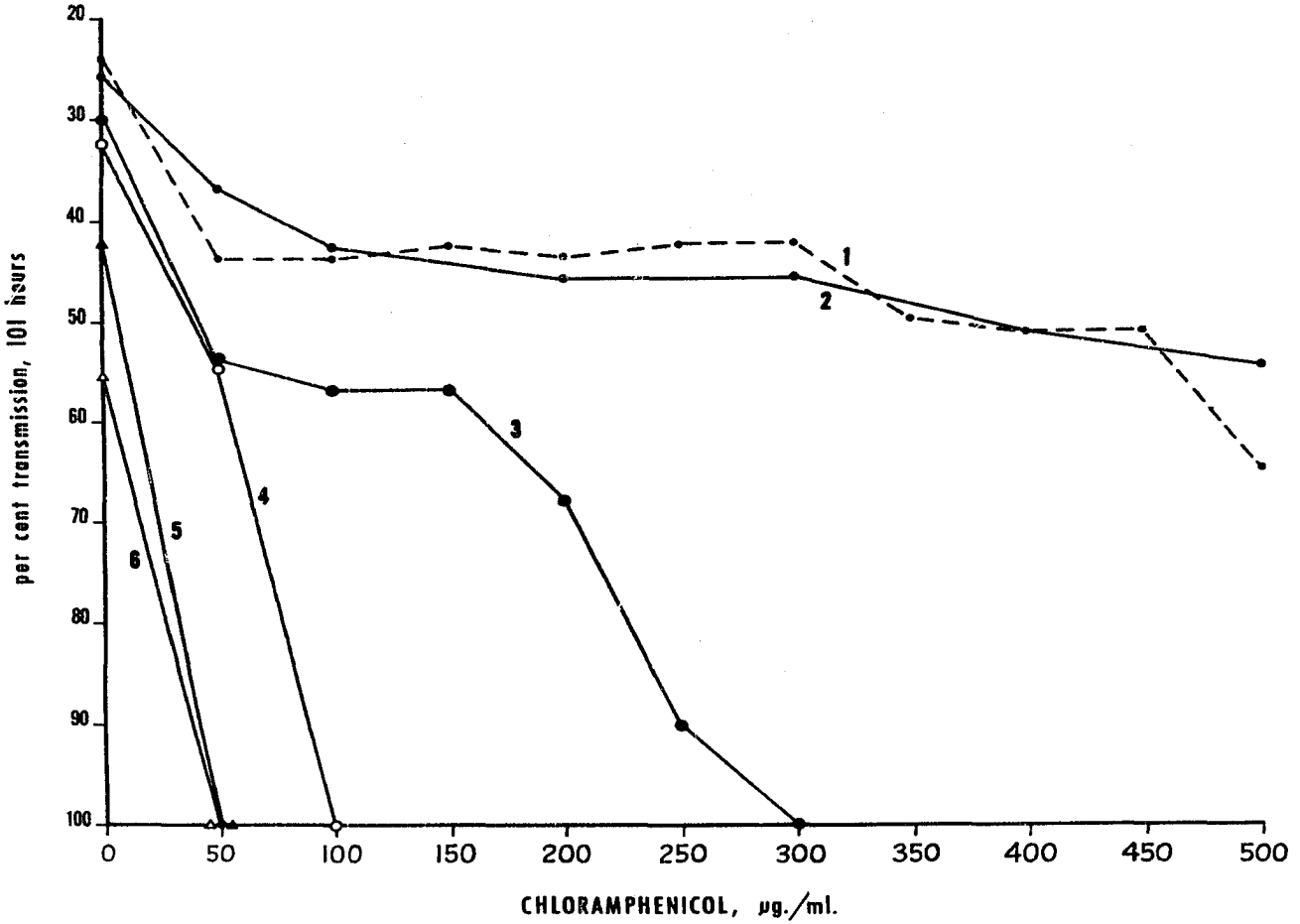



Figure 3. The Response of Strain R to Chloramphenicol Before and After Enhancement of Tolerance to Potassium Chloride. 1. Strain R after nine transfers in basal medium (control). 2. Strain R (stock strain). 3. Strain R after nine transfers in increasing concentrations of potassium chloride. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M and 2.0 M added potassium chloride, respectively.

in a marked decrease in chloramphenicol resistance. Strain R was only slightly inhibited by 450 ug of chloramphenicol per ml, but the sodium chloride tolerant strain was markedly inhibited at this concentration and the potassium chloride tolerant strain was completely inhibited by 300 ug per ml. Control cultures, transferred nine times in the absence of salts demonstrated no significant alteration in chloramphenicol resistance. Since Strain R had been shown to retain resistance to chloramphenicol after 25 transfers in AC broth in the absence of the drug (Ramsey and Padron, 1954) it would appear that transfer in the presence of salt results in selection of a strain with increased salt tolerance and a parallel decreased resistance to chloramphenicol.

Figures 2 and 3 also indicate a striking combined effect of chloramphenicol and salts upon the salt tolerant strains. This observation, as well as the failure of attempts to enhance salt tolerance in the presence of subinhibitory concentrations of chloramphenicol, suggested that their combined effect should be investigated. Experiments designed to test this effect were performed by measuring the growth of Strains S and R in various combinations of both salts and chloramphenicol (Tables I and II). The effects of the two inhibitory agents appear to be additive in most concentrations and are often synergistic. For example, 0.8 M potassium chloride failed to inhibit Strain S and 2 ug chloramphenicol per ml resulted in only 4 per cent inhibition; a combination of 0.8 M potassium chloride and 2 ug chloramphenicol per ml inhibited growth by 42 per cent at 36 hours. It should be noted, however, that these effects are relative to time of incubation since the same concentrations suppressed growth by only 8 per cent after 90 hour incubation. It would appear,

TABLE I

THE COMBINED EFFECTS OF SODIUM CHLORIDE AND CHLORAMPHENICOL ON

MICROCOCCUS PYOGENES var. AUREUS

STRAIN S

| | Chloramphenicol concentration, ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 2 | | | 4 | | |
| M. NaCl | 0.0 | 0.8 | 1.4 | 0.0 | 0.8 | 1.4 | 0.0 | 0.8 | 1.4 |
| Time in hours | | | | | | | | | |
| 30 | 0* | 0 | 21 | 6 | 48 | 100 | 45 | 100 | 100 |
| 47 | 0 | 0 | 9 | 3 | 15 | 63 | 13 | 82 | 100 |
| 71 | 0 | 0 | 6 | 6 | 17 | 24 | 14 | 26 | 86 |
| 95 | 0 | 0 | 7 | 7 | 8 | 20 | 15 | 19 | 58 |

STRAIN R

| | Chloramphenicol concentration, ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 100 | | | 200 | | |
| M. NaCl | 0.0 | 0.6 | 1.2 | 0.0 | 0.6 | 1.2 | 0.0 | 0.6 | 1.2 |
| 30 | 0 | 11 | 40 | 40 | 30 | 100 | 73 | 100 | 100 |
| 47 | 0 | 12 | 32 | 27 | 27 | 100 | 35 | 51 | 100 |
| 71 | 0 | 13 | 28 | 21 | 24 | 75 | 27 | 36 | 100 |
| 95 | 0 | 15 | 28 | 23 | 25 | 56 | 28 | 33 | 100 |

* per cent inhibition

TABLE II

THE COMBINED EFFECTS OF POTASSIUM CHLORIDE AND CHLORAMPHENICOL ON MICROCOCCUS PYOGENES var. AUREUS

STRAIN S

| | Chloramphenicol concentration, ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 2 | | | 4 | | |
| M. KCl | 0.0 | 0.8 | 1.4 | 0.0 | 0.8 | 1.4 | 0.0 | 0.4 | 1.2 |
| Time in hours | | | | | | | | | |
| 36 | 0* | 0 | 23 | 4 | 42 | 100 | 65 | 92 | 100 |
| 42 | 0 | 1 | 14 | 1 | 30 | 100 | 41 | 84 | 100 |
| 68 | 0 | 0 | 8 | 5 | 5 | 45 | 9 | 15 | 100 |
| 90 | 0 | 8 | 13 | 3 | 8 | 18 | 13 | 13 | 100 |

STRAIN R

| | Chloramphenicol concentration, ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 100 | | | 200 | | |
| M. KCl | 0.0 | 0.4 | 0.6 | 0.0 | 0.4 | 0.6 | 0.0 | 0.4 | 0.6 |
| 36 | 0 | 16 | 25 | 40 | 90 | 100 | 70 | 100 | 100 |
| 42 | 0 | 16 | 22 | 35 | 70 | 100 | 55 | 100 | 100 |
| 68 | 0 | 8 | 20 | 22 | 29 | 90 | 29 | 57 | 100 |
| 90 | 0 | 7 | 18 | 22 | 30 | 47 | 27 | 35 | 90 |

* per cent inhibition

therefore, that both agents exert their primary effect on the initial stationary phase of growth.

## Combined Effects of Polymyxin B and Chloramphenicol

It was reported by Szybalski and Bryson (1952) that with E. coli, strain B/r, a 300 fold increase in chloramphenicol resistance was accompanied by a 100 fold decrease in resistance to polymyxin. The strains of Micrococcus studied here did not show the "collateral" sensitivity. Both strains were equally resistant to a high concentration of polymyxin (90 ug per ml) when this antibiotic was tested alone. However, polymyxin B, in combination with chloramphenicol, gave different results. In experiments similar to those just described it was observed that the chloramphenicol tolerance of Strain R was more drastically reduced by polymyxin than that of Strain S (Table III).

These results suggested that a comparative study of the permeability mechanisms of Strains S and R might be of value. The studies of Gale (1947) indicated that lysine enters gram positive organisms by free diffusion, whereas glutamic acid enters the cells only under conditions where an energy source is available and is considered to be an active transport mechanism.

## Permeability of Strain S and Strain R to Amino Acids

In an attempt to develop a satisfactory quantitative assay for lysine and glutamic acid, twenty chromatography experiments quantitated by densitometry proved this method to be unsatisfactory. Aliquots of supernatants and cell extracts were spotted on sheets of Whatman No. 1 filter paper and ascending chromatography was carried out using a butanol-

TABLE III

THE COMBINED EFFECTS OF POLYMYXIN B AND CHLORAMPHENICOL

ON MICROCOCCUS PYOGENES var. AUREUS

STRAIN S

| | Chloramphenicol concentration ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Polymyxin ug/ml | | | | | | | | | |
| 0 | 0* | 11 | 12 | 17 | 31 | 22 | 28 | 40 | 100 |
| 10 | 8 | 9 | 12 | 20 | 30 | 40 | 100 | 100 | 100 |
| 40 | 16 | 17 | 19 | 39 | 100 | 100 | 100 | 100 | 100 |
| 80 | 9 | 16 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

STRAIN R

| | Chloramphenicol concentration ug/ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 |
| Polymyxin ug/ml | | | | | | | | | |
| 0 | 0 | 12 | 17 | 21 | 26 | 17 | 17 | 28 | 28 |
| 10 | 0 | 21 | 28 | 100 | 100 | 100 | 100 | 100 | 100 |
| 40 | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

* per cent inhibition

acetic acid-water mixture (80:20:20) in closed chambers. Chromatograms were developed in a water-saturated butanol solution of 0.2 per cent ninhydrin. This method was not sufficiently sensitive to detect the small change in quantity of amino acid in solution after exposure to a heavy bacterial suspension. The quantity which could be extracted from cells by boiling was not measurable consistently.

Attempts to employ microbiological assays also yielded equivocal results. In four experiments in which Leuconostoc mesenteroides P-60 was used for lysine assay and Lactobacillus arabinosus 17-5 for glutamic the results obtained were no more consistent than that obtained by chromatography.

The use of isotopically labeled ($C^{14}$) amino acids produced excellent results, and allowed the measurement of radioactivity in whole cells as well as in extracts.

## Lysine

Sixteen experiments, conducted under a variety of conditions, indicated that following an initial lag of 90-105 minutes Strain R, assimilated considerably more lysine than Strain S particularly in the presence of glucose. The rate and quantity of lysine absorption were the same for both strains in only 3 instances. Moreover, the incorporation of lysine as measured by bound amino acid content, was consistently higher in Strain R (Figure 4B). At 0 C, with or without glucose, neither strain assimilated or bound a significant amount of lysine. It would seem that an active transport mechanism is responsible for the transfer of lysine into cells. This is supported by the observation

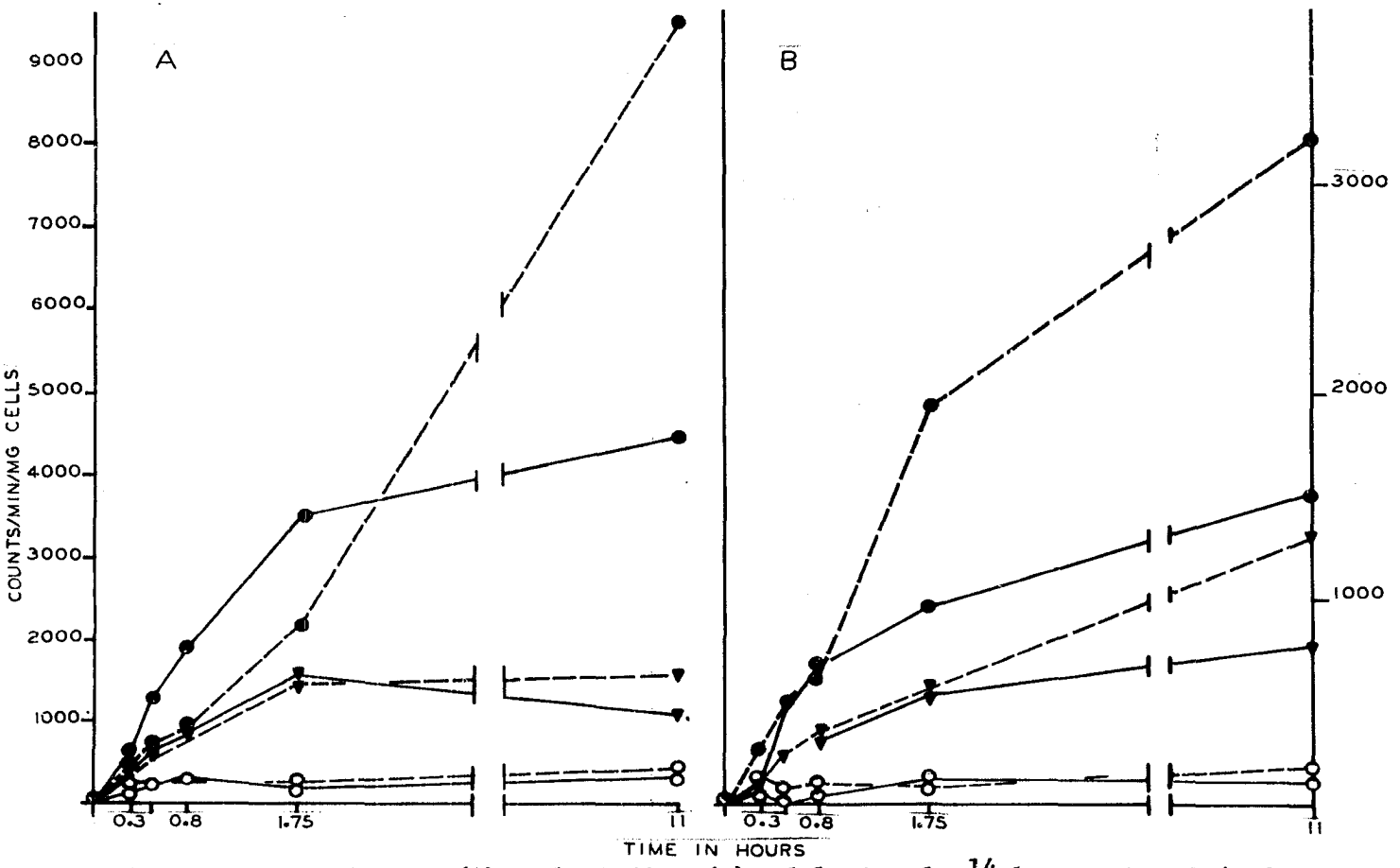



Figure 4. The Assimilation (A) and Binding (B) of lysine-1-$C^{14}$ by Strains S (solid lines) and R (broken lines) of Micrococcus pyogenes var. aureus. ▼——▼, diffusion salts mixture alone at 37 C; ●——●, diffusion salts mixture with 0.5 per cent glucose at 37 C; O——O, diffusion mixture with 0.5 per cent glucose at 0 C.

that 2,4-dinitrophenol, an agent which uncouples energy-yielding reactions from synthetic reactions, was inhibitory to lysine incorporation. The effect of dinitrophenol was more pronounced with Strain R and might indicate that a more active transport mechanism had been developed concomitantly with chloramphenicol resistance (Figure 5).

Assimilation and incorporation of lysine also occurred in the absence of glucose but at a lower level (Figure 4). Here the rate is approximately the same in both strains. This accumulation in the absence of an exogenous energy source may be due partially to diffusion, as stated by Gale (1947). However it would seem that here, too, active uptake is the main method of assimilation. This assimilation is probably the result of endogenous metabolism since, at 0 C, the rate and total amount of lysine incorporation is greatly reduced indicating, once again, the necessity of a source of energy (Figure 4). This interpretation is supported by the fact that lysine accumulation at 37 C, both in the presence and in the absence of glucose, is decreased by dinitrophenol (Figure 5).

## Glutamic Acid

Seventeen experiments were performed in order to measure the intracellular accumulation and binding of glutamic acid. Strain R invariably assimilated and incorporated more glutamic acid than Strain S under all conditions tested (Figure 6). This difference was slight in the absence of glucose, but very marked in its presence. According to Gale (1947) no glutamic acid is assimilated in the absence of an energy source. The significant accumulation without glucose which was

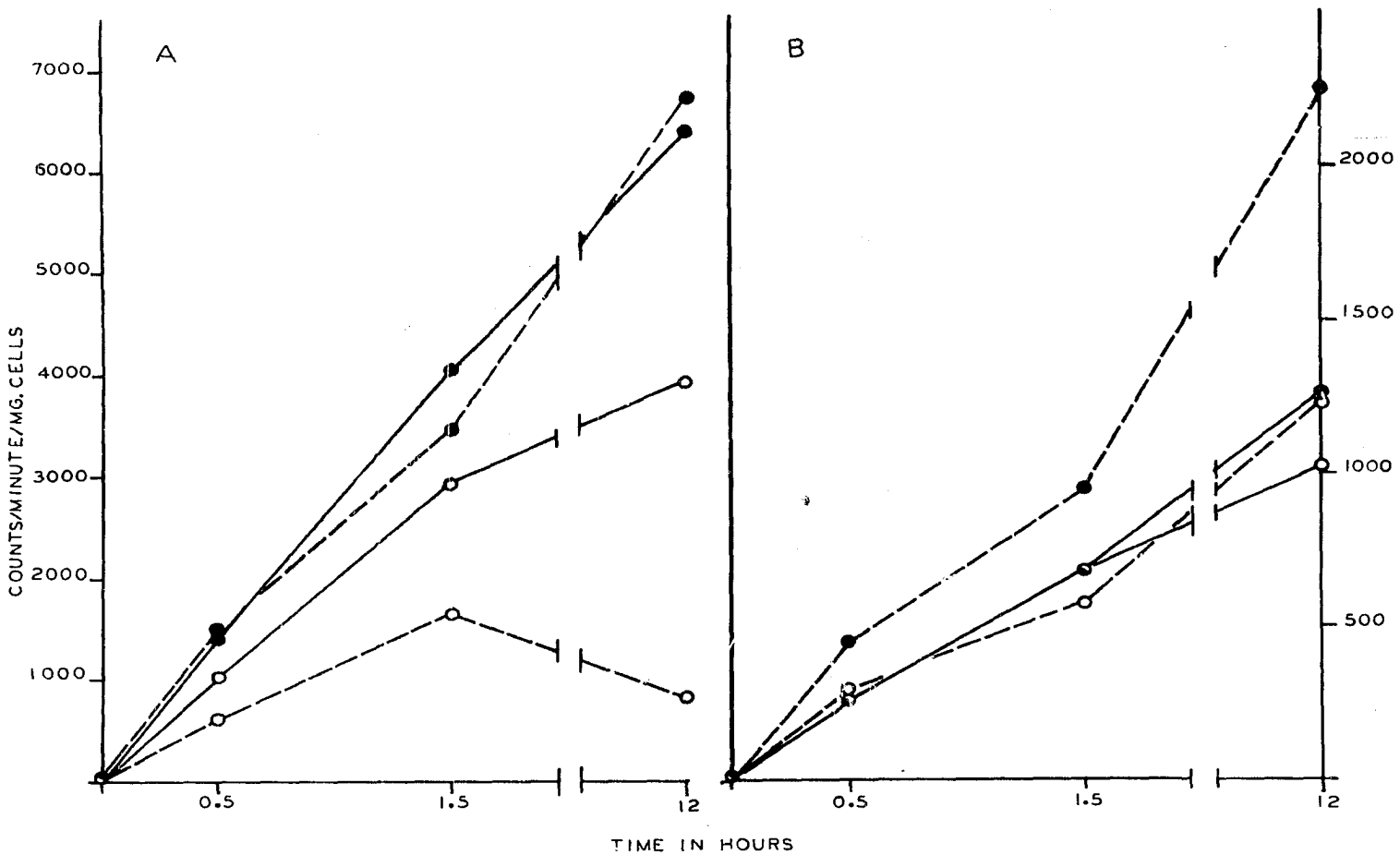



Figure 5. The Effect of 2,4-Dinitrophenol on the Assimilation (A) and Binding (B) of Lysine-1-$C^{14}$ by Strains S (solid lines) and R (broken lines) of Micrococcus pyogenes var. aureus. ●——●, diffusion salts mixture with 0.5 per cent glucose; O——O, diffusion salts mixture with 0.5 per cent glucose and 75 ug 2,4-dinitrophenol per ml. Incubation at 37 C.

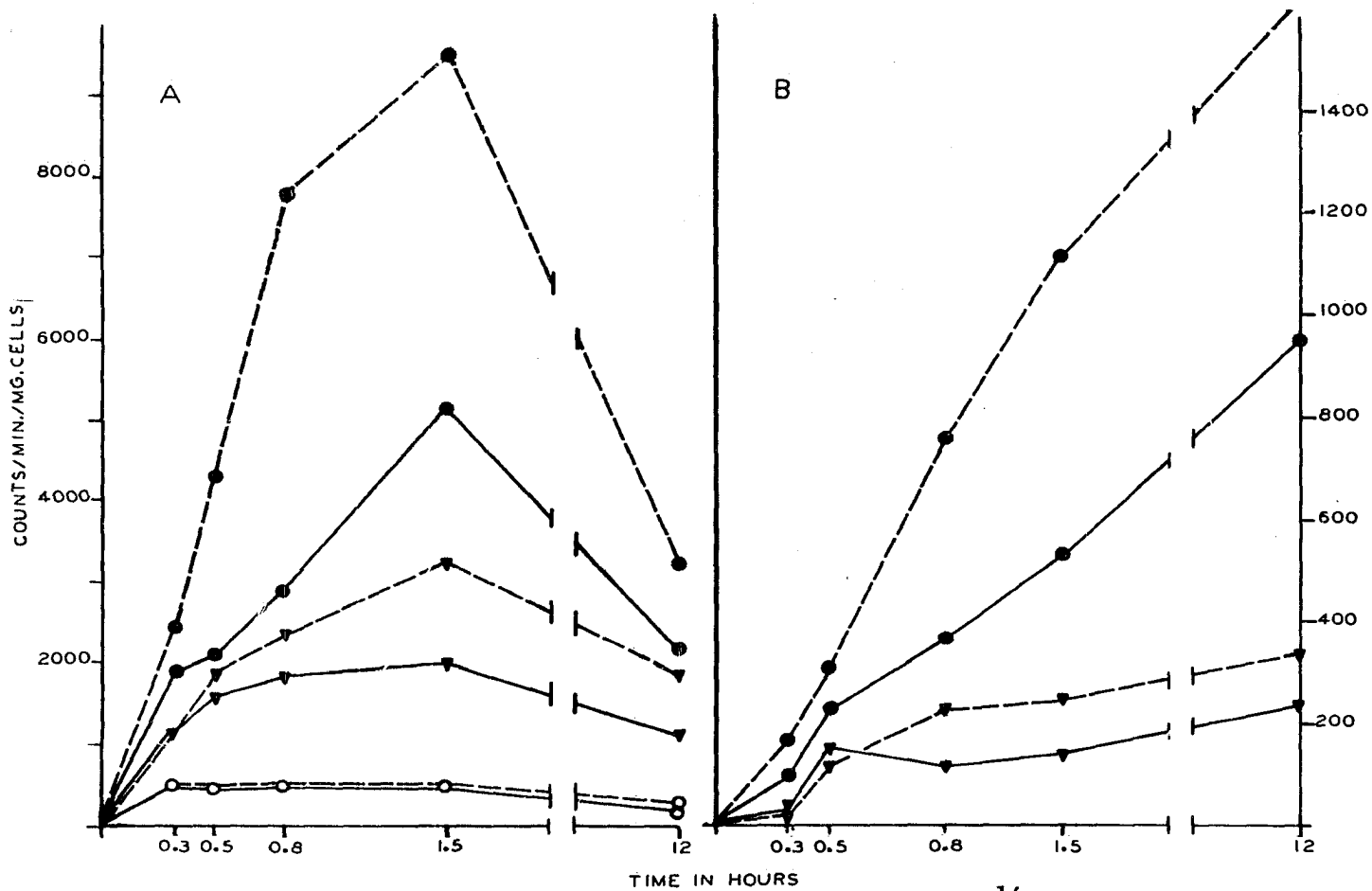



Figure 6. Assimilation (A) and Binding (B) of Glutamic Acid-2-$C^{14}$ by Strains S (solid lines) and R (broken lines) of Micrococcus pyogenes var. aureus. ▼——▼, diffusion salts mixture alone; ●——●, diffusion salts mixture with 0.5 per cent glucose at 37 C; O——O, diffusion salts mixture with 0.5 per cent glucose at 0 C.

observed here was probably due to endogenous metabolism or to the metabolism of glutamic acid itself. The marked disappearance of glutamic acid from the suspending medium toward the end of the experimental period points toward its metabolism in the absence of glucose. Starving and aeration of suspensions of cells in the salt solution prior to addition to the amino acid solutions did not alter the rate or total amount of assimilation.

It may be seen that a maximum concentration of free intracellular glutamic acid was reached at approximately 1½ hours. Following this maximum, a decrease in concentration occurs so that at 12 hours the free amino acid pool concentration was only one third that of the maximum amount. This is probably the result of oxidative decarboxylation of glutamic acid since a corresponding increase in radioactivity appeared in the suspending medium. Gale (1947) noted a similar occurrence in Strep. faecalis, but only in the presence of an energy source, whereas less than 5 per cent of the internal glutamic acid was lost from M. pyogenes var. aureus in media containing glucose (Gale, 1953). It is probable that the two mechanisms are different, since in experiments not described herein it was found that Strains S and R oxidize glutamic acid.

In agreement with Gale (1951), 2,4-dinitrophenol decreased both the assimilation and incorporation of glutamic acid in the presence of glucose (Figure 7). In addition, a depression also occurred in the absence of glucose and was probably due to an inhibition of endogenous energy-yielding mechanisms.

Chloramphenicol, at a concentration of 1000 ug per ml, exerted only a slight inhibitory effect on the accumulation of glutamic acid

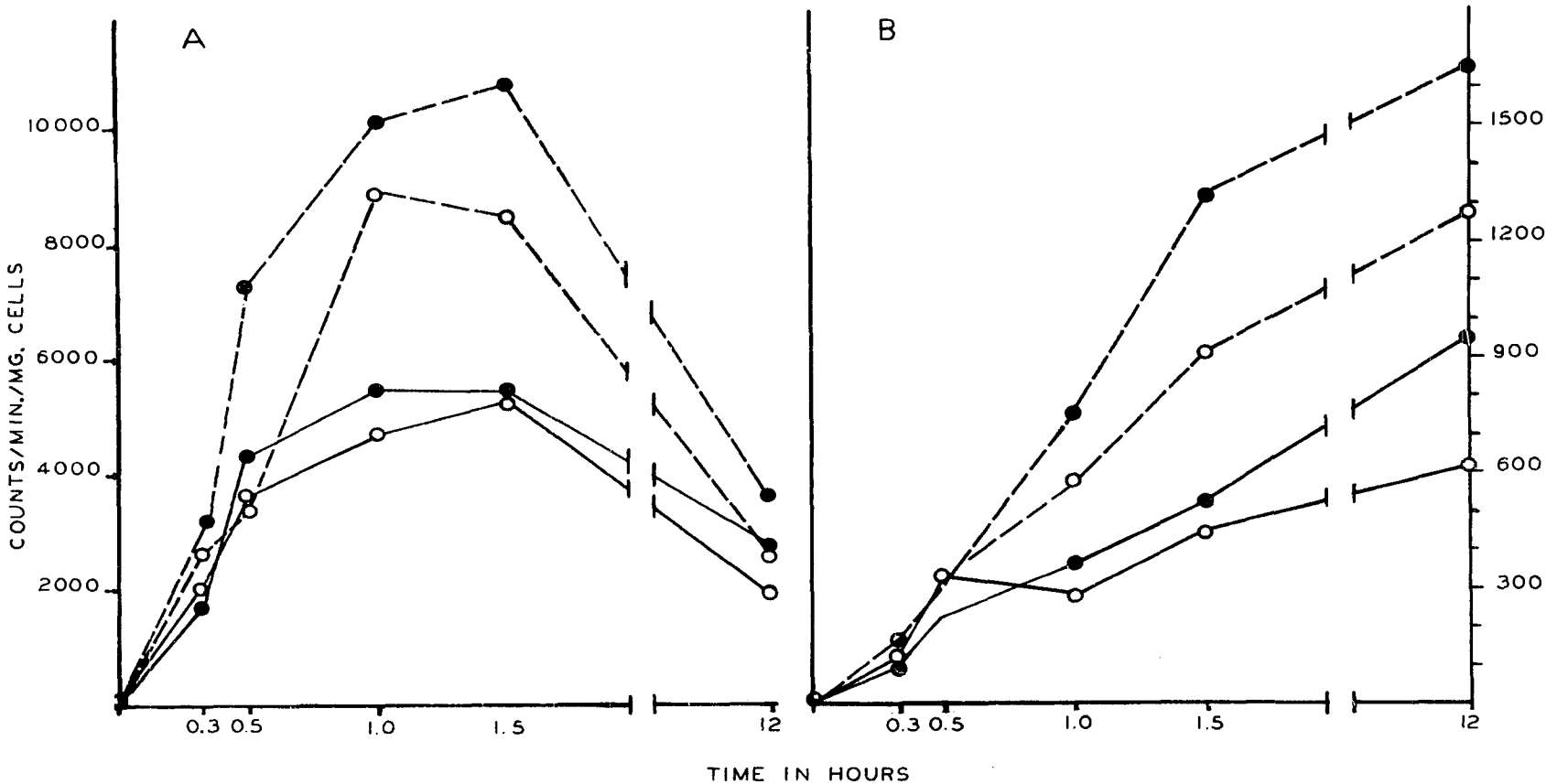



Figure 7. Effect of 2,4-Dinitrophenol upon the Assimilation (A) and Binding (B) of Glutamic Acid-2-$C^{14}$ by Strains S (solid lines) and R (broken lines) of Micrococcus pyogenes var. aureus. ●——● , diffusion salts mixture with 0.5 per cent glucose; O——O, diffusion salts mixture with 0.5 per cent glucose and 75 ug 2,4-dinitrophenol per ml.

(Figure 8A). However, a more pronounced inhibition of incorporation of the amino acid into the water-insoluble fraction of the cells was observed under these conditions (Figure 8B). This inhibition by chloramphenicol was more apparent in Strain R than in Strain S. This observation would indicate that chloramphenicol resistance is not related to an altered metabolism, involved in glutamic acid assimilation or incorporation, which is less susceptible to the antibiotic.

It may be observed that Strain S and Strain R appear to remove no chloramphenicol from solutions in the growth medium or in the diffudion salts mixture of Gale (1947) (Table IV). Under the conditions used, neither cellular uptake nor difference in uptake could be demonstrated. Therefore, it seems unlikely that an exclusion of chloramphenicol is the mechanism of resistance in Strain R.

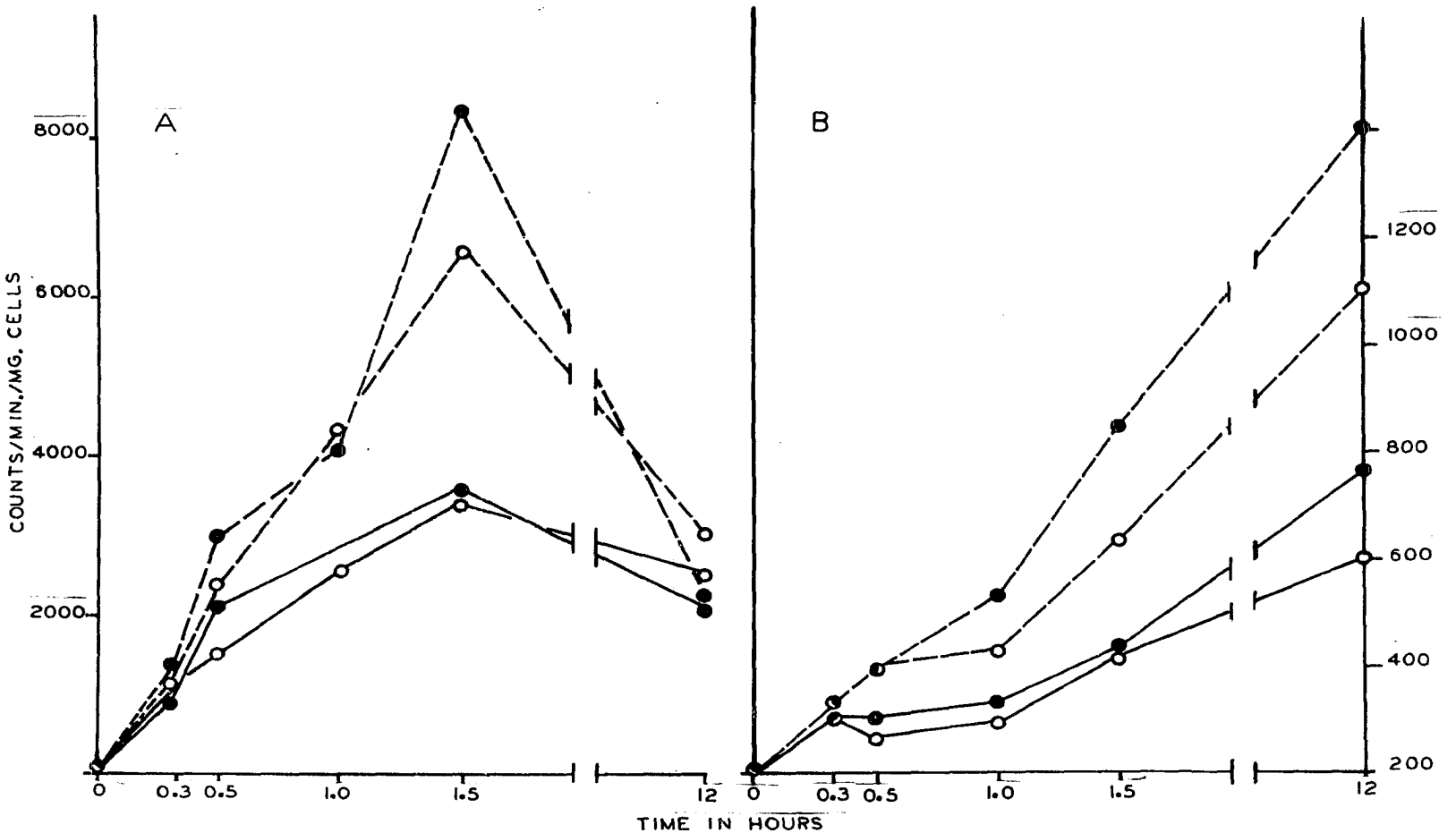



Figure 8. Effect of Chloramphenicol upon the Assimilation (A) and Binding (B) of Glutamic Acid-2-$C^{14}$ by Strains S (solid lines) and R (broken lines) of Micrococcus pyogenes var. aureus. ●——●, diffusion salts mixture with 0.5 per cent glucose; ○——○ diffusion salts with 1000 ug chloramphenicol per ml. Incubation at 37 C.

TABLE IV

THE ABSORPTION OF CHLORAMPHENICOL BY STRAINS S AND R

OF MICROCOCCUS PYOGENES var. AUREUS

| | Exp. A# | | | | Exp. B# | Exp. C+ |
|---|---|---|---|---|---|---|
| | 18 hrs. | | 25 hrs. | | 26 hrs. | 28 hrs. |
| Control | 8.5* | 145 | 8.5 | 145 | 95 | 6.0 |
| Strain S | 11.0 | 150 | 9.5 | 157 | 95 | 7.2 |
| Strain R | 11.0 | 155 | 9.7 | 150 | 100 | 7.2 |

* ug chloramphenicol per ml in control and test solutions following exposure to heavy suspensions of cells.

\# incubation mixture: diffusion salts

\+ incubation mixture: basal medium

# CHAPTER IV

## DISCUSSION

The inverse relationship between salt tolerance and chloramphenicol resistance is interesting and worthy of emphasis. This inverse relationship has been demonstrated in two separate ways. First, Strain S is noticeably more resistant to salt than its chloramphenicol resistant counterpart, Strain R. Second, cultivation of Strain R in increasing concentrations of salt results in an increased tolerance for salt which is accompanied by a decreased resistance to chloramphenicol. There are at least two possible mechanisms whereby such a phenomenon could occur. First, it might be anticipated that chloramphenicol interferes with some enzymatic sequence which is relatively resistant to salt. Alteration of this enzyme sequence as a result of chloramphenicol resistance could be accompanied by a decreased resistance to salt. Alternatively, the inverse resistance patterns might be due to altered permeability mechanisms. If salt and chloramphenicol gain access to the internal portion of the cell by different means, an increased activity of one mechanism might well be accompanied by a decreased activity of the other. This latter possibility is considered to be the more plausible since it has been demonstrated that Strain R has a more highly developed active transport mechanism than does Strain S. If one postulates that salt is

transported into bacterial cells by an energy-linked mechanism, this highly developed mechanism would actually work to the disadvantage of the organism in the presence of high concentrations of salt.

The mechanism of toxicity of salts for bacteria is only poorly understood. Sodium, potassium, and ammonium ions are the least toxic of all cations for E. coli (Winslow and Hotchkiss, 1922) and Hotchkiss (1923) found these cations stimulatory in low concentrations (0.25 M); toxic concentrations were 2.0 M for potassium and sodium chloride and 1.0 M for ammonium chloride.

Winslow and Falk (1923) and Winslow and Dolloff (1923), working with E. coli, observed that combinations of cations may be either additive or antagonistic. This work was expanded by Winslow and Haywood (1931) who believed that growth stimulation by low concentrations of cations was associated with increased permeability and the inhibition by higher concentrations was associated with decreased permeability to essential metabolites. (For a thorough review of earlier work, see Falk, 1923.)

While osmotic pressure may play a role in the toxicity of salt solutions, Spiegelberg (1944) believed this to be an inadequate explanation. He noted that Clostridium pasteurianum was inhibited by a sodium chloride solution exerting an osmotic pressure of only 18 to 38 atmospheres, whereas a sugar solution of 60 to 70 atmospheres was required for inhibition. Thus, he ascribes additional toxic characteristics to salts.

It has been recognized for some time that gram positive cocci are capable of growth in comparatively high salt concentrations. Hill

and White (1929) suggested culture media including sodium chloride in concentrations of 2 to 20 per cent for use in the selective isolation of gram positive cocci. Koch (1943) recommended that agar containing 7.5 per cent sodium chloride or broth containing 15 per cent sodium chloride be used as a selective medium for isolation of all "staphylococci." Chapman (1945) employed proteose-lactose agar containing 7.5 per cent sodium chloride for the isolation of micrococci from feces and found this to be superior to other methods. Furthermore, the maintenance of stock cultures on this medium resulted in enhancement of chromogenesis and coagulase production; also, degeneration and dissociation of cultures were less likely. Maitland and Martyn (1948) recommended a medium containing 10 per cent sodium chloride for the isolation of "staphylococci." Haynes, et al., (1954) reported that media containing less than about 0.04 per cent potassium were deficient for the growth of M. pyogenes var. aureus.

There is no satisfactory explanation for salt tolerance. Ingram (1947) has suggested that the proteins of extreme halophiles are less readily salted out. An example of this is the glycerol dehydrogenase of Pseudomonas salinaria which requires high concentrations of sodium or potassium chloride for maximum enzymatic activity (Baxter and Gibbons, 1954). Robinson and Katznelson (1953) described a transaminase from the same species which was most active at high salt concentrations. On the other hand the moderate halophile, Micrococcus halodenitrificans, maintains a low intracellular salt concentration by means of an energy-dependent mechanism (Robinson, et al., 1952). Baxter and Gibbons (1956),

using the same species, demonstrated several dehydrogenases which were most active at low salt concentrations. They suggested that the intracellular salt concentration of this organism was about 0.5 M, and could be maintained against a gradient. They designated as "moderate halophiles" organisms which grow in concentrations of 1 or 2 to about 20 per cent sodium chloride.

The results reported here confirm the tolerance of a strain of M. pyogenes var. aureus to high concentrations of potassium and sodium ions. These strains could be considered to be moderate halophiles. Further, it is shown that this tolerance is decreased with acquisition of resistance to chloramphenicol but can be restored easily by adaptation in the absence of chloramphenicol. The restoration is accompanied by a concomitant decrease in chloramphenicol resistance.

McVeigh and Hobdy (1952) found that two penicillin-resistant strains of M. pyogenes var. aureus had lost the ability to grow in proteose-lactose agar containing 7.5 per cent sodium chloride, while strains resistant to streptomycin, aureomycin, chloramphenicol, and subtilin retained the capacity to grow in this medium. The effect of restoring salt tolerance was not investigated.

The enhanced inhibitory action of combinations of sodium chloride, and chloramphenicol noted herein indicate that their separate actions may not be the same. No reports of synergism between salts and antibiotics have been found although there are several reports of the converse. For example, Weinberg (1954) noted that the inhibition of Ps. aeruginosa by oxytetracycline was strongly reversed by several multivalent cations; the inclusion of subinhibitory concentrations of

the antibiotic in the medium resulted in a slight reversal of the toxicity of bivalent ions. Berti (1950) found that calcium and magnesium ions antagonized the effect of streptomycin on E. coli and Soncin (1953) reported a similar antagonism between magnesium and aureomycin, terramycin and chloramphenicol. Grossowicz, et al., (1955) observed that sodium and potassium antagonized the inhibition of M. pyogenes var. aureus by spermine and spermidine. The action of tetracycline on Micrococcus pyogenes var. aureus and var. albus was reversed by manganese (Hamburger, et al., 1957).

It should be noted in the results presented here that even at salt concentrations which are slightly stimulatory, the addition of chloramphenicol results in more inhibition than the drug could produce alone. According to Winslow and Haywood (1931), stimulatory concentrations of salts are associated with increased permeability of cells. This could explain the enhancement of inhibition by chloramphenicol at low salt concentrations. However, the decreased permeability at high concentrations of salts, postulated by Winslow and Haywood, would not explain the greater enhancement of chloramphenicol activity.

It is believed that polymyxin exerts its bactericidal action as a surface active agent. Cohen, et al., (1954) noted that polymyxin had the same inhibitory action as quarternary ammonium detergents upon esterases derived from mycobacteria. Also, polymyxin-sensitive strains absorbed greater quantities of polymyxin than resistant strains (Few and Schulman, 1953), and a leakage of materials occurs from cells treated with polymyxin; this leakage is related to the percentage of killed cells. It

was concluded that polymyxin combined with and disorganized structures responsible for maintenance of the permeability barriers of the cells. These observations were confirmed and extended by Newton (1953, 1954) with Ps. aeruginosa, and Sussman and Lowry (1955) and Lowry and Sussman (1956) with ascospores of Neurospora.

Since polymyxin is a surface active agent and interferes with permeability, the observation that subinhibitory concentrations of this antibiotic enhance chloramphenicol activity is of interest. It should be noted that this effect is more drastic with Strain R. What relationship, if any, this altered permeability has to altered salt tolerance is unknown because reconstruction experiments using combinations of salt and polymyxin were not performed. It is of interest, however, that once again a distinct difference in permeability has been noted between Strains S and R. It is tempting to assume that this alteration in permeability is a direct reflection of chloramphenicol resistance. However it must be pointed out that definitive evidence for this is lacking.

Evidence for other differences in permeability between Strains S and R may be observed in the data from the amino acid assimilation experiments. Evidence of such a permeability barrier to chloramphenicol has recently been reported by Kushner ($1955_{a,b}$), who found that succinate oxidation by intact sensitive cells of Ps. fluorescens was easily inhibited by chloramphenicol whereas intact resistant cells were not inhibited. However, cell-free extracts of both strains were equally susceptible to the drug indicating that resistance depended upon a structural integrity of the cell. He also observed that polymyxin increased the

permeability of cells to chloramphenicol.

The lysine-assimilation experiments described herein point to an energy-linked transport mechanism and are somewhat at variance with the earlier work of Gale (1947) in which assimilation was attributed entirely to passive diffusion since the presence of glucose inhibited assimilation. It was also reported that dinitrophenol was without effect upon the entry of lysine into M. pyogenes var. aureus or Strep. faecalis (Gale, 1953). However, in later work using isotopically labeled lysine, it was concluded that lysine accumulation did increase in the presence of an energy source (glucose) and this increase was inhibited by dinitrophenol (Gale, 1954). Using resting yeast cells, this finding was confirmed by Davies, et al., (1953) who observed that lysine accumulation did not occur in the absence of glucose.

The enhanced accumulation and binding of lysine by Strain R points to an alteration in the metabolism and probably the permeability of this strain. Thus, by a third method, distinct differences in permeability mechanisms have been shown to exist in chloramphenicol sensitive and resistant organisms. The insignificant effect of chloramphenicol upon both assimilation and incorporation of lysine casts doubt that this alteration is directly related to chloramphenicol resistance. Gale and Folkes ($1953_a$) have said that chloramphenicol interferes with protein synthesis at an advanced stage of the process. By whatever mechanism lysine is bound, that mechanism is not affected to any appreciable extent by chloramphenicol and therefore probably does not represent true protein synthesis. It would be of interest to determine whether or not a storage mechanism, similar to that of the polyglutamate forms of folic

acid, might also exist for lysine.

The results with glutamic acid agree with many of Gale's observations. Here, again, it should be noted that Strain R has the ability to accumulate and bind considerably more glutamic acid than Strain S. This is in contrast to penicillin-resistant strains of M. pyogenes wherein a decreased ability to concentrate glutamic acid has been observed (Gale and Rodwell, 1949). The continuation of assimulation in the presence of growth inhibitory concentrations of chloramphenicol is of interest, but because protein synthesis was not measured, it can not be related to growth inhibition as Gale and Folkes (1953b) have done. Gale and Paine (1951) reported a 90 per cent inhibition of protein synthesis and a 10 per cent inhibition of glutamic acid accumulation in the presence of a quantity of chloramphenicol greatly in excess of that required for inhibition of growth. Using isotopically labeled glutamic acid, Gale and Folkes (1953b) observed that some "incorporation" of glutamic acid into cell protein occurred when M. pyogenes var. aureus was incubated with salts, glucose and the amino acid. These conditions do not allow net protein synthesis and the incorporation was attributed to exchange reactions between the cellular protein and the glutamic acid of the medium. This exchange was less sensitive to chloramphenicol than the uptake of glutamic acid under conditions which allowed net protein synthesis. Chloramphenicol is markedly inhibitory to protein synthesis, but only moderately inhibitory to the accumulation of glutamic acid (Gale and Paine, 1951). On the other hand, penicillin inhibits the accumulation of glutamic acid by M. pyogenes var. aureus but apparently

not its utilization (Gale and Taylor, 1947).

Working with young growing cells of M. pyogenes var. aureus Gale (1948) detected an increased concentration of free intracellular glutamic acid which occurred simultaneously with sulfathiazole resistance. This was ascribed to a decreased rate of protein synthesis which allowed accumulation of the amino acid. Clapper and Heatherman (1950) reported that no free glutamic acid could be shown in nine strains of viridans streptococci. After "training" to resistance to sulfathiazole, aureomycin and penicillin, free glutamic acid could be isolated from growing cells. It was suggested that accumulation occurred because the resistant strains had an altered metabolism which obviated the use of free glutamic acid from the environment and therefore it accumulated within the cells. Clapper and Heatherman based their suggestion on the previous observation of Harrison and Clapper (1950) that sulfathiazole-resistant strains of Streptococcus mitis required pteroylglutamic acid, whereas the parent strains did not. This was thought to be a possible explanation for part of the increase in glutamic acid, since the resistant strains may have lost the ability to synthesize pteroylglutamic acid from free glutamic acid. It was not known whether these strains utilized glutamic acid from the environment.

As has been discussed, the moderate halophiles grow in high salt concentrations by maintaining a low intracellular salt concentration. This process has been termed "active exclusion" (Robinson, et al., 1952). In the present studies, it may well be that Strain R is deficient in this mechanism while Strain S is not. It is interesting to note that when halotolerance is restored in Strain R by adaptation to

higher salt concentration, chloramphenicol resistance is decreased. This may indicate that permeability to salts or "active exclusion" is lowered in chloramphenicol resistance while permeability to the antibiotic is not changed.

The observations on active and passive transport of amino acids revealed more efficient mechanisms for concentrating and binding these compounds in Strain R than in Strain S. These mechanisms are slightly inhibited by chloramphenicol, but continue to operate in growth-limiting concentrations of the drug. It is concluded, therefore, that chloramphenicol does not exert its growth inhibiting effect by prevention of passage of the amino acid across the cell wall, concentration of the amino acid intracellularly or its incorporation into the water-insoluble fraction of the cell.

It has been shown by Foster and Pittillo (1953) that inhibition of growth by chloramphenicol can be "reversed" by the presence of a variety of complex materials such as Basamine, Wilson Liver B. Wilson and Ramsey (1955) have suggested that this reversal is not a direct antagonism of chloramphenicol by these substances, but results indirectly from the stimulation of growth of the organisms. It is tempting to theorize that chloramphenicol resistance of Strain R is due, in part at least, to its increased permeability and active transport mechanisms. The increased cellular permeability could result in a greater accessibility of possible stimulatory nutrients to the intracellular environment. Such a mechanism would endow the cells with a greater concentration and wider variety of preformed nutrients. Further, it could permit an increased rate of protein synthesis which would require a higher

concentration of chloramphenicol for inhibition. In support of this idea is the unpublished observation of Wilson that Strain R synthesizes inducible enzymes at a higher rate than Strain S. The slower growth of Strain R seems contradictory to this idea, but little is known about growth initiation and it may be that this phase of growth is not stimulated by the same material(s) as that promoting resistance to chloramphenicol. Such a possibility seems reasonable in view of the fact that an increase in cellular mass may proceed in the absence of cell division and that the two processes appear to be quite distinct (Nickerson and Sherman, 1952).

# CHAPTER V

## SUMMARY

A chloramphenicol-resistant strain of Micrococcus pyogenes var. aureus (Strain R) has been found less tolerant to salts than its chloramphenicol-sensitive counterpart (Strain S). The restoration of salt tolerance in Strain R by serial subculture in increasing salt concentrations resulted in a decrease in chloramphenicol resistance.

The combined inhibitory effects of chloramphenicol and sodium chloride or potassium chloride on both strains were additive in most concentrations and frequently synergistic.

The combined effects of chloramphenicol and polymyxin B were synergistic; the synergism was more pronounced with Strain R than with Strain S.

Strain R was found to be more active in assimilating and binding isotopically labeled lysine and glutamic acid, especially in the presence of an energy source (glucose). This activity was inhibited by 2,4-dinitrophenol and by incubation at 0 C suggesting that active transport of lysine may be more important than previously reported. The assimilation and binding of glutamic acid were moderately inhibited by chloramphenicol while assimilation and binding of lysine were inhibited only slightly, if at all.

No evidence for removal of chloramphenicol from solution by resting and growing cells of both strains could be obtained.

It is concluded that Strain R exhibits greater permeability and more highly developed active transport mechanisms than Strain S, and may owe its resistance to chloramphenicol, in part, to this feature.

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