GENETIC STUDIES IN PSEUDOMONAS AERUGINOSA

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GENETIC STUDIES IN PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

Enrichment Techniques in Bacterial Genetics

Selective or enrichment cultures are so elementary as a concept and many times as a technique that often little attention is paid to their fundamental significance. The techniques of selection of microorganisms by simulating environmental selection were developed by the two most prominent originators of enrichment techniques, Beijerinck and Winogradsky, during a time when the theory of evolution by natural selection presented the main force in the rapid growth of biology (Schlegel and Jannasch, 1967). It is the aim and the art of the enrichment culture technique to achieve selective conditions which quickly and reproducibly lead to the predominance in the population of one special organism, thereby facilitating its isolation.

The selection and isolation of microorganisms is dependent upon a few usually rather simple procedures. Microorganisms are continually selected by environmental factors which determine their natural distribution. The environmental conditions required by a particular microorganism may exist only after microbial alteration of

pre-existing conditions and many times require a series of microbial predominances (van Niel, 1955). Thus, the source of an enrichment culture inoculum initially determines its success.

The inoculum usually consists of a community of competing metabolic types in which the desired microorganism, if present, may be predominant or be a minority member; however, its presence even at very low concentrations in the inoculum should be adequate if the enrichment technique for its selection is reasonably specific. In order to be sufficiently selective, the enrichment technique must require the phenotypic expression of some of the microorganism's most unique properties and metabolic potentials, thus excluding most or all of its competitors. Enrichment techniques are therefore only an extension of the principles of natural selection to an artificial environment and require the expression of those capacities developed for survival and growth in a natural environment.

Direct selection refers to the enrichment of a specific microorganism or a genetic variant by growth in a selective environment. The selection of a particular microorganism from a mixed population or the selection of a microbial variant possessing a unique metabolic capability is directly dependent upon the expression of this particular property. Mutants resistant to bacteriostatic and bactericidal agents or to adverse physical conditions can be enriched and isolated without difficulty. Large numbers of cells of the

parent population are plated or grown under the proper environmental conditions which act selectively in favor of certain mutant cells and suppress the growth of the parent cells. This method has been used to screen for mutants resistant to antibiotics, bacteriophages, irradiation, and many toxic compounds (Witkin, 1950). It is also an indispensable technique for selection of revertants and suppressed mutants.

Constitutive mutants of a catabolic pathway may be selected by employing growth on alternating substrates since these mutants will actively grow and divide on the substrate of interest, without a lag for induction (Cohen-Bazire, and Jolit, 1953). Mutants of this nature may also be selected by growth in the chemostat when the corresponding substrate is chosen as the growth-limiting nutrient (Novick and Horiuchi, 1961; Horiuchi et al., 1962) or by growth in the presence of structural analogues and related compounds which exert an inhibitory effect on the induction of catabolic enzymes in the presence of their corresponding substrates (Buttin, 1963; Müller-Hill et al., 1964; Torriani and Rothman, 1961).

Mutants less sensitive or insensitive to catabolite repression have been isolated by enrichment techniques similar to those applied by Neidhardt (1960). In this instance, Aerobacter aerogenes was required to grow in glucose-histidine minimal medium with histidine being the sole source of nitrogen. Cells in which a glucose catabolite repressed

histidine degradation received a limited source of nitrogen; while, those mutants which could degrade histidine while utilizing glucose produced their nitrogen source via histidine catabolism and grew rapidly.

Adelberg (1958) devised an enrichment technique for isolating control-defective mutants of anabolic pathways by using plates containing an antimetabolite. Many structural analogues of normal metabolites are bacteriostatic since they are able to mimic the feedback inhibition of an end product, but are unable to fulfill the biosynthetic functions of this metabolite. Cell growth ceases as a consequence of "false feedback inhibition" or "pseudo-feedback inhibition" (Moyed, 1960). Some of these resistant colonies are surrounded by a halo of secondary colonies which can grow by utilizing the metabolite excreted by the resistant clone. The excretion of p-aminobenzoic acid by strains of Staphylococcus aureus resistant to sulfonamides had been observed earlier by Oakberg and Luria (1947). Numerous mutants resistant to different amino acid analogues have since been isolated (Cohen, 1965; Schlegel and Jannasch, 1967).

The painstaking efforts which may be involved in the isolation of mutants pertain mainly to those types of mutants which are differentiated from the wild type by auxotrophy or impaired utilization of substrates. The major difficulty in enrichment is the inability to design systems in which the mutants will grow more rapidly than the wild

type, thus allowing direct selection. This difficulty has been overcome by applying indirect selection or counter-selection techniques for mutant enrichment. A cell population consisting of a mixture of wild type and mutants is placed in an environment selective for the growth of wild type cells, i.e., inadequate for mutant growth. This is usually simply the most minimal medium capable of supporting growth of the prototroph. One or more bactericidal agents which affect only growing cells are added to the enrichment medium to selectively kill the growing cells thereby increasing the mutant to prototroph ratio. Although the theory and, for some bacteria, the application of this procedure is rather simple, much difficulty has been encountered in attempting to apply these techniques to the more penicillin-resistant gram negative microorganisms.

The procedure routinely in use for the isolation of auxotrophic and catabolic mutants comprises a number of steps during which the mutant population is increased by mutagenization and intermediary cultivation for complete phenotypic expression, enriched by antibiotic treatment, selected on plates and screened prior to final identification. Although methods for mutagenization (Tatum, 1946), plate selection (Lederberg and Tatum, 1946) and mutant screening (Lederberg, 1946) were in existence, bacterial genetics was greatly advanced in 1948 by the independent development in two laboratories of a broth enrichment technique for mutant selection (Davis, 1948; Lederberg and

Zinder, 1948). The enrichment technique utilized the selective activity of penicillin to kill growing cells and thereby increase the frequency of cells which could not grow in the minimal medium. The selective effect of penicillin on growing cells had been reported by Hobby et al. (1942) and Chain and Duthie (1945).

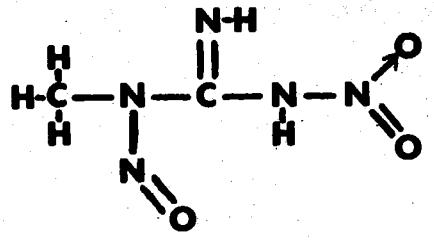
In order to utilize a more appropriate enrichment inoculum for mutant isolations, a bacterial culture is usually treated with a mutagen to increase the number of genetic variants in the population prior to mutant selection; this is analogous to the selection of a proper inoculum for a direct selection enrichment. Since the initial report of its mutagenicity by Mandell and Greenberg (1960), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG or nitrosoguanidine), Figure 1, has been widely used in bacterial genetics to induce point mutations in the bacterial genome. It acts primarily on replicating DNA (Barker and Tessman, 1968) and preferentially mutagenizes the replication point (Cerdia-Olmedo et al., 1968). This and other bacterial mutagens have been extensively reviewed (Witkin, 1950; Adelberg et al., 1965; Drake, 1969; Fishbein et al., 1970). The mutagenized culture is cultivated in a supplemented or nutrient medium to allow complete phenotypic expression of induced mutations (Newcombe, 1948; Davis, 1949). Although the importance of the mutagenization and intermediary cultivation methods cannot be overemphasized, apparently few attempts have been made to optimize these techniques as was

Figure 1. Structural Formulae of N-Methyl-N′-Nitro-N-Nitrosoguanidine, Penicillin, and Geraniol

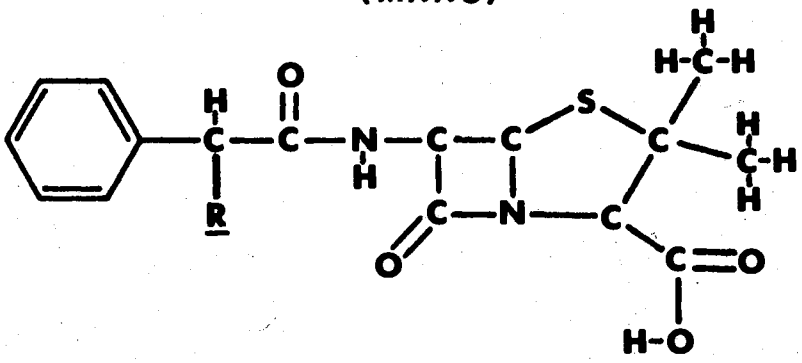
N-Methyl-N′-nitro-N-nitrosoguanidine is an alkylating agent widely used as a mutagen in bacterial genetics and useful in Pseudomonas aeruginosa genetics.

Penicillin G modified by carboxylating the α-carbon yields carbenicillin a more effective inhibitor of cell wall synthesis in Pseudomonas aeruginosa.

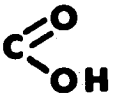
Geraniol, a hydrophobic terpene, is a rather unique substrate for Pseudomonas aeruginosa.

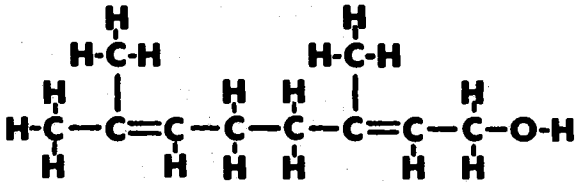


N-methyl-N′-nitro-N-nitrosoguanidine
(MNNG)



Penicillin-G R = H
(Benzylpenicillin)

Carbenicillin R = 
(α-Carboxybenzylpenicillin)



Geraniol

done by Adelberg, et al. (1965), Escherichia coli K12 using MNNG.

The penicillin broth enrichment technique developed independently by Davis (1948) and Lederberg and Zinder (1948) utilized minimal medium containing 300 units per ml penicillin G as the mutant enrichment system. A mutagenized culture of E. coli which had been grown in a complete medium, washed and resuspended in minimal medium was subjected to penicillin treatment for four to twenty-four hours prior to plating. The viable cell count per ml of culture was reduced by two to four logs, 10^{-2} to 10^{-4}, and the mutant to prototroph ratio proportionately increased during the penicillin treatment. Mutants were obtained by this method for several Salmonella strains; other antibiotics which were not inhibitors of cell wall biosynthesis were tested and found to have no selective value (Lederberg and Zinder, 1948).

Modifications of this enrichment technique have utilized methacillin (Bolt and Lundgren, 1962), dihydrostreptomycin (Ishida et al., 1966), kanamycin (Evans, 1966), glycine (Liu and Takahashi, 1964), carbenicillin (Green, 1969), D-cycloserine (Lessie and Whiteley, 1969), D-cycloserine and penicillin (Ornston et al., 1969) and D-cycloserine and carbenicillin (Heath, 1971). Gorini and Kaufman (1960) utilized hypertonic media, 20 per cent sucrose (Lederberg, 1956) to prevent osmotic lysis and cross-feeding during the penicillin treatment and penicillin

plates were used by Adelberg and Myers (1953) for mutant enrichment. Although numerous methods for the broth enrichment of mutants have been reported, the most successful are rather simple techniques utilizing antibiotics which inhibit cell wall biosynthesis (Schlegel and Jannasch, 1967).

Following the antibiotic treatment for mutant enrichment, the survivors are diluted and plated. Lederberg and Tatum (1946) reported a rather complicated plate procedure for detecting biochemical mutants in which four agar layers were poured; the final layer was poured after prototrophic colonies had formed in the minimal medium and this layer contained the nutrients required for development of mutant clones. Davis (1949b) utilized limited nutrient supplementation to produce readily distinguishable small mutant colonies on minimal medium plates. The ability to distinguish a mutant clone on a plate bearing numerous prototrophic colonies can be a significant selective advantage and its value should not be underestimated in developing efficient techniques for mutant selection.

Plate techniques for the rapid screening and identification of bacterial mutants have been reported by Lederberg (1946) and Holliday (1956). These plate methods or a modification thereof, used in conjunction with the replica plating procedure of Lederberg and Lederberg (1952) provide the bacterial geneticist with efficient methods for mutant screening and initial identification.

Antibiotics Useful in Selection of Bacterial Mutants

Fleming (1929) reported the antibiotic activity of a substance from a colony of the mold Penicillium notatum contaminating a culture of Staphylococcus aureus. The inhibitory substance, which he named penicillin, seemed too unstable to isolate and, thus, the problem was set aside. Modern antibacterial chemotherapy was launched six years later at the I. G. Farben industry in Germany, where Domagk developed a dye, Prontosil, that dramatically cured streptococcal infections, although it was inactive in vitro. A year later Trefouel, in France, demonstrated the in vitro activity of sulfonamide, a colorless product excreted by patients to which the dye had been administered (Davis et al., 1967). Chain and his colleagues (1940), at Oxford, undertook the purification of penicillin and demonstrated it to be reasonably stable once purified and dried.

The microscopic observations of Gardner (1940) indicated the possible interference of penicillin in the structural integrity of a bacterial cell, and in 1942 Hobby and his colleagues (1942) demonstrated that penicillin exerts little killing effect except during active growth. The bacteriostatic effect of sulfanilamide was explained when D. D. Woods (1940) found that it could be competitively reversed by a structurally related, but previously unknown, bacterial metabolite, p-aminobenzoic acid.

Although Duguid suggested that penicillin specifically

interfered with the formation of the outer supporting cell wall as early as 1945 (Salton, 1960), experimental evidence to verify this conclusion began accumulating only seven years later after the isolation of the "Park nucleotides" (Park, 1952). Duguid also concluded that all bacteria appear to be susceptible to penicillin in some degree and that, therefore, the cellular component upon which penicillin exerted its effect must be possessed by all bacterial species. Park demonstrated the accumulation in penicillin-inhibited cells of S. aureus of peptide derivatives of uridine nucleotides; these nucleotides and the nucleotides which accumulated during the inhibition of growth by other antibiotics provided an important key to the understanding of cell wall biosynthesis and the action of the antibiotics which inhibit these biosynthetic enzymes.

In 1954, a new antibiotic was independently discovered in four laboratories using different species of Streptomyces (S. lavendulae, Shull and Sardinas, 1955; S. orchidaceus, Harned et al., 1955; S. grayphalus strain 106-7, Harris et al., 1955; S. roseochromogenus, Kurihara and Chiba, 1956). The antibiotic was given the generic name D-cycloserine. Structural studies showed that the isolated compound was D-4-amino-3-isoxazolidone (Kuehl et al., 1955; Hidy et al., 1955).

D-Cycloserine is unstable in acid (Stammer, 1962), dimerizes to 3, 6 (diaminoxymethyl)-2, 5-diketopiperazine at neutral pH (Hidy et al., 1955) and is relatively stable

in alkali (Kuehl et al., 1955). In an aqueous solution at 37°C, the reaction of pyridoxal phosphate with D-cycloserine is quite complex (Roze and Strominger, 1963; Roze, 1964).

In testing D-cycloserine, it was observed to be more effective in *in vivo* tests than in *in vitro* tests (Cuckler et al., 1955; Lillick et al., 1956). Later, it was demonstrated that certain agar media after heating contained a significant concentration of D-alanine which antagonized the action of D-cycloserine (Hoeprich, 1963).

D-Cycloserine was found to have a synergistic effect when used in combination with penicillin, streptomycin, or oxytetracycline in experimental staphylococcal infections (Cuckler et al., 1955). A synergistic effect was observed with both gram-positive and gram-negative bacteria when combinations of D-cycloserine and penicillin, bacitracin, oxytetracycline, chlortetracyline, or chloramphenicol were used (Harris et al., 1955). Although, in general, it is more effective against gram-positive bacteria than against gram-negative bacteria, D-cycloserine is a "broad spectrum" antibiotic.

One of the clues to the mode of action of D-cycloserine was the discovery that D-alanine reversed the inhibitory effects of D-cycloserine. Pittillo and Foster (1954) observed that the effects of antibiotic 106-7 were reversed by D-alanine, L-alanine, and DL-α-amino-n-butyric acid; D-alanine was more effective than L-alanine. Bondi

et al. (1957) demonstrated that alanine inhibits the antibacterial activity of D-cycloserine in S. aureus. On the basis of these results, it was concluded that D-cycloserine interferes with the metabolism of alanine. Ito et al. (1958) confirmed the antagonism between D-cycloserine and alanine in several strains of bacteria. Independently, Buogo et al. (1958) and Park (1958a) proposed that D-cycloserine may prevent the incorporation of D-alanine into the bacterial cell wall. Evidence supporting these proposals was obtained a year later (Shockman, 1959).

Alanine is a major component of the peptidoglycan (mucopeptide) and teichoic acid moieties of bacterial cell walls (Salton, 1964). Part of the alanine in the cell wall is present as the D-isomer (Toennies and Shockman, 1959; Strominger, 1959; Ikawa and Snell, 1960). Salton (1961) proposed that the occurrence of D-amino acids in the cell wall renders the bacterium more resistant to proteolytic enzymes.

D-Alanine and D-glutamic acid are rare in biological materials. As a result of the specific location of these amino acids in the cell wall and the fact that the integrity of the wall is essential to bacterial survival, it was suggested by Park (1958b) that compounds which are analogues of these cell wall components would be useful as chemotherapeutic agents.

Park (1958b) and Ciak and Hahn (1959) observed a marked accumulation of UDP-N-acetyl-amino sugar when either

S. aureus or *E. coli* was grown in the presence of D-cycloserine. A direct relation between the accumulation of UDP-N-acetyl-amino sugar and the inhibition of peptidoglycan synthesis was established by Park (1958b; 1960). The structure of the UDP-N-acetyl-amino sugar which accumulates in the presence of D-cycloserine was elucidated by Strominger et al. (1959) and shown to be UDP-N-acetyl-muramyl-L-ala-D-glu-L-lys (Figure 2). The "Park nucleotides" which accumulate in penicillin-inhibited *S. aureus* cells were shown to be UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala, UDP-N-Ac-muramyl-L-ala and UDP-NAc-muramic acid (Park and Strominger, 1957).

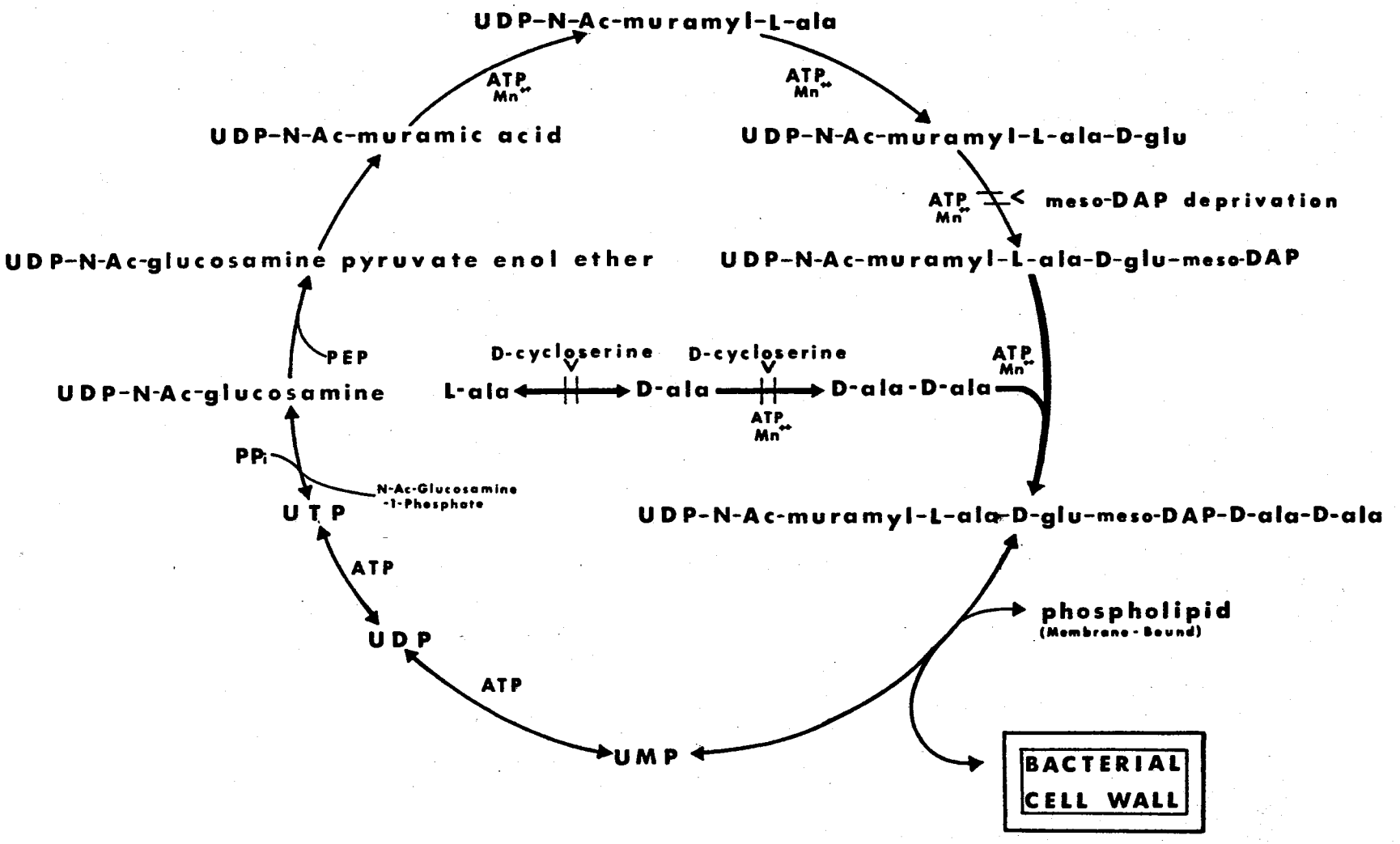
Since the peptide in the largest nucleotide precursor of cell wall biosynthesis has the sequence L-ala-D-glu-L-lys-D-ala-D-ala, and D-alanine was observed to reverse the effect of D-cycloserine, Strominger et al. (1960) examined the metabolism of D-alanine. D-cycloserine was found to be a competitive inhibitor of alanine racemase.

L-ALANINE $\xleftarrow{\text{alanine racemase}}$ D-ALANINE

Alanine racemase was discovered and purified by Wood and Gunsalus (1951) and was found to require pyridoxal phosphate as a coenzyme. Since L-alanine was not effective in the reversal of D-cycloserine inhibition in *S. aureus*, Strominger and his colleagues considered a second site of inhibition possible and found that D-cycloserine competitively inhibits D-alanyl-D-alanine synthetase which they discovered during this investigation.

Figure 2. Cytoplasmic Synthesis of Uridine Nucleotides for Cell Wall Synthesis in Escherichia coli

Uridine diphospho-N acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid cannot be synthesized during diaminopimelic acid deprivation resulting in osmotic fragility. D-Cycloserine competitively inhibits alanine racemase and D-alanyl-D-alanine synthetase depriving the cell of D-alanyl-D-alanine required for synthesizing uridine diphospho-N acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl--D-alanine and resulting in osmotic fragility. The end-product, UDP-N acetylmuramyl-L-ala-D-glu-meso-DAP-D-ala-D-ala, is bound by a membrane phospholipid prior to further synthesis.


UDP-N-Ac-muramyl-L-ala
ATP Mn++
ATP Mn++
UDP-N-Ac-muramic acid
UDP-N-Ac-muramyl-L-ala-D-glu
ATP Mn++
meso-DAP deprivation
UDP-N-Ac-glucosamine pyruvate enol ether
UDP-N-Ac-muramyl-L-ala-D-glu-meso-DAP
PEP
D-cycloserine
D-cycloserine
ATP Mn++
UDP-N-Ac-glucosamine
L-ala
D-ala
D-ala-D-ala
ATP Mn++
PPi
N-Ac-Glucosamine -1-Phosphate
UDP-N-Ac-muramyl-L-ala-D-glu-meso-DAP-D-ala-D-ala
UTP
ATP
phospholipid
(Membrane - Bound)
UDP
ATP
UMP
BACTERIAL CELL WALL

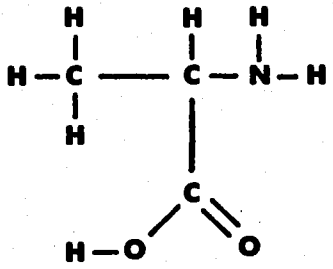
$$\text{D-ALANINE} + \text{D-ALANINE} + \text{ATP} \xrightarrow[Mg^{++},K^{+}]{\text{D-alanyl-D-alanine synthetase}} \text{D-ALANYL-D-ALANINE}$$

Strominger (1962) proposed that the substrate on the enzyme surface has the conformation of D-cycloserine. Experimental support for this proposal was found in an independent series of investigations (Neuhaus and Lynch, 1964). Molecular model sketches of D-cycloserine and D-alanine are compared in Figure 3.

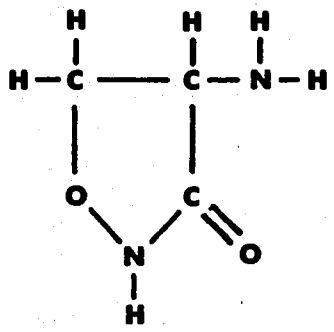
The inhibition of alanine racemase and D-alanyl-D-alanine synthetase deprives the bacterium of the D-alanyl-D-alanine required for the biosynthesis of the complete uridine nucleotide required for cell wall synthesis, UDP-N-Ac-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala. This results in the accumulation of UDP-N Ac-muramyl-L-ala-D-glu-L-lys and the inhibition of peptidoglycan synthesis. In growing cells, osmotic fragility results when peptidoglycan synthesis is inhbited. Spheroplast formation similar to that observed by Lederberg (1956) in penicillin-inhibited cells of E. coli and S. typhimurium has been observed during D-cycloserine inhibition of E. coli (Buogo et al., 1958), Alcaligenes faecalis (Lark and Schichtel, 1962), S. faecalis (Shockman and Lampen, 1962), and Proteus mirabilis (Plapp and Kandler, 1965). Protoplast formation is dependent upon growth during D-cycloserine inhibition (Ciak and Hahn, 1959); omission of sources of carbon or nitrogen abolish the effect of D-cycloserine.

Figure 3. A Comparison of Structural Formula and Molecular Model Sketches of D-Alanine and D-Cycloserine

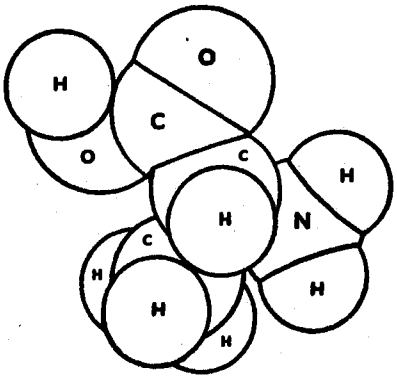
A comparison of the structural formulae and molecular models of D-alanine and D-cycloserine led Strominger (1962) to propose that the substrate on the enzyme surface has the conformation of D-cycloserine.

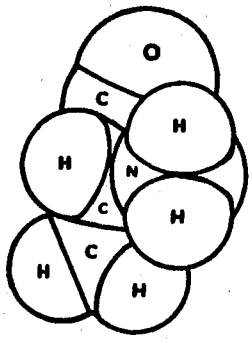


D-Alanine

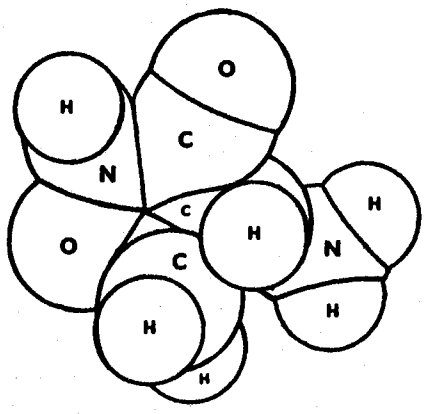


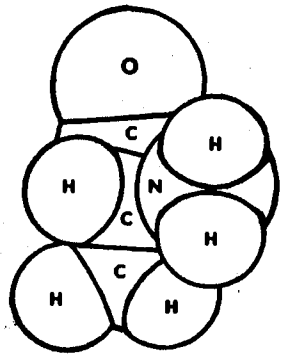
D-Cycloserine





D-Alanine





D-Cycloserine

Leach and Snell (1960) established the existence of a transport system for D-alanine and L-alanine in Lactobacillus casei and Mora and Snell (1963) observed that D-cycloserine inhibits the permease in S. faecalis which is responsible for the transport of L-alanine and D-alanine. In investigating D-alanine transport, it was established that D-cycloserine is transported by the D-alanine transport system in E. coli (Kessel and Lubin, 1965). D-Cycloserine has been found to competitively inhibit uptake of glycine and D-alanine in E. coli (Wargel et al., 1970).

The isolation of D-cycloserine-resistant mutants of S. aureus in multiple steps was reported by Howe et al. (1964); only stepwise mutations were observed. An analysis of D-cycloserine-resistant mutants of Streptococcus strain Challis revealed three mutant groups. One group had elevated levels of alanine racemase; another group had elevated levels of both alanine racemase and D-alanyl-D-alanine synthetase. The third group had a defective transport system for L-alanine and D-alanine (Reitz et al., 1967).

Bacterial cell walls are synthesized in three distinct stages which occur at three different sites in the bacterial cell. The uridine nucleotide precursors of the bacterial cell wall, UDP-N-Ac-muramyl-pentapeptide and UDP-N-Ac-glucosamine, are synthesized in the cytoplasmic fraction of the cell (Figure 2). Since D-cycloserine inhibits enzymes in the cytoplasmic fraction, its transport into the cell aids this inhibition.

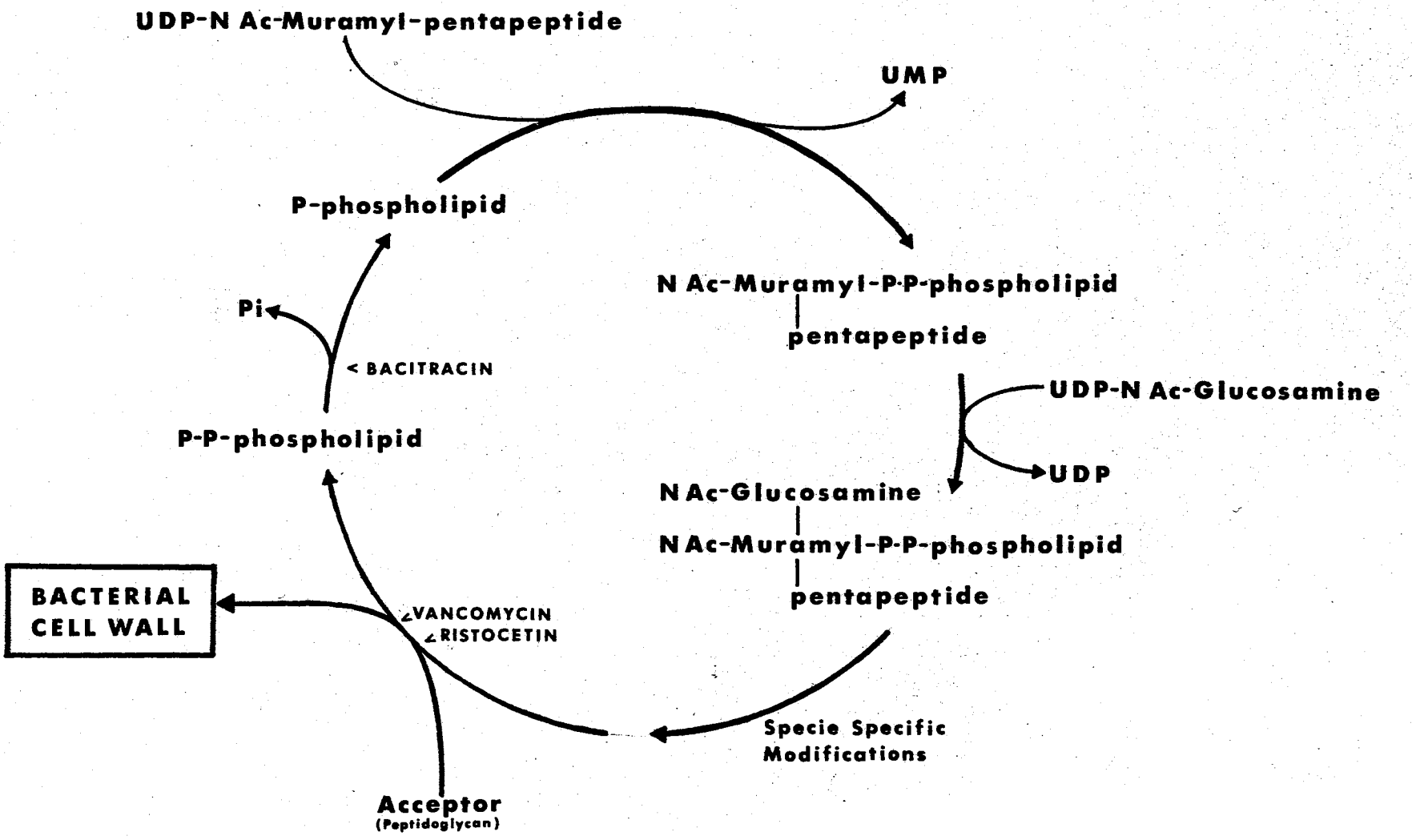
The second stage of cell wall synthesis is the utilization of these uridine nucleotide precursors for the introduction of new disaccharide-pentapeptide units into a growing peptidoglycan in the cell wall. In this complex sequence, the sugar fragments of the nucleotides are first transferred to a membrane-bound phospholipid carrier with the formation of disaccharide-pentapeptide-P-P-phospholipid (Dietrich et al., 1965). The disaccharide-pentapeptide moiety may then be modified in a manner which depends on the particular bacterial specie. In S. aureus, this modification includes amidation of the α-carboxyl group of glutamic acid (Dietrich et al., 1966) and addition of a pentaglycine chain attached to the ϵ-amino group of lysine (Chatterjee and Park, 1964). The disaccharide-pentapeptide moiety is transferred to the growing peptidoglycan with the release of P-P-phospholipid (Figure 4). The latter compound is dephosphorylated to form P-phospholipid and inorganic phosphate. Vancomycin and ristocetin are specific inhibitors of the utilization of lipid intermediates for peptidoglycan synthesis (Anderson et al., 1965; Anderson et al., 1966). Bacitracin is a specific inhibitor of the dephosphorylation of P-P-phospholipid (Siewert and Strominger, 1967). The lipid moiety has been identified as a C_{55}-isoprenoid alcohol (Higashi et al., 1967).

The third phase in bacterial cell wall synthesis is the cross-linking of the linear peptidoglycan strands which are formed by the mechanism just described. This cross-linking

Figure 4. General Membrane-Bound Reactions in Cell Wall Synthesis

Uridine diphospho-N acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine is bound to a membrane phospholipid with the release of uridine monophosphate. N-Acetylglucosamine is added to form a membrane bound disaccharide-pentapeptide which is then modified according to specie-specific enzymatic reactions prior to bonding the cell wall subunit to a growing peptidoglycan strand.

Vancomycin and ristocetin inhibit the transfer of the cell wall subunit to a growing peptidoglycan strand. Bacitracin inhibits the dephosphorylation of the membrane-bound phospholipid.


UDP-N Ac-Muramyl-pentapeptide
UMP
P-phospholipid
N Ac-Muramyl-P-P-phospholipid
pentapeptide
UDP-N Ac-Glucosamine
UDP
Pi
< BACITRACIN
P-P-phospholipid
N Ac-Glucosamine
N Ac-Muramyl-P-P-phospholipid
pentapeptide
BACTERIAL CELL WALL
VANCOMYCIN
RISTOCETIN
Specie Specific Modifications
Acceptor
(Peptidoglycan)

reaction is a transpeptidation in which two linear peptidoglycan strands interact to form an interpeptide peptide bond with the elimination of D-alanine (Figure 5). This is an extracellular reaction catalyzed by peptidoglycan transpeptidase (Araki et al., 1966a; Araki et al., 1966b; Izaki et al., 1966). The reaction is extracellular in the sense that it occurs outside the cell membrane, and no external energy source, such as ATP, is required. The terminal D-alanine of the second peptide strand is removed by D-alanine carboxypeptidase.

Martin (1964a; 1964b) hypothesized the inhibition of peptidoglycan cross-linking by penicillin while investigating penicillin-induced spheroplasts of P. mirabilis. When a cell-free preparation which catalyzed transpeptidation was obtained from E. coli two years later, it was possible to demonstrate directly that this enzyme, peptidoglycan transpeptidase, was irreversibly inactivated by low concentrations of penicillin G and other penicillins (Izaki et al., 1966). It was further hypothesized that penicillin is an analog of the D-alanyl-D-alanine at the end of the linear peptidoglycan strand (Tipper and Strominger, 1965; Strominger and Tipper, 1965). Molecular models revealed a striking similarity in structure between penicillins and the end of an uncross-linked peptidoglycan strand (Figure 6). In particular, it was pointed out that the highly reactive CO - N bond in the β-lactam ring of penicillin is the analog of the peptide bond in D-alanyl-D-alanine

Figure 5. The Penicillin Sensitive Reactions of Cell Wall Synthesis

Peptidoglycan transpeptidase (1) cross-links two peptidoglycan strands by breaking one D-alanyl-D-alanine peptide bond and attaching the carboxyl group of the remaining acyl-D-alanine to the free ϵ-amino group of the diaminopimelic acid of another peptidoglycan strand in Escherichia coli. The remaining D-alanyl-D-alanine peptide bond is hydrolyzed by carboxypeptidase (2) releasing D-alanine. Peptidoglycan transpeptidase and carboxypeptidase are sensitive to penicillins.

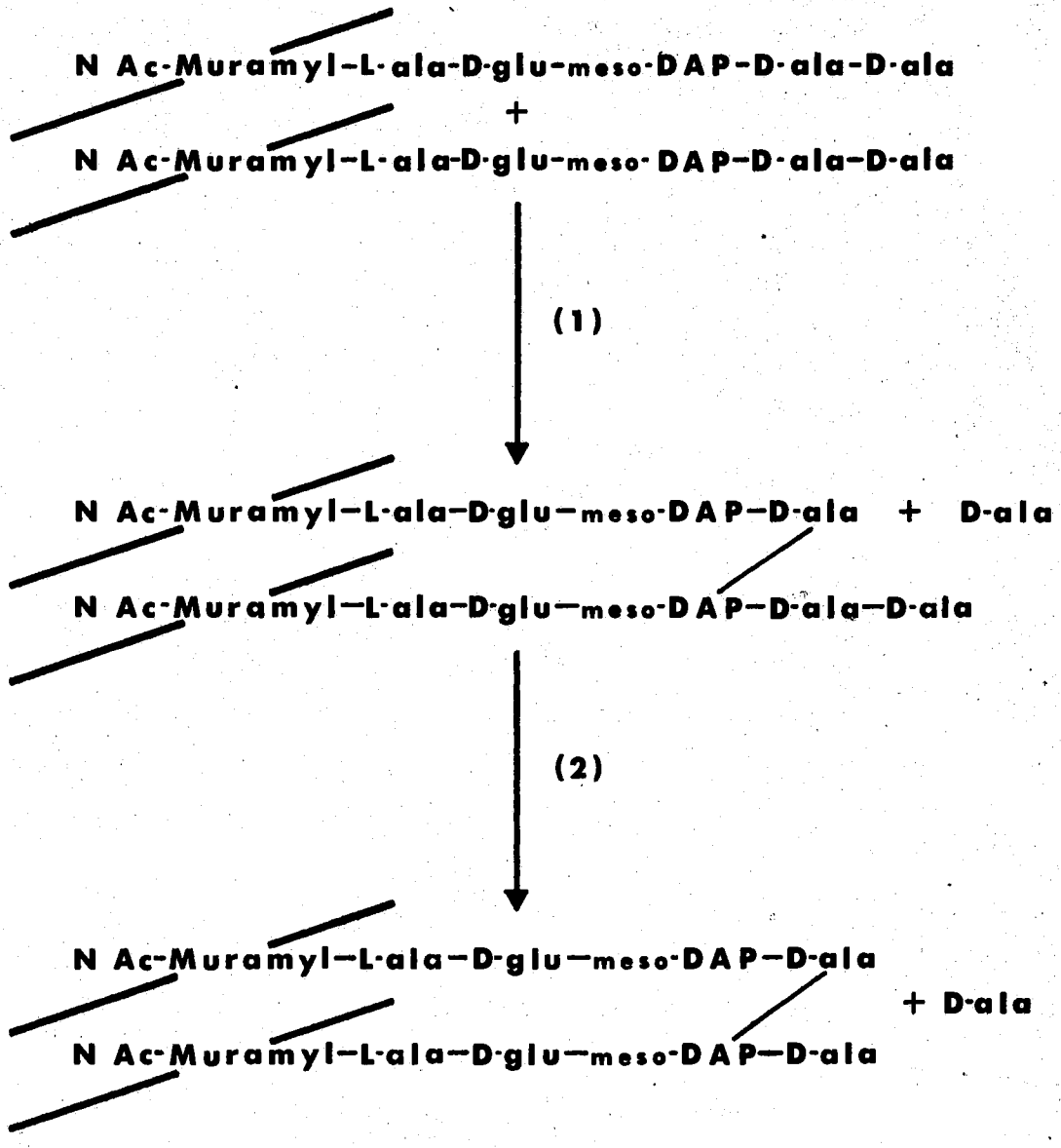
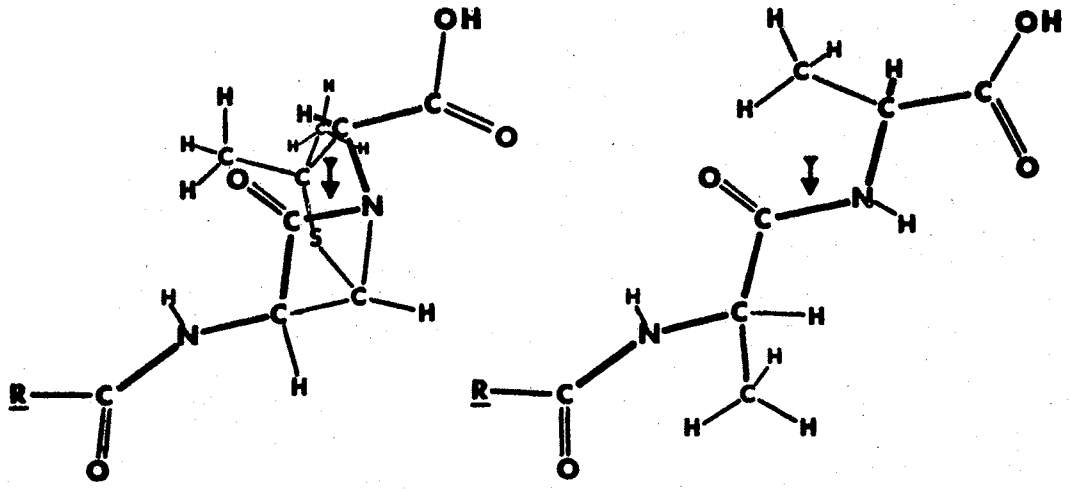

N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala-D-ala
+
N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala-D-ala
(1)
N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala + D-ala
N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala-D-ala
(2)
N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala
+ D-ala
N Ac-Muramyl-L-ala-D-glu-meso-DAP-D-ala

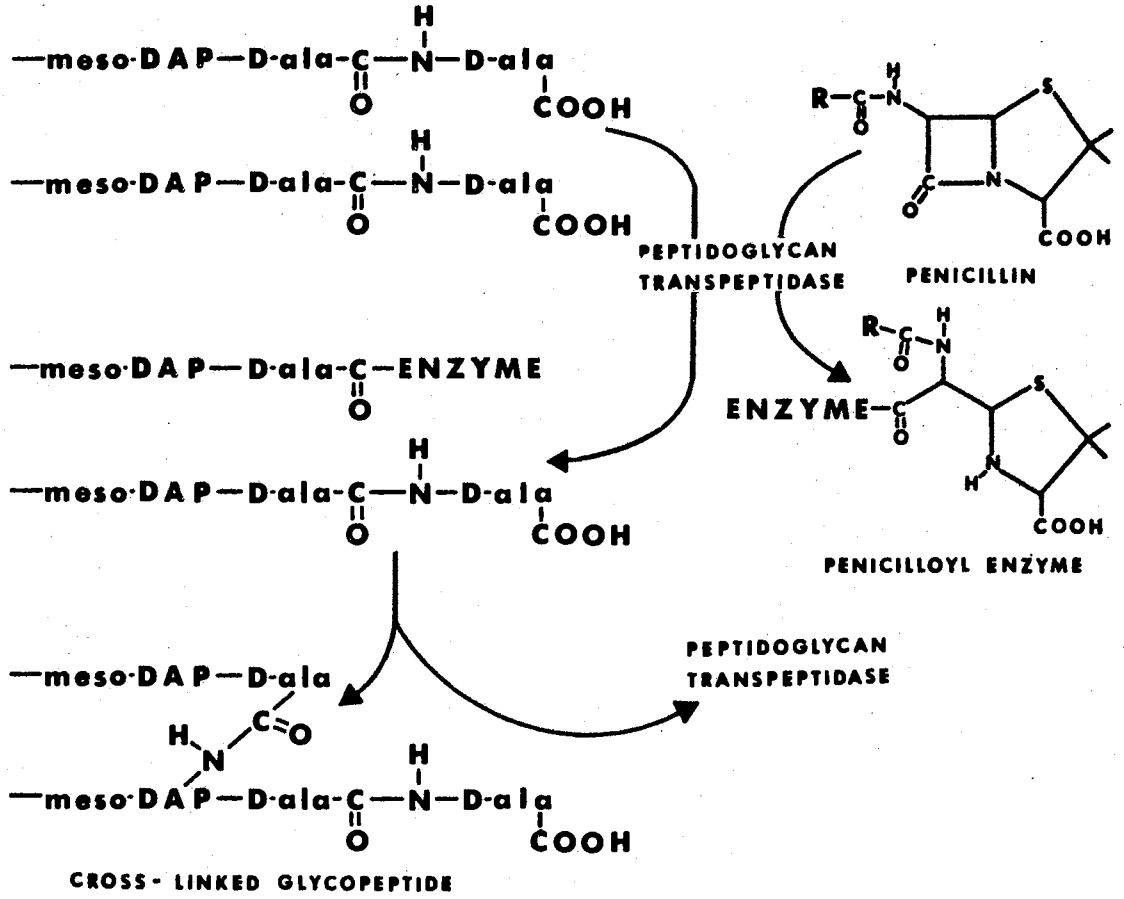
Figure 6. A Comparison of the Structural Formulae of Penicillin and Acyl-D-Alanyl-D-Alanine

Molecular models reveal a striking similarity in structure between penicillins and the acyl-D-alanyl-D-alanine end of an uncross-linked peptidoglycan strand. The structural similarity between the highly reactive C(O)-N bond in the β-lactam ring of penicillin and the peptide bond in acyl-D-alanyl-D-alanine involved in transpeptidation is noted (arrows).

Figure 7. A Proposed Mechanism of Peptidoglycan Transpeptidase Inactivation by Penicillin



PENICILLIN ACYL-D-ALANYL-D-ALANINE



which is involved in the transpeptidation. A reaction mechanism for the transpeptidation was proposed (Figure 7) and it was suggested that penicillin acylates the transpeptidase through the β-lactam ring at the active site involved in transpeptidation. Penicillin has been shown to be bound as a penicilloyl derivative and the data indicate the formation of a thiol ester of penicilloic acid and a functional thiol group in the enzyme (Lawrence and Strominger, 1970).

Pseudomonas aeruginosa was reported to be sensitive to a new derivative of benzylpenicillin, carbenicillin (α carboxylbenzylpenicillin, see Figure 1), by Acred et al. (1967). Carbenicillin was found to have a broad spectrum of activity against both gram-positive and gram-negative bacteria and a synergistic effect when used with gentamicin on strains of P. aeruginosa (Rolinson and Sutherland, 1968). The minimal inhibitory concentration of carbenicillin is dependent upon the cell concentration, and resistance regularly develops on exposure of P. aeruginosa to subinhibitory concentrations (Holmes et al., 1970). Resistance to carbenicillin, as to penicillin, is a step-wise development and has been observed to occur in most carbenicillin-treated cultures (Brumfitt et al., 1967; Bell and Smith, 1969; Lowbury et al., 1969; Stephenson, 1969). Carbenicillin has been used in combination with D-cycloserine to isolate mutants of P. aeruginosa (Heath, 1971).

Stanier et al. (1966) in a taxonomic study of the

aerobic pseudomonads have greatly advanced the understanding of the metabolic capacities and differences of several strains of aerobic pseudomonads. P. aeruginosa was shown to be capable of utilizing a minimum of 73 of the 146 different substrates tested; some strains could catabolize as many as 82 of these for carbon and energy. Many rather unique properties were noted, such as the ability of P. aeruginosa to utilize geraniol (Figure 1) vapor as a source of carbon and energy, and a number of characteristics were noted to be of value in differentiating strains of fluorescent pseudomonads.

Pseudomonas and related genera have been reviewed by deLey (1964) and the genetics of Pseudomonas has recently been reviewed by Holloway (1969).

CHAPTER II

MATERIALS AND METHODS

Bacteria and Bacteriophage

Pseudomonas aeruginosa, strain 1, (designated PA-1) and its transducing phage, F 116, were originally obtained from B. W. Holloway, Monash University, Clayton, Australia. A *P. aeruginosa* soil isolate, strain 2, (designated Soil-2) was isolated and identified in this laboratory by E. T. Gaudy and R. Meganathan. All mutants are derivatives of PA-1 unless otherwise indicated.

Media

Minimal medium was a modification of M-9 medium (Roberts et al., 1957) and contained per liter: $Na_2H\ PO_4 \cdot 7\ H_2O$, 8.2g; KH_2PO_4, 2.7g; NH_4Cl, 1.0g; $FeSO_4$, 0.1 per cent solution, 0.5 ml; distilled water to volume. Sterile $MgSO_4 \cdot 7H_2O$ (final concentration 0.4g per liter) and carbon source (final concentration 0.5 per cent) were added from separately sterilized concentrated solutions, following the sterilization of minimal medium by autoclaving at 15 psi (121°C) for 15 minutes. Carbon sources and other medium supplements were autoclaved or filter-sterilized separately as concentrated solutions and added as indicated.

Nutrient broth (Difco) was rehydrated as recommended by the manufacturer.

Acetate phosphate medium contained 0.05 M sodium acetate plus 3.0g per liter KH_2PO_4 and 6.0g per liter Na_2HPO_4; the pH of the buffer was adjusted to 6.0 with HCl. Acetate buffer contained 0.05 M sodium acetate and was adjusted to pH 6.0 with HCl.

A concentrated amino acid mixture contained 1.0 mg per ml of each of the following: leucine, isoleucine, valine, arginine, proline, cysteine, serine, glycine, alanine, methionine, lysine, threonine, histidine, phenylalanine, tyrosine, and tryptophan. All amino acids were the L-isomers. The solution was stored frozen at -20°C.

Plates contained 25 ml of medium solidified with 2.0 per cent Bacto-agar (Difco) or 0.9 per cent Ionagar No. 2 (Oxoid) unless otherwise indicated. Sufficient Bacto-agar was added to nutrient agar (Difco) to give a final concentration of 2.0 per cent agar. Nutrient agar slants for mutant storage were supplemented with yeast extract, 3.0g per liter.

Fifteen different plate sets were utilized to identify presumptive auxotrophs. Glucose minimal medium solidified with 0.9 per cent Ionagar No. 2 was supplemented with 20µg per ml of each of the following L-amino acids: (a) methionine, threonine, lysine (MTL); (b) leucine, isoleucine, valine (LIV); (c) arginine, proline, serine, cysteine, glycine (APSCG); (d) histidine, phenylalanine,

tyrosine, tryptophan (H Aro); (e) methionine, threonine (MT); (f) methionine (Meth); (g) threonine (Thr); (h) lysine (Lys); (i) leucine (Leu); (j) arginine (Arg); (k) proline (Pro); (l) serine, cysteine, glycine (SCG); (m) histidine (His); (n) phenylalanine, tyrosine, tryptophan (Aro); and (o) tryptophan (Tryp).

Media for Pseudomonas phage propagation and storage have been described by Holloway et al. (1962). Phage broth contained per liter: nutrient broth, 8.0g; yeast extract, 5.0g; NaCl, 5.0g; distilled water to volume. Phage plates contained 35 ml of bottom layer agar, which was phage broth solidified with 1.1 per cent Bacto-agar. Top layer phage agar (soft agar) contained per liter: NaCl, 5.0g; Bacto-agar, 6.0g; distilled water to volume. Soft agar was dispensed in 3.0 ml portions into 13 mm tubes and sterilized by autoclaving.

Lactate phage agar (Sutter et al., 1963) was used for phage titrations and contained per liter: NaCl, 5.0g; $MgSO_4 \cdot 7H_2O$, 0.2g; $NH_4H_2PO_4$, 1.0g; K_2HPO_4, 1.0g; sodium lactate, 60 per cent syrup, 2.0 ml; distilled water to volume. The medium was solidified with 1.1 per cent Bacto-agar and dispensed in quantities of 35 ml per plate.

Transduction plates contained minimal medium plus 5.0g per liter of NaCl and were solidified with 0.9 per cent Ionagar No. 2. The carbon and energy source was added to a final concentration of 1.0 per cent.

Growth of Bacteria

Stock cultures were maintained on nutrient agar slants by periodic transfer; inoculated slants were incubated overnight at room temperature prior to storage at 5°C. Liquid cultures were grown in 18 mm tubes containing 7.0 ml of medium, 20 mm tubes containing 8.0 ml of medium or in flasks containing a volume not greater than one-tenth the total flask volume unless otherwise indicated. Liquid cultures were aerated on a reciprocal shaker at 37°C. Growth was measured as optical density at 540 nm against an appropriate blank with a Coleman Junior Spectrophotometer, Model D, or as viable cells per ml of experimental medium, determined by plate count.

Minimal salts medium was used for diluting and washing bacterial suspensions except for the final wash prior to a treatment, for which the treatment medium was used. Bacterial suspensions were harvested and washed by centrifugation at 8,000 rpm and 0°C for 20 minutes unless otherwise indicated.

D-Cycloserine Survival Curves

Fresh slants of PA-1 and six arginine auxotrophs (PA-921, PA-923, PA-926, PA-927, PA-928, and PA-933) were soaked with 1.0 ml of nutrient broth per slant and 0.5 ml portions of each of the six suspensions of mutants were mixed in a tube. Two separate 18 mm tubes, each containing 6.8 ml of nutrient broth, were inoculated with 0.2 ml of the

suspension of PA-1 and the mutant mixture, respectively. The cultures were aerated on a reciprocal shaker until both cultures contained more than 3.0×10^9 cells per ml as determined by optical density measurements. The cultures were harvested, washed twice and resuspended in minimal medium to a concentration of 3.0×10^9 cells per ml and a 10^{-2} dilution of the mutant mixture was prepared (3.0×10^7 cells per ml).

The experimental system was inoculated with 0.1 ml of each culture, giving a final concentration of 3.0×10^7 cells per ml for PA-1 and 3.0×10^5 cells per ml for the mutant population.

Samples of the antibiotic-treated culture were diluted and spread on glycerol minimal medium supplemented with 5µg per ml of arginine. The plates were incubated at 37°C for approximately 50 hours at which time the prototrophic colonies were much larger than the small auxotrophic colonies. The large colonies were counted as wild type clones and the small colonies as mutant clones; thirty large colonies and thirty small colonies were randomly selected from each experiment and tested on glucose minimal medium and glucose minimal medium supplemented with 25µg per ml of arginine.

Mutagenesis

A modification of the procedure of Adelberg et al. (1965) was used for N-methyl-N′-nitro-N-nitrosoguanidine

treatment. Nutrient broth-grown cells were harvested when the culture reached a density of approximately 5.0×10^8 cells per ml, washed and resuspended in pH 6.0 acetate phosphate buffer. A solution of MNNG containing 1.0 to 8.5 mg/ml was prepared fresh in acetate phosphate buffer and 0.8 ml was added to 7.2 ml of the cell suspension to give a final MNNG concentration of 100µg to 850µg per ml. The culture was incubated on a reciprocal shaker at 37°C for 15 to 20 minutes prior to harvesting and washing in minimal medium.

Phenotypic Lag and Nuclear Segregation

The mutagenized culture was resuspended in nutrient broth and aerated on a reciprocal shaker at 37°C for two to four hours. The culture was then washed and resuspended in minimal medium in preparation for antibiotic treatment.

Screening and Testing Presumptive Mutants

Auxotrophs

After four to five days of incubation at 37°C, presumptive auxotrophs were selected as small to minute colonies on glycerol minimal medium supplemented with 150 ng per ml of each amino acid in the amino acid mixture and inoculated as a small spot onto a nutrient agar plate. After growth, each colony was used to inoculate a rectangular area on nutrient agar master plates; approximately 50 to 60 rectangles would fill a plate. The plates were incubated at 37°C for six to eight hours prior to replicating onto TML,

H Aro, LIV, APSCG, and nutrient agar plates. These plates were incubated at 37°C for 24 to 36 hours. After comparing the plates of a set, areas of growth which appeared on one plate but were absent on the other three glucose plates were picked from the nutrient agar plate and inoculated in rectangles on nutrient agar master plates for each of the four groups. After growth, the TML group was replica-plated onto MT, Meth, Thr, Lys, and nutrient agar plates; the H Aro group was replicated onto His, Tryp, Aro, and nutrient agar plates. The APSCG group was replicated onto Arg, Pro, SCG, and nutrient agar plates and the LIV group was replica-plated onto LIV, Leu, and nutrient agar plates. The plates were incubated at 37°C for 24 to 36 hours and the plate sets compared. Each rectangular growth area was then tentatively classified and inoculated onto nutrient agar slants from the nutrient agar plate. At the same time, a loop inoculum from the nutrient agar plate was used to inoculate a tube of glucose minimal medium to slight turbidity. The glucose minimal broth tubes were aerated at 37°C on a reciprocal shaker and observed for 150 hours. Strains growing in the minimal medium prior to 100 hours were discarded.

Glycerol-Negative Mutants

After five to eight days incubation at 37°C, glycerol mutants on 0.15 per cent glycerol minimal medium with four to five drops of geraniol in the lid were selected as thin flat colonies and spotted onto nutrient agar plates. After

each spot formed a small colony, the colonies were spread in a rectangular area and incubated for three to six hours. These nutrient agar master plates were replica-plated onto glycerol, glucose, and lactate minimal media, solidified with 0.9 per cent Ionagar No. 2, followed by a nutrient agar plate. The plates were incubated 48 hours prior to comparison. Glycerol negative, glucose and lactate positive strains were used to inoculate nutrient agar slants and a tube of glycerol minimal medium to slight turbidity. The glycerol minimal medium was then aerated on a reciprocal shaker at 37°C and observed for five days. Strains growing prior to 80 hours were discarded.

Isolation of Pseudomonas aeruginosa

Soil samples were washed with 0.2 per cent acetamide minimal medium and the supernatant fluid decanted after the soil had settled. Eight ml samples of the soil washings were placed in 50 ml flasks. Pond water and sewage water samples were diluted with an equal volume of double strength acetamide minimal medium and 8 ml placed in 50 ml flasks. Each enrichment culture was aerated on a reciprocal shaker at 42.5°C until grown. Lactate minimal plates were spread or streaked with a 0.1 ml sample of each enrichment and incubated at 37°C for 24 hours.

Each lactate plate was examined by a mineralite or comparable UV light source in the dark and fluorescent colonies or areas marked. Each fluorescent area was then

sampled with a spreading needle and streaked on minimal medium plates containing no substrate. Four drops of geraniol were placed in the petri dish lid and the plates incubated at 37°C for four to seven days prior to sampling the large very thin colonies for slant storage and testing. Adipate, azelate, sebacate, L-mandelate, pelargonate, benzoate, p-hydroxybenzoate, arginine, betaine, and sarcosine are a few substrates which could be utilized instead of acetamide (Stanier et al., 1966).

Preparation and Titration of Phage Plate Stocks

Phage plate stocks were prepared by a modification of the procedure of Swanstrom and Adams (1951). One drop of a culture of the desired host strain grown in nutrient broth to approximately 1.0×10^9 cells per ml, and 0.1 ml of phage suspension containing 2.0×10^6 to 1.0×10^7 plaque-forming units per ml were mixed in 3.0 ml of molten (47°C) soft agar and poured over the bottom layer phage agar. After overnight incubation at 37°C, the phage were soaked off by flooding the plates with 5.0 ml of phage broth. After 30 minutes, the phage broth was pipetted from the plates; contaminating cells and soft agar were removed by room temperature centrifugation at 5,000 rpm for 15 to 20 minutes. The supernatant fluid was immediately harvested by Millipore filtration (HA, 0.45μm pore size).

Phage plate stocks were titered by the soft agar

overlay method described above. Lactate phage agar was used since the plaques were more readily visible than on nutrient phage agar. PA-1 was the host for all phage titrations and phage plate stocks ranged from 1.0×10^{10} to 1.0×10^{11} plaque-forming units per ml for most hosts. Phage suspensions were diluted in phage broth for titering and were maintained at 5°C. Phage suspensions were tested for bacterial contamination by spotting several drops on a nutrient agar plate immediately following Millipore filtration.

Transduction

A modification of the spot plate transduction procedure of Murphy and Rosenblum (1964) was used. Recipient cells were washed from fresh nutrient agar slants with 1.1 ml of minimal medium, and 0.1 ml aliquots were spread on glycerol minimal transduction plates. One drop of each donor phage plate stock was spotted at a marked location on the surface of each plate. Phage suspensions were also spotted on nutrient agar and glycerol minimal transduction plates without cells to test the sterility of the phage stocks. The plates were inverted and incubated after the spots had soaked into the agar. Transductants were counted after approximately three to four days of incubation.

Chemicals

Carbenicillin was a generous gift from Beecham Pharmaceuticals. N-Methyl-N′-nitro-N-nitrosoguanidine was

obtained from Aldrich Chemical Company. Geraniol was purchased from Eastman Kodak Company and dihydrostreptomycin was obtained from Pfizer and Company. Ionagar No. 2 was the product of Colab Laboratories. Sodium acetate and acetamide were purchased from J. T. Baker Chemical Company. Sodium lactate, glycerin, and D-fructose were obtained from Fisher Scientific Company; aminoacetic acid (glycine), L-isoleucine, D-alanine and DL-alanine were products of Nutritional Biochemicals Corporation.

The following chemicals were obtained from Mann Research Laboratories: L-alanine, L-arginine, D-cycloserine, allo-cystathionine, L-cysteine, DL-homocysteine thiolactone HCl, DL-homoserine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. Penicillin G, L-methionine, L-histidine, and α, ϵ-diaminopimelic acid were obtained from Calbiochem.

All other chemicals were of the highest quality commercially available.

CHAPTER III

EXPERIMENTAL RESULTS

Experiments described in this chapter are presented primarily in chronological order. Initial attempts to isolate specific types of amino acid auxotrophs, which had not previously been done with this organism, indicated that a thorough study of methods of mutant isolation with *P. aeruginosa* was needed. Therefore, the study was designed to determine optimum conditions for several of the most critical steps in isolating mutants. In several instances, results of experimentation with one step of the procedure necessitated revision of other steps, and a chronological description of these experiments seems most appropriate.

Mutant Isolation by Direct Plating

Several months were spent in attempting to isolate arginine auxotrophs using various combinations of ultraviolet light (UV), ethylmethane sulfonate (EMS), acriflavin or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) as mutagens and penicillin G or carbenicillin as the antibiotic system for mutant selection. These experiments yielded completely negative results.

A low yield of arginine auxotrophs was obtained by

mutagenizing log phase cells with 850µg per ml MNNG in 0.05M acetate buffer at pH 6.0. The cells were aerated on a reciprocal shaker at 37°C for 20 minutes. After this incubation, the cells were harvested and washed by centrifugation prior to resuspension in minimal medium. A 0.1 ml aliquot was used to inoculate 8.0 ml of nutrient broth which was then replaced on the shaker and incubated until grown. The culture was then diluted in minimal salts medium and spread on glucose minimal plates containing 1.0µg per ml of arginine. The plates were incubated at 37°C for 36 hours at which time small colonies were picked for testing as possible arginine auxotrophs. This procedure was utilized in sixty attempts to isolate arginine auxotrophs, which yielded a total of 356 mutants requiring arginine for optimum rate of growth in glucose minimal broth. However, all of these grew within 100 hours incubation in glucose minimal medium on a reciprocal shaker at 37°C. Approximately 80 per cent reached full growth within 36 hours under these conditions. Therefore, none of the mutants seemed sufficiently stable or non-leaky for enzyme studies and transduction experiments.

Thus, after spending considerable time in isolating a few stable auxotrophs, a decision had to be made. One could spend several years utilizing the known techniques and perhaps map one pathway in P. aeruginosa or one could attempt to develop selective methods for mutant isolation and, if successful, possess the techniques necessary for

mapping any P. aeruginosa pathway in a much shorter length of time. The latter route was chosen and studies of prototroph and auxotroph survival in minimal medium containing antibiotics were initiated.

Survival Curves and Reconstruction Experiments

It was considered imperative that a broth enrichment procedure be developed to increase the mutant to prototroph ratio (M:P ratio) and to eliminate the more leaky mutants so that stable mutants might be isolated efficiently in high enough numbers to allow genetic analysis. Lederberg and Zinder (1948) designed reconstruction experiments to monitor mutant and prototroph survival in penicillin enrichments for mutants of E. coli. In experiments of this nature, two cell populations, prototroph and mutant, are mixed at a constant M:P ratio (1:100 or 1:1,000), exposed to selective conditions, and the final M:P ratio determined. This allows the investigator to determine more reliably the effects of a selective procedure on both the mutant and prototroph populations.

A modification of this procedure in the form of viable count curves, or survival curves, was designed to monitor a test system in greater detail. Rather than determine the effects of selective compounds, e.g., antibiotics, on the separate prototroph and auxotroph populations, a controlled system which would more closely mimic actual broth

enrichments was considered to possess many advantages.

Amino acid auxotrophs were utilized in all reconstruction experiments and survival curves. Since catabolic pathways utilize milligram quantities of a substrate and auxotrophs require nanogram to microgram quantities of most nutrients, the auxotroph system was considered a more sensitive monitor of syntrophic growth and perhaps of the quality of mutants which could be isolated.

A mixture of six previously isolated auxotrophs was utilized as the mutant population. Initially, two auxotrophs requiring methionine, two requiring phenylalanine, one requiring tryptophan and one requiring leucine composed the mutant population. In later experiments, the mixture was changed to six mutants requiring arginine.

An M:P ratio of 1:100 was established by mixing nutrient broth-grown auxotrophs in equal proportions, washing with minimal medium by centrifugation and resuspending to a given optical density with minimal medium. In later experiments, equal portions of a 1.0 ml slant wash of each mutant were mixed prior to growth in nutrient broth. A nutrient broth-grown prototroph culture was likewise washed and resuspended. The prototroph culture was inoculated into the test system to a final concentration of 3.0×10^7 cells per ml and a 1 to 100 dilution of the mutant population was likewise inoculated to a final concentration of 3.0×10^5 cells per ml. A 10^{-4} dilution of the test system was prepared and duplicate 0.1 ml samples were spread on nutrient

agar plates to check the initial viable cell count.

The survival of the prototroph and auxotroph (mutant) populations was monitored by diluting 0.1 ml samples of the experimental system and spreading 0.1 ml of appropriate dilutions in duplicate on two plate sets; one plate set was designed to support the growth of both the mutant and prototroph populations and the other plate set was designed to support only prototroph growth, i.e., it did not supply the nutrients required by the auxotrophs present in the experimental system. As the work progressed, it became apparent that the first plating system used was not satisfactory for all antibiotics used in reconstruction experiments. Therefore, the plate sets were changed as explained in a following section.

Penicillin Survival Curves

The survival of prototroph and auxotroph populations was monitored by spreading 0.1 ml of appropriate dilutions in duplicate on nutrient agar and glucose minimal plates. The difference between the average viable counts on nutrient agar and glucose minimal medium was the auxotroph determination since the auxotrophs could not form colonies on a minimal medium, whereas all strains grew well on the nutrient agar plates. The auxotroph determination could not be considered reliable until it amounted to approximately 10 per cent or more of the total NA colony count and, thus, each auxotroph curve shown is initially void of determinations

until the prototroph viable count decreases.

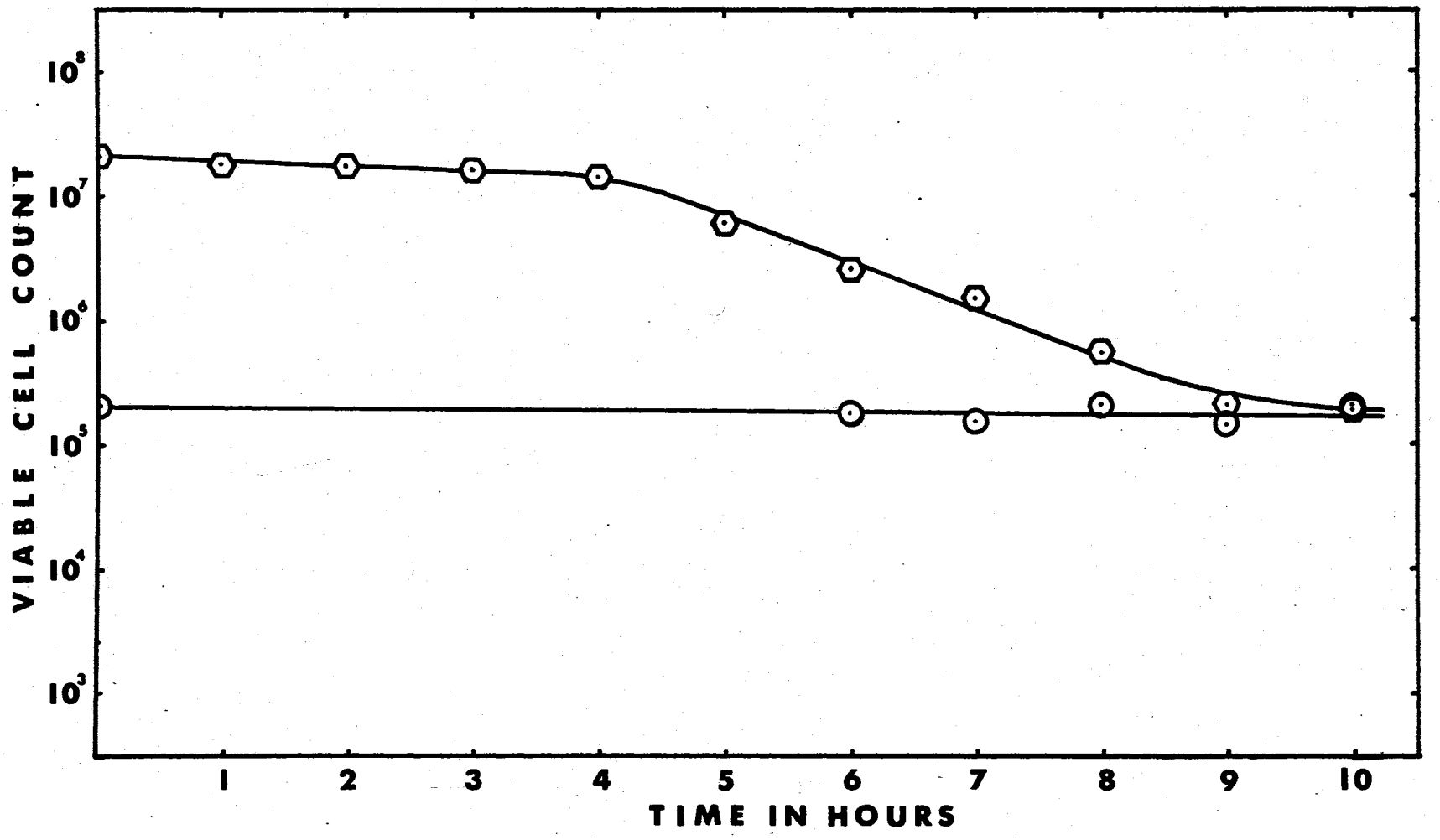
A typical penicillin G survival curve utilizing 50,000 units of antibiotic per ml is shown in Figure 8. Prototroph decay began at approximately four hours and continued for another five hours, during which time the auxotroph to prototroph ratio was increased by two logs (100X). The auxotroph curve was quite stable. These data indicate that penicillin G is a good antibiotic for selection but possesses only moderate enrichment potential even when used in very high concentrations.

Both the original broth enrichments for E. coli utilized 300 units per ml of benzylpenicillin (Davis, 1948; Lederberg and Zinder, 1948), as compared to 50,000 units per ml in this experiment (Bruce, 1965), in obtaining two to three logs of selective enrichment in reconstruction experiments. This is an indication of the possible magnitude of the generally recognized resistance of P. aeruginosa to antibiotics.

In considering possible ways to improve the selective value of penicillin G for further experimentation, several points were noted. Penicillin G did selectively kill only the growing bacterial population and auxotroph recovery was adequate. A quantity of the antibiotic could have been destroyed during the initial four-hour lag prior to prototroph decay. Auxotroph stability indicated the possibility of further selection by a second antibiotic addition at eight or nine hours without auxotroph decay.

Figure 8. Survival of PA-1 and Mutant Mixture Populations in Glucose Minimal Medium Containing Penicillin G

Nutrient broth-grown cells were harvested, washed, and resuspended to 3.0×10^7 cells per ml for the prototroph, PA-1, population. The mutant mixture was added to a final total concentration of 3.0×10^5 cells per ml in 10 ml of 0.5 per cent glucose minimal medium containing 50,000 units per ml penicillin G in a 20 mm test tube. Viable cell counts were determined by diluting a 0.1 ml sample each hour and plating appropriate dilutions in duplicate on nutrient agar and glucose minimal plates. The prototroph curve was determined by viable counts on glucose minimal medium and the auxotroph curve (mutant mixture) by determining the difference in the average viable count on nutrient agar and on glucose minimal plates. The viable cell count per ml of enrichment broth is plotted against time for PA-1 (⊙) and the mutant mixture (⊙).


VIABLE CELL COUNT
10^8
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

An experiment was run to determine the effect of a second penicillin addition and more vigorous aeration. Identical cell concentrations and media were used; however, the 10 ml volume was shaken in a 125 ml flask for more vigorous aeration. A second penicillin G addition of identical concentration was added when, according to Figure 8, 80 per cent of the prototroph population had died; this was determined to be at approximately six hours.

As can be seen in Figure 9, two logs of auxotroph decay were observed while four logs of prototroph decay occurred. The auxotroph determinations at hours five and six indicate loss of auxotroph viability prior to the second antibiotic addition. Since this did not occur under the conditions previously used, it suggested that excessive aeration in the presence of a carbon and energy source might be the primary factor in the linear auxotroph decay. Linear decay in the presence of substrate under starvation conditions has been described previously (Postgate and Hunter, 1962). Another observation of importance was the effect of the second antibiotic addition on auxotroph decay. No effect was observed on the rate of auxotroph decay; however, a second phase of prototroph decay nearly as extensive as the initial phase of decay was immediately initiated.

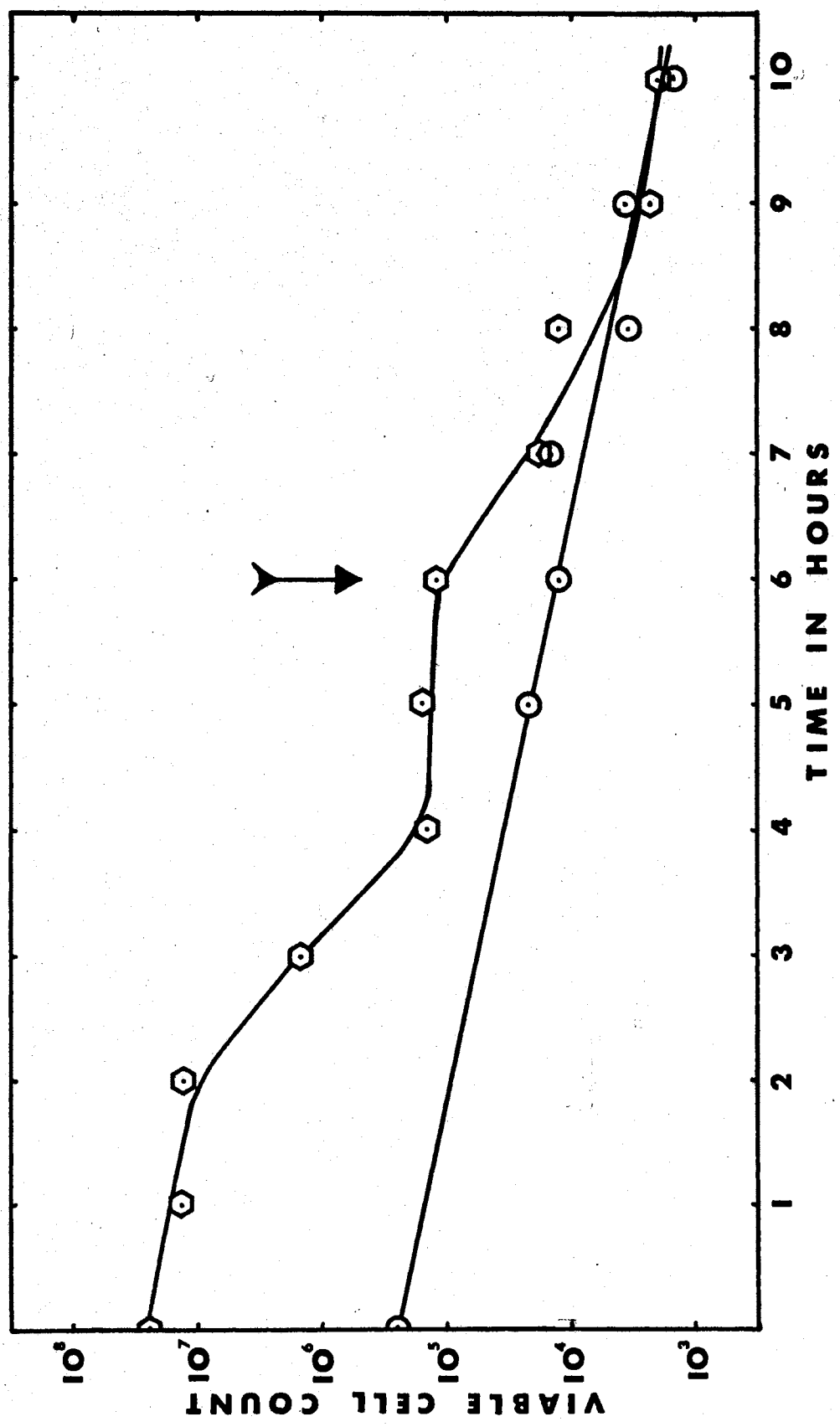
Plate Systems for Monitoring

Viable Cell Count

Initially the two plate sets used for monitoring

Figure 9. Survival of Prototroph and Mutant Mixture Populations in Highly Aerated Glucose Minimal Medium Containing Penicillin G With a Second Penicillin G Addition

Nutrient broth-grown cells were harvested, washed, and resuspended to 3.0×10^7 cells per ml for PA-1 and the mutant mixture was added to a final concentration of 3.0×10^5 cells per ml in 10 ml of 0.5 per cent glucose minimal medium containing 50,000 units per ml penicillin G. The culture was aerated in a 125 ml flask to which an additional 50,000 units per ml penicillin G was added at six hours. Viable cell counts were determined on nutrient agar and glucose minimal medium as described in Figure 4. All viable counts are in units of cells per ml of enrichment broth and are plotted against time for PA-1 (⊙) and the mutant mixture (⊙).


VIABLE CELL COUNT
10^8
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

survival curves were nutrient agar (NA) and 0.5% glucose minimal medium. These were found to be adequate when penicillin G was being tested as a selective antibiotic. However, experiments with the next antibiotic to be tested, carbenicillin (α-carboxybenzylpenicillin), revealed problems which can occur if a culture is grown on a rich medium after being subjected to most antibiotics (Table I). Although the experiment was a complete failure in terms of its design, one of the most important clues as to why mutants may not be isolated even though they are present in the broth enrichment may be observed in these data.

In this experiment, carbenicillin was added to a final concentration of 200μg per ml in minimal medium containing 1.0 per cent glucose and 20.0 per cent sucrose. The total volume was 10 ml in a 125 ml Ehrlenmeyer flask. As shown in Table I, the initial viable count was approximately 1.9×10^7 cells per ml and the viable count from glucose minimal plates at one hour was 1.80×10^7 cells per ml, indicating little if any prototroph death. However, the viable count determined from nutrient agar plates at one hour was 1.34×10^7 indicating a 30 per cent loss in viable count as opposed to a 5 per cent loss as determined from glucose minimal plates. Since the first hour plating errors are approximately 11 (colonies per plate) for NA and 13 for GM, the duplicate plate counts vary by less than the plating error, both plate sets were spread from the same dilution tube at approximately the same time, and the average

TABLE I

DATA FROM A CARBENICILLIN TREATED CELL SURVIVAL CURVE INDICATING CELL DEATH AFTER PLATING ON NUTRIENT MEDIUM

Time in Hours	Viable Colony Counts on Nutrient Agar	Average	Viable Colony Counts on Glucose Minimal Medium	Average	Total Dilution
1	130 138	134	176 184	180	10^{-5}
2	102 91	96	137 115	126	10^{-5}
3	82 76	79	94 90	92	10^{-5}
4	47 49	48	59 59	59	10^{-5}
5	202 250	226	416 410	413	10^{-4}
6	267 234	247	188 187	187	10^{-4}
7	241 237	239	197 268	232	10^{-4}
8	243 290	266	409 394	401	10^{-4}
9	73 43	58	74 77	75	10^{-4}
Initial Viable Count	192 187	189	-	-	10^{-5}

Glucose minimal medium containing 200μg per ml carbenicillin, a prototroph population of 1.9×10^{7} cells per ml and an auxotroph mixture of approximately 1.9×10^{5} cells per ml was the experimental system.

difference in the two plate sets is 46, one can only conclude that more death had occurred on the nutrient agar plates than on the glucose minimal plates. If death occurs on the nutrient agar plates, one must question whether the glucose minimal plates indicate the actual viable count as the sample was taken or a lower value due to some death as growth initiates on these plates. It can also be seen that viable counts on the two plate sets for hours 2, 3, 4, 5, 8, and 9 reveal the same phenomenon. Thus, the NA plates were no longer used and 0.5 per cent glucose minimal medium enriched with 45μg per ml acid-hydrolyzed casamino acids plus 2μg per ml tryptophan (ESG) was substituted. These plates were tested for plating antibiotic-treated cultures in an experiment using 400μg per ml dihydrostreptomycin plus 25,000 units per ml penicillin G (Table II). Viable cell counts were again lower on the supplemented glucose minimal medium than on the glucose minimal medium.

In an attempt to increase plating efficiency, a less readily metabolizable carbon source, glycerol, was substituted for the glucose in the minimal and enriched minimal plates. These plates were found acceptable for use in experiments with antibiotic-treated cultures with few exceptions. In enrichment systems containing dihydrostreptomycin, the toxicity problems were solved by also utilizing glycerol instead of glucose as the carbon and energy source in the enrichment medium as explained in the following section.

TABLE II

DATA FROM A DIHYDROSTREPTOMYCIN PENICILLIN TREATED CELL SURVIVAL CURVE INDICATING CELL DEATH ON CASAMINO ACID ENRICHED GLUCOSE PLATES

Time in Hours	Viable Cell Counts on Enriched Glucose Minimal Plates		Viable Cell Counts on Glucose Minimal Plates		Total Dilution
2	45 48	46.5	53 47	50.0	10^{-5}
4	368 418	393.0	536 512	524.0	10^{-3}
6	138 163	150.5	156 190	173.0	10^{-3}
8	57 43	50.0	70 87	78.5	10^{-3}
10	50 66	58.0	56 75	65.5	10^{-3}

Initial Cell Concentration Determined by Optical Density:
Prototroph Population 2.95×10^{7} cells per ml
Mutant Mixture 2.90×10^{5} cells per ml

Glucose minimal medium containing 400µg per ml dihydrostreptomycin, 25,000 units per ml penicillin G, a prototroph population of approximately 3.0×10^{7} cells per ml and auxotroph mixture of approximately 3.0×10^{5} cells per ml was the experimental system.

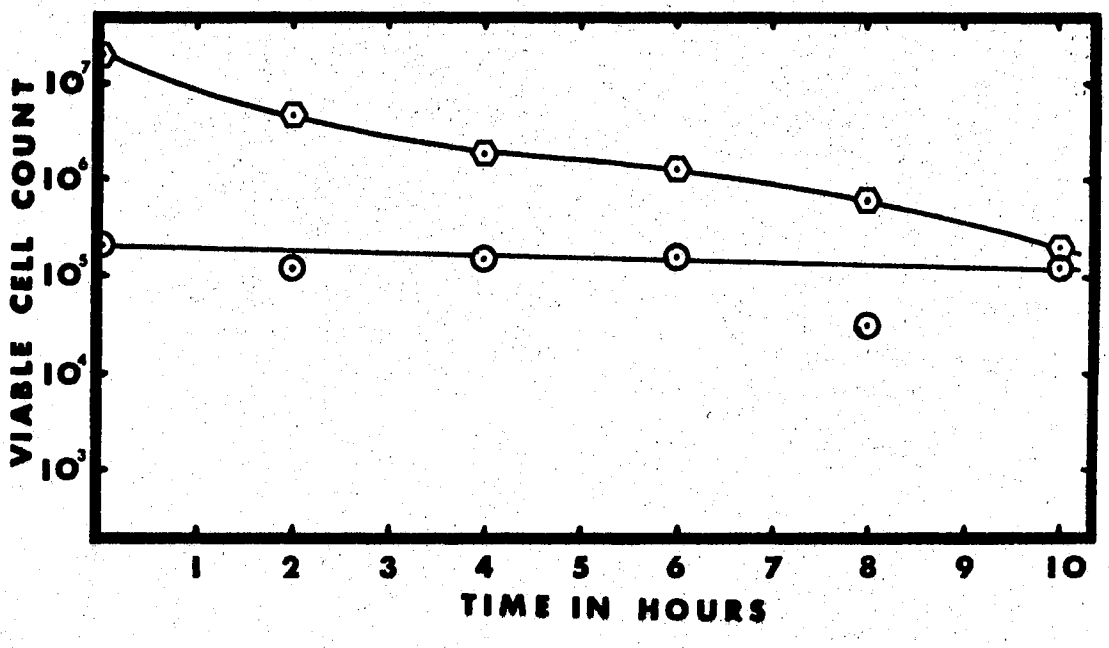
Dihydrostreptomycin Studies

Dihydrostreptomycin was thoroughly tested under various conditions. Its latent toxicity, i.e., its antibacterial activity on cells growing after removal of the antibiotic by washing or diluting and plating, indicated that it would probably have little value as a selective antibiotic. However, the use of this antibiotic was important in the development of an adequate plate system for monitoring viable cell counts under slightly to highly toxic conditions.

When dihydrostreptomycin was tested for selection of auxotrophs in glucose minimal medium, followed by plating on glucose minimal medium and enriched glucose minimal medium, the prototroph and auxotroph decay rates appeared identical after two hours and problems with cell death on supplemented glucose minimal plates occurred. The use of 20 per cent sucrose in the enrichment medium decreased the rate of decay of both prototroph and auxotroph curves; however, the rate of decay of each population still appeared identical after two hours. Harvesting, washing, and resuspension in fresh enrichment medium every two hours improved the selective properties of dihydrostreptomycin; however, the experiments lacked adequate reproducibility. Lowering the temperature of incubation during enrichment had little if any effect on selection. Substituting glycerol for glucose as the carbon and energy source for the enrichment culture greatly decreased the rate of auxotroph decay, while maintaining an adequate prototroph decay rate (Figure 10). Substituting

Figure 10. Survival of Prototroph and Auxotroph Populations in Glycerol Minimal Medium Containing Dihydrostreptomycin

Nutrient broth-grown cells were harvested, washed, and resuspended to 3.0×10^7 cells per ml for PA-1 and the mutant mixture was added to a final concentration of 3.0×10^5 cells per ml in 10 ml of 0.5 per cent glycerol minimal medium containing 400µg per ml dihydrostreptomycin. Viable cell counts were determined by diluting a 0.1 ml sample every two hours and plating appropriate dilutions in duplicate on glycerol minimal medium enriched with 45µg per ml acid hydrolyzed casamino acids plus 2µg per ml tryptophan and on glycerol minimal medium. The prototroph curve, PA-1, was determined by viable counts on the glycerol minimal plates and the auxotroph curve by subtracting the average prototroph viable count from the average total viable counts on the enriched glycerol minimal plates. The viable cell count per ml of enrichment broth is plotted against time for PA-1 (⊙) and the mutant mixture (⊙).


VIABLE CELL COUNT
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

glycerol for glucose in the plate sets for monitoring viable cell count improved the survival curves, but inadequate curves were obtained unless glycerol or a carbon and energy source used at a similar metabolic rate by the cell was employed as the carbon and energy source for both the enrichment system and the monitoring system. Some auxotroph death occurred under even the most protective conditions. Therefore, this antibiotic was not considered to be particularly useful for selection of mutants of P. aeruginosa.

Use of D-Cycloserine in Enrichment

D-cycloserine is an antibiotic possessing the necessary properties for use in the broth selection of P. aeruginosa mutants. It competitively inhibits two enzymes in the bacterial cell wall biosynthetic pathway. It is transported by the D-alanine transport system, being concentrated intracellularly where the synthesis of uridine nucleotide precursors for cell wall synthesis occurs.

Initially, D-cycloserine was used in several reconstruction experiments to determine its potential as an antibiotic for broth enrichments and to establish inhibitory concentration levels. The standard prototroph and auxotroph cell concentrations, 3.0×10^7 and 3.0×10^5, respectively, were utilized; however, six arginine auxotrophs were introduced as the mutant mixture.

Several dilutions of eight-hour samples were plated in duplicate on glycerol minimal medium and glycerol minimal

medium supplemented with 5.0μg per ml arginine. It was intended to determine the auxotroph concentration by subtracting the viable count on glycerol minimal plates from the plate count on glycerol minimal plus arginine. However, after incubation, the viable cell counts were approximately equivalent on the enriched and minimal plates, with very small colonies beginning to appear. The two colony sizes were especially noted in three of the sixteen plate sets due to the predominance of small colonies. The large colony morphology was that normally observed in previous enrichment experiments.

The initial impression was that dissociation had occurred in some of the experimental systems since the large colony counts on all plates were approximately equal and the small colony counts were likewise approximately equal on all plates at a given dilution. However, one observation did seem important; the three experimental systems having small colony predominance were those which had been treated with the highest concentrations of D-cycloserine, i.e., 500μg per ml, 600μg per ml, and 700μg per ml of D-cycloserine.

A glycerol minimal plate and three glycerol-arginine plates containing approximately 150 to 200 colonies per plate, with the small colony predominating, were replicated onto glucose minimal medium and nutrient agar plates. After one day of incubation at 37°C, the large colony type appeared to be prototrophs and the small colony type auxotrophs. Eleven colonies of each size were picked from

eleven different plates and inoculated onto nutrient agar slants. After incubation at room temperature for one day, the slants were soaked with 2.0 ml of minimal medium and one drop used to inoculate glucose minimal and glucose arginine liquid media. These were aerated on a reciprocal shaker at 37°C for one day prior to scoring for growth optically. The small colonies grew in glucose minimal plus arginine but did not grow in glucose minimal medium, while the large colonies grew in both media. The plates were then counted as indicated by the preceding broth tests, the small colonies being counted as auxotrophs and the large colonies as prototrophs.

Several studies were made using penicillin G and D-cycloserine in combination for enrichment. Of the various penicillin-D-cycloserine combinations and D-cycloserine concentrations, D-cycloserine in the range 500μg per ml to 700μg per ml provided the better enrichments with 600μg per ml of D-cycloserine yielding three logs of selective enrichment and 700μg per ml appearing more selective than 500μg per ml. Thus, a series of survival curves was considered the most informative route in determining the D-cycloserine concentration of greatest selective value, with little or no auxotroph decay, and in determining the best time interval for plating.

In a series of survival curve experiments testing 580, 600, 620, 640, 660, and 680μg per ml D-cycloserine concentrations, two experiments were run in which concentrations of 620μg per ml and 640μg per ml of D-cycloserine were

initially added and 60μg per ml were added to each at six hours. The sixth hour was chosen simply because it was the time of first sampling and the influence of these additions could thereby be observed with one viable count determination prior to the addition. The effect of the addition could be determined using the six-hour counts as a reference in comparing the two curves of identical initial D-cyloserine concentration. The survival curves were obtained by diluting and plating 0, 6, 8, 9, and 10-hour samples on glycerol minimal medium containing 5μg per ml of arginine and counting the small colonies as auxotrophs and the large colonies as prototrophs. Thirty large and thirty small colonies were picked at random from each experimental system and tested in broth by direct loop inoculation into glucose minimal medium, followed by aeration on a reciprocal shaker at 37°C for 24 hours. All small colonies tested failed to grow and all large colonies tested were completely grown in 24 hours. The tubes failing to grow in 24 hours were removed, supplemented with 20μg per ml of arginine from a sterile concentrated solution and reincubated for an additional 24 hours at which time growth had occurred in all tubes.

Survival curves for initial D-cycloserine concentrations of 580μg per ml and 600μg per ml demonstrated a variance in average viable counts for prototroph. This seemed to indicate a drop in antibiotic effectiveness of adequate dimension to allow some prototroph growth

(Figures 11 and 12). A gradual increase in auxotroph concentration also occurred after eight hours. This could be a demonstration of syntrophic growth (cross-feeding or utilization of compounds released by lysis) after the effective antibiotic concentration has decreased and/or auxotroph growth as a reflection of the very slightly leaky property demonstrated by each of the six arginine auxotrophs present. Since these auxotrophs appear as small colonies on glycerol minimal plus arginine plates only after incubation for 3.5 days at 37°C, it was considered probable that both phenomena might be contributing to the observed increase in the number of auxotrophs.

Increased stability in both the auxotroph and prototroph curves was noted as the initial D-cycloserine concentration was increased to 680µg per ml (Figures 11, 12, 13, 14, 15, 16, 17, 18). It might also be noted that the two curves were more stable when 60µg of D-cycloserine was added per ml at six hours (Figures 13 and 14, and 16 and 17). The stabilization of both the auxotroph and prototroph curves was considered most important in developing a reproducible broth enrichment. A special significance was placed on the stability of the auxotroph curve as it was hoped that a broth enrichment could yield mutants of good quality, i.e., not significantly leaky, by lysing those strains which would attempt to double during the course of an enrichment.

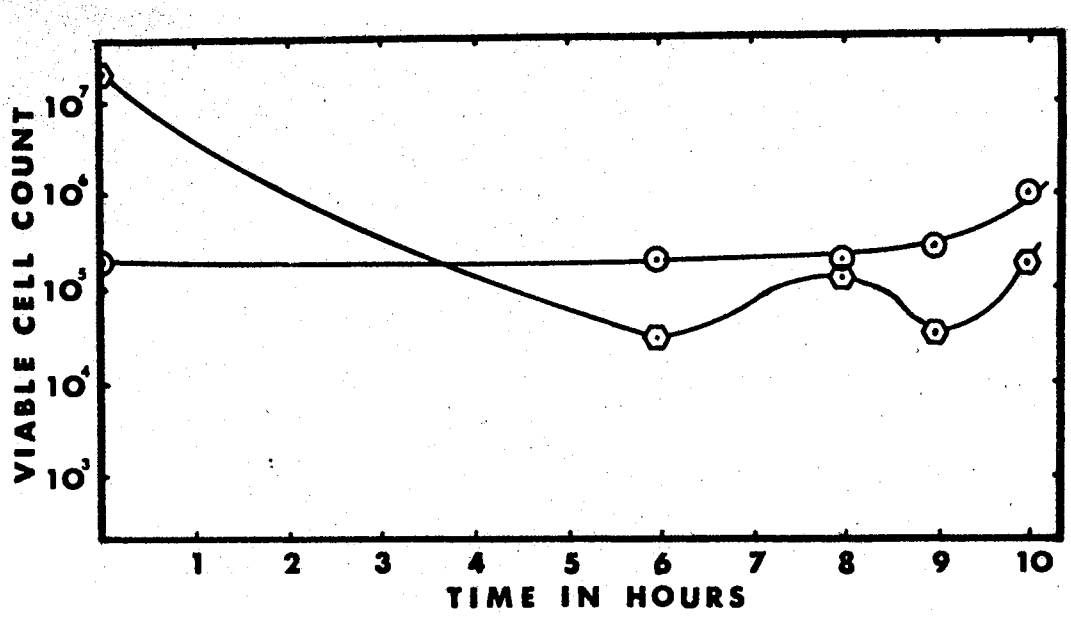
Although an initial D-cycloserine concentration of 680µg per ml produced the most stable auxotroph and

Figure 11. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 580μg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as described for D-cycloserine curves in Materials and Methods. Glucose minimal medium containing 580μg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 12. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 600μg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as described in Materials and Methods. Glucose minimal medium containing 600μg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

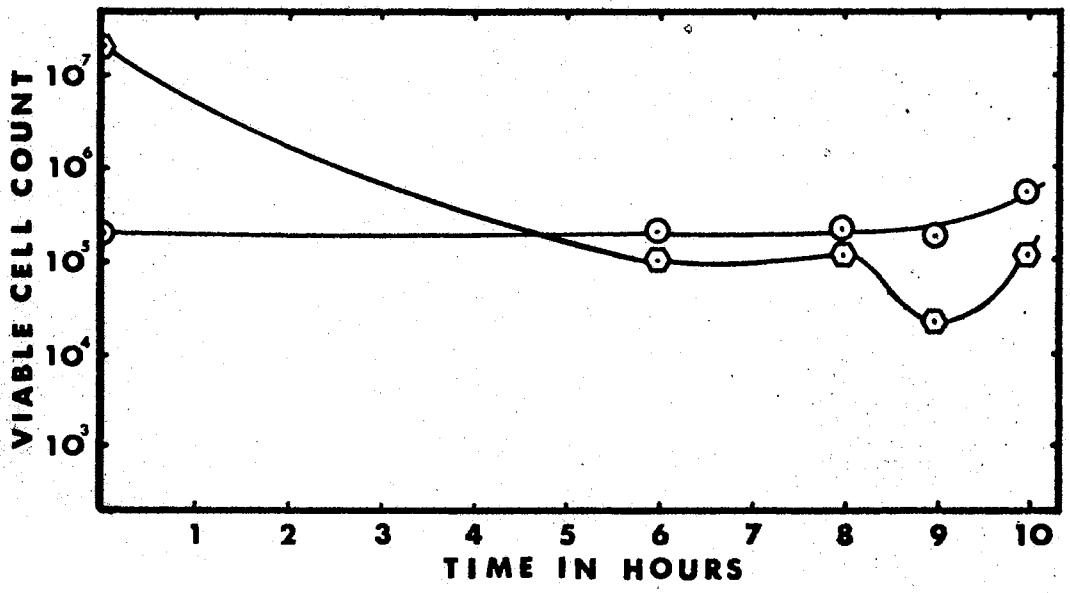
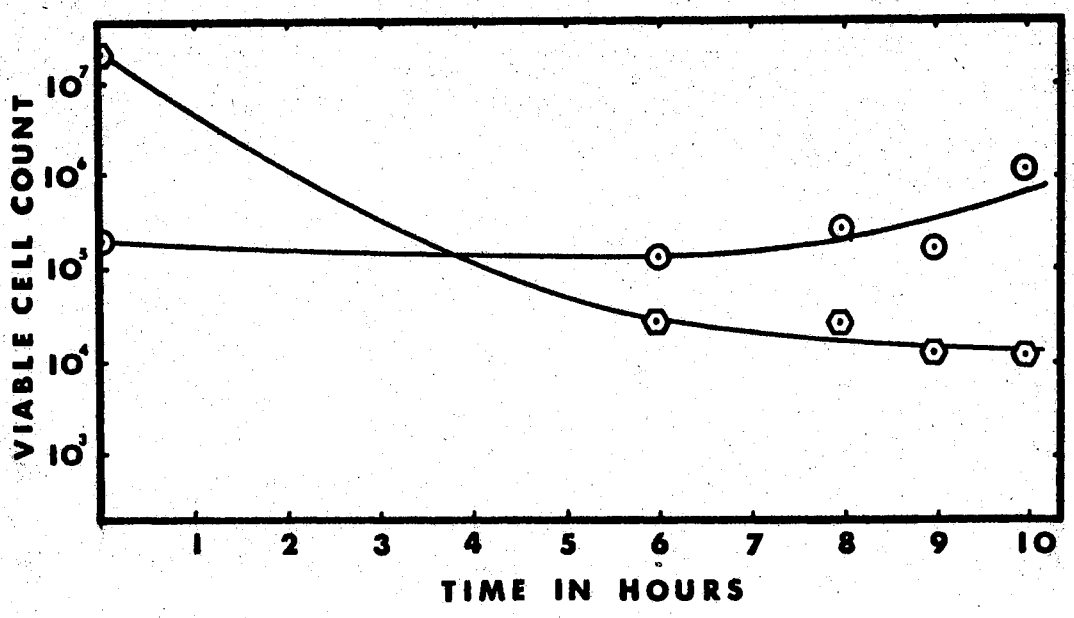

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

Figure 13. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 620μg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 620μg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 14. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 620μg per ml D-Cycloserine With a 60μg per ml D-Cycloserine Addition at Six Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 620μg per ml D-cycloserine with a 60μg per ml D-cycloserine addition at six hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

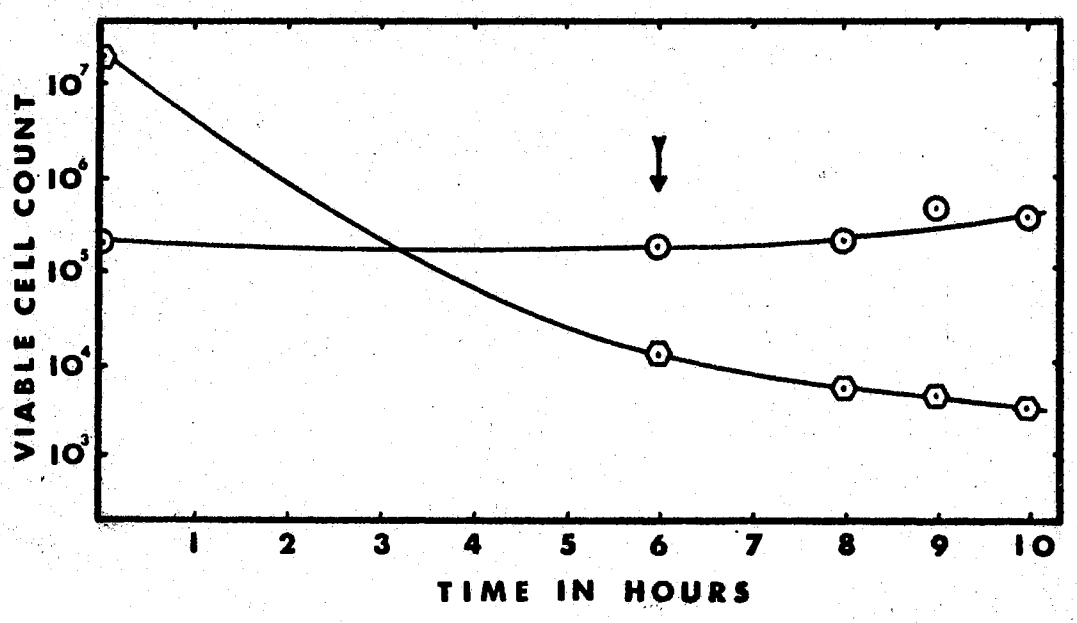
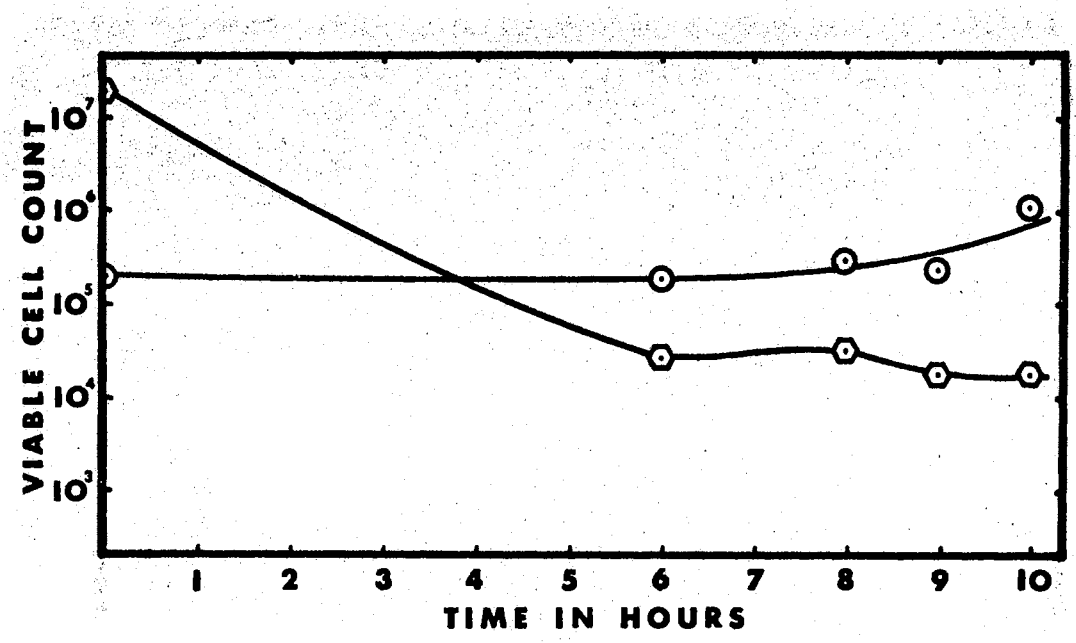

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

Figure 15. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 640µg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 640µg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 16. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 660µg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 660µg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

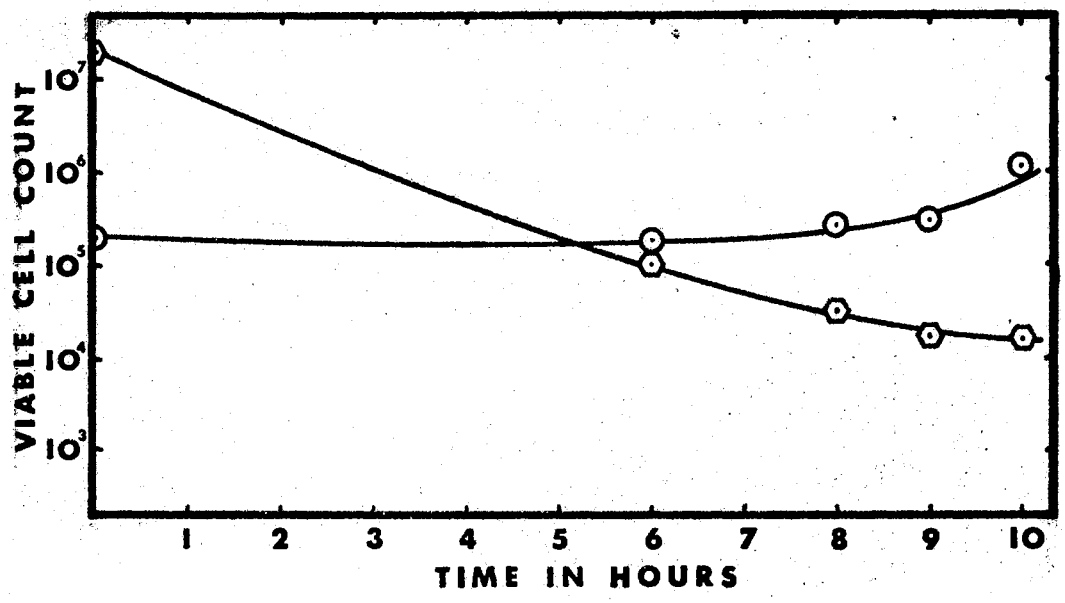
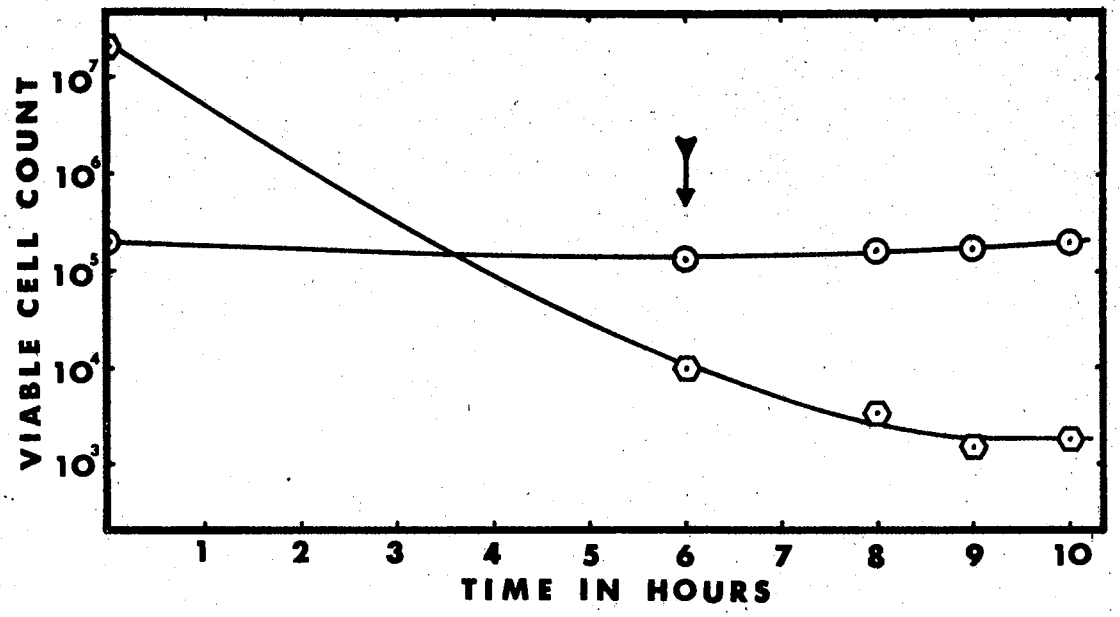

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

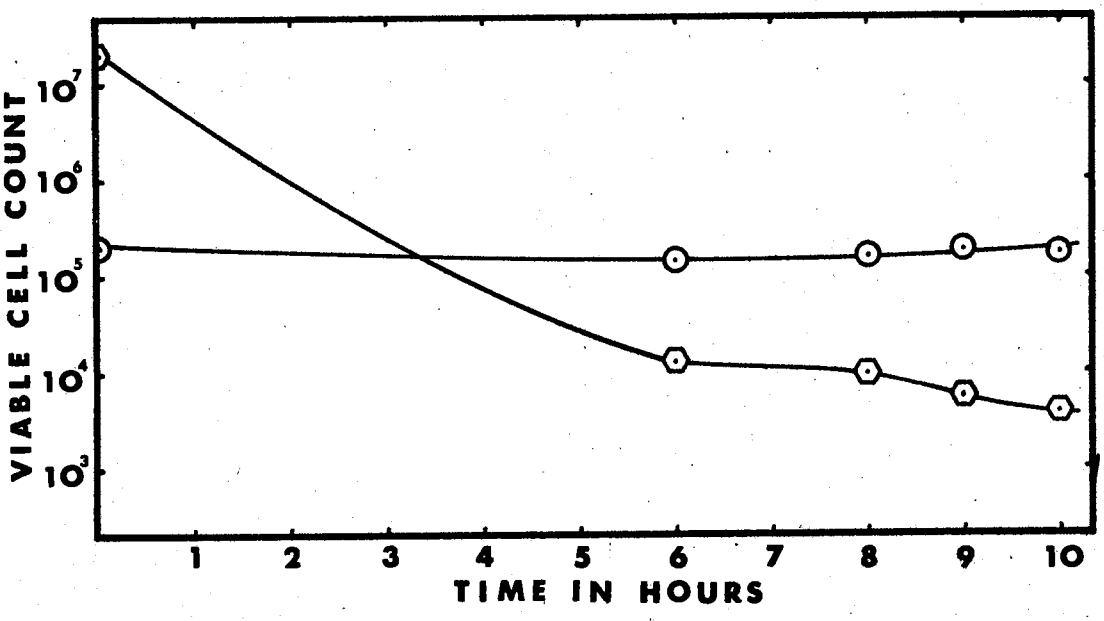
Figure 17. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 640μg per ml D-Cycloserine With a 60μg per ml D-Cycloserine Addition at Six Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 640μg per ml D-cycloserine with 60μg per ml D-cycloserine addition at six hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 18. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 680μg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 680μg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 6, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

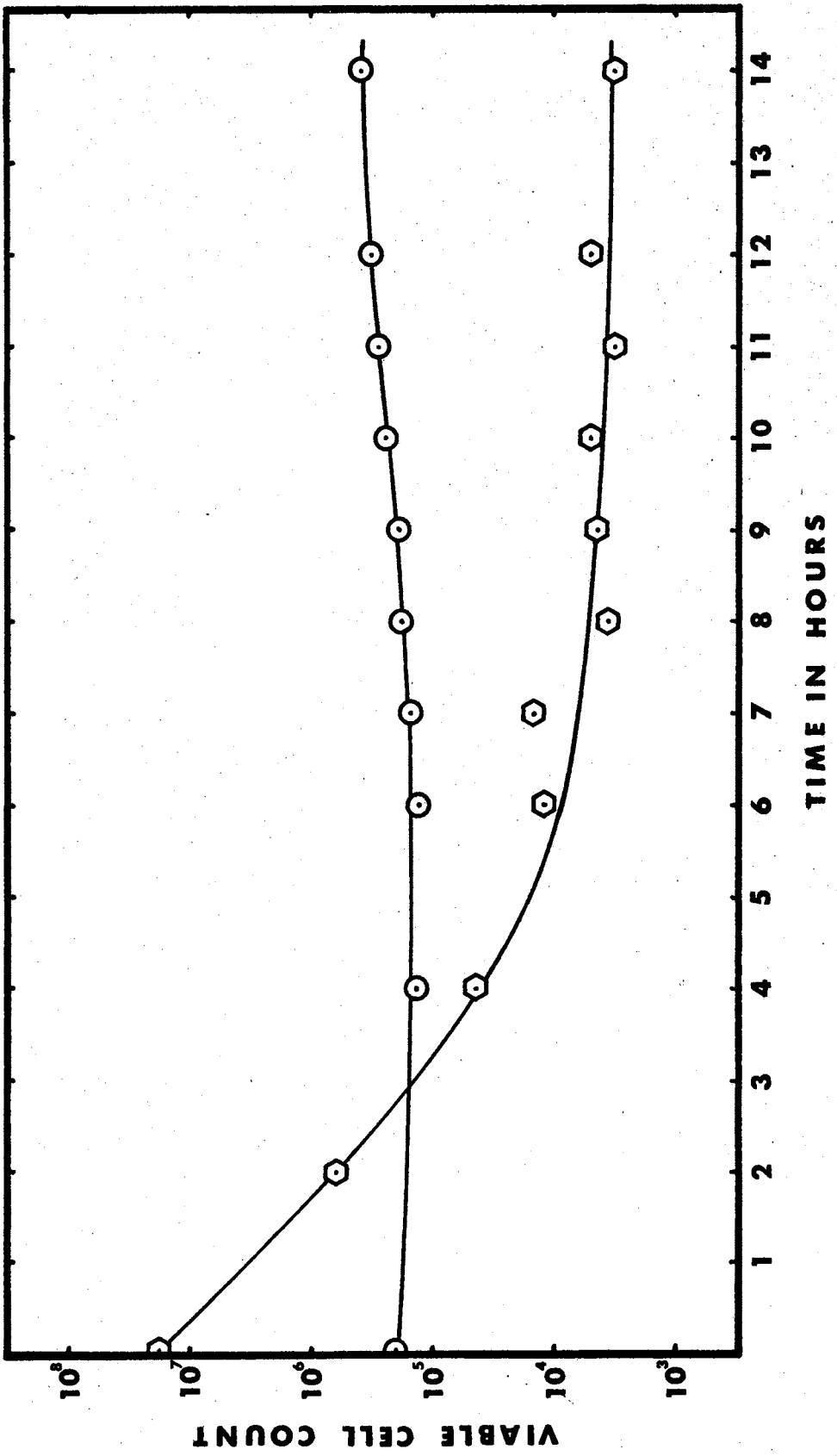
prototroph curves, a slightly inadequate D-cycloserine concentration, 650µg per ml, was selected for use in further testing the effects of second antibiotic additions since both the curve stability and the decrease in viability could serve as monitors in indicating the desirability of a particular system. An initial D-cycloserine concentration of 650µg per ml was chosen by comparing Figures 11 and 12. However, prior to determining the effects of adding various concentrations of D-cycloserine during the 650µg per ml enrichment, it was necessary to estimate the most desirable time for these additions. Two survival curves, at 640µg per ml and 660µg per ml, were run to determine the rates of initial prototroph decay, the approximate time of reaching a plateau for the prototroph curve, and the prototroph curve stability at extended time intervals. The curves for 660µg per ml are shown in Figure 19.

Greater than one log of prototroph decay was noted in both curves for the two-hour sample and a decrease in the rate of prototroph decay occurred in both after four hours. For this reason, four hours was selected as the time for the second antibiotic addition. Auxotroph and prototroph curve stability was also observed through 14 hours in the 660µg per ml curve. This stability from 8 to 14 hours was considered quite adequate in allowing considerable freedom in the time of plating broth enrichments.

With four hours considered an experimentally sound time for a second antibiotic addition, a series of survival curve

Figure 19. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 660µg per ml D-Cycloserine

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 660µg per ml D-cycloserine was the experimental system. Viable cell counts per ml of enrichment broth at 2, 4, 6, 7, 8, 9, 10, 11, 12, and 14 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10^8
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
TIME IN HOURS

experiments was run utilizing 650µg per ml of D-cycloserine as the initial antibiotic concentration and 150, 200, 250, 300, 350, and 400µg per ml of D-cycloserine as the antibiotic concentrations for the four-hour addition. Samples were diluted and plated on arginine enriched glycerol minimal medium at 0, 6, 7, 8, 9, and 10 hours (Figures 20, 21, 22, 23, 24).

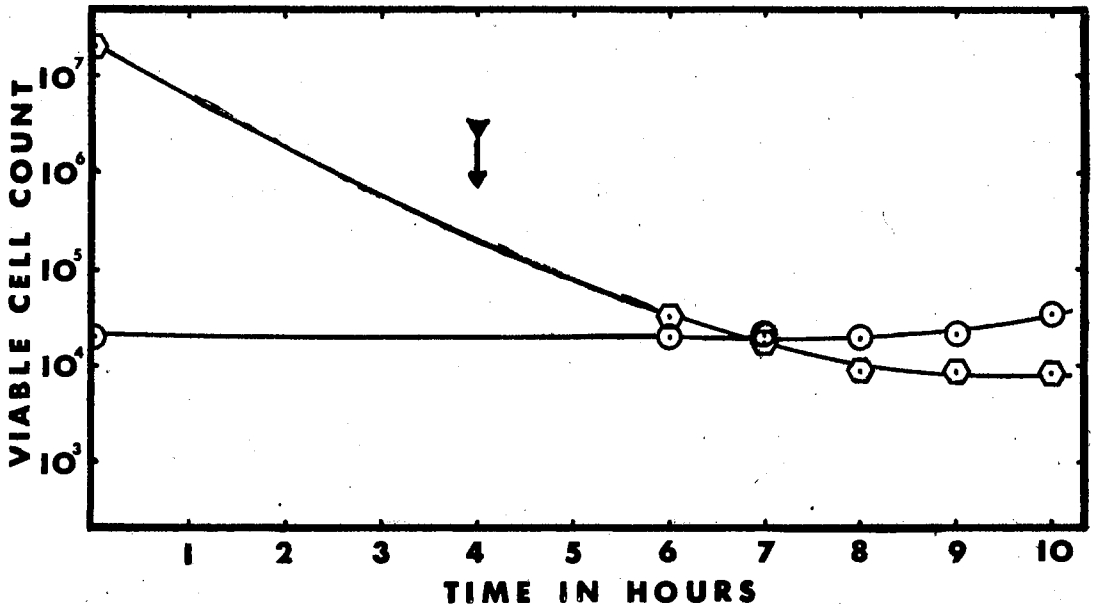
An increase in prototroph decay from three logs of selective killing to approximately four logs was observed when the second antibiotic addition was 350µg per ml or 400µg per ml of D-cycloserine with a slight tendency for the auxotroph curve to increase after eight hours of enrichment (Figure 24). A curve with a second addition of 200µg per ml D-cycloserine at three hours was also included and indicated the possibility of greater curve stability if the second antibiotic addition was earlier than the fourth hour (Figures 21 and 25). Therefore, two survival curves were run using 660µg per ml D-cycloserine as the initial concentration and second D-cycloserine additions of 350µg per ml were made at 2.5 and 3.5 hours, respectively (Figures 26 and 27). No greater curve stability was observed by an earlier second addition; however, 2.5 hours was chosen as the time for the second D-cycloserine additions merely for convenience. All double addition D-cycloserine enrichments hereafter utilized the conditions of the experiment shown in Figure 26, i.e., 660 and 350µg per ml concentrations, with the 350µg per ml being added at 2.5 hours.

Figure 20. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 150µg per ml D-Cycloserine Addition at Four Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 650µg per ml D-cycloserine with a 150µg per ml D-cycloserine addition at four hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 21. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 200µg per ml D-Cycloserine Addition at Four Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 650µg per ml D-cycloserine with a 200µg per ml D-cycloserine addition at four hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

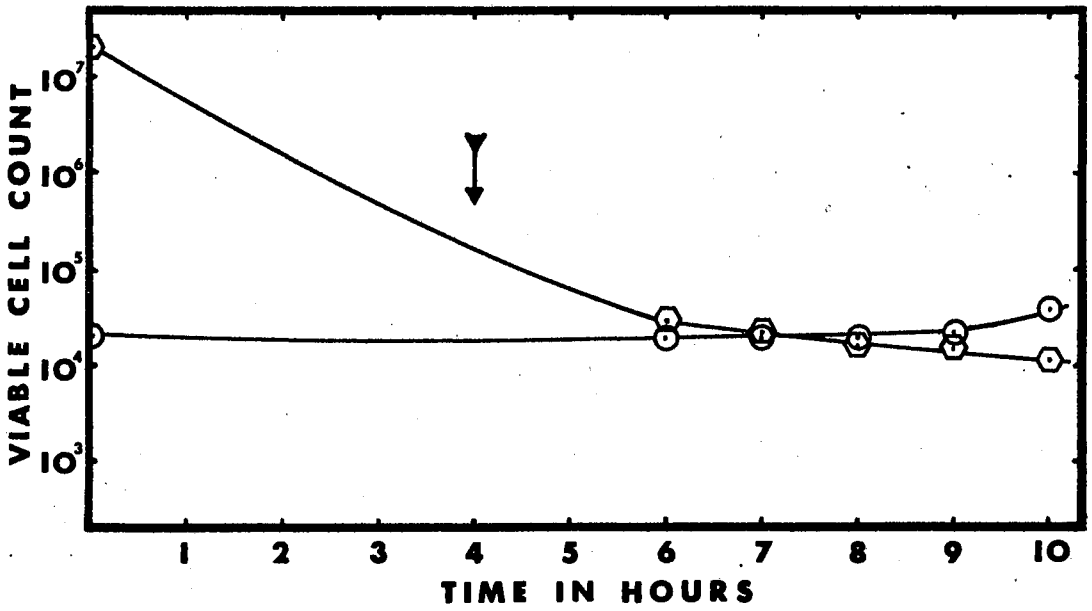
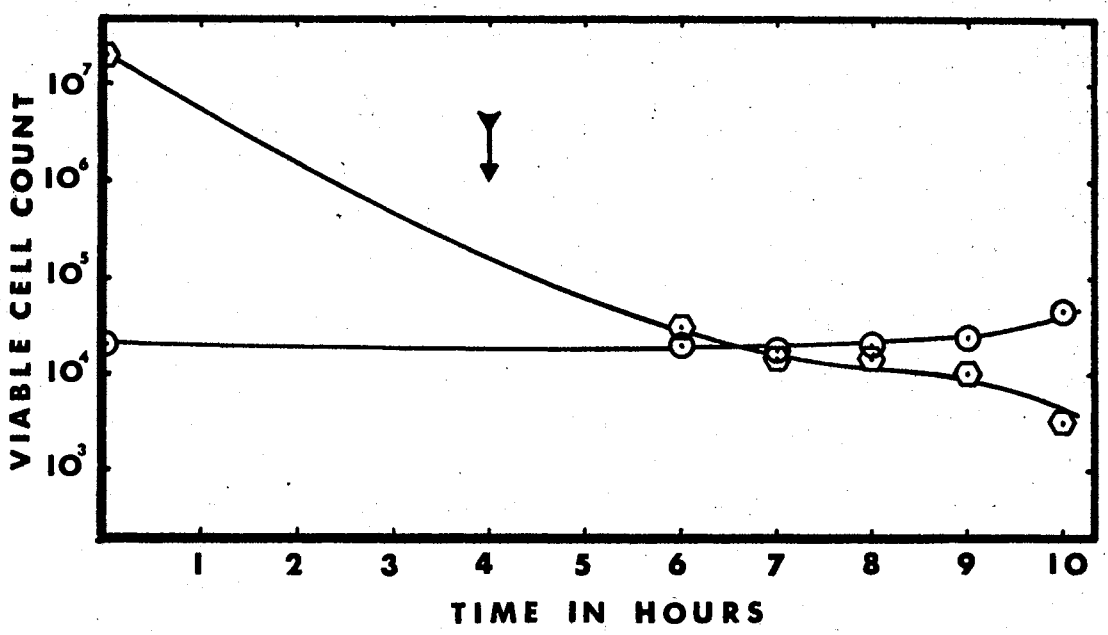

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1
2
3
4
5
6
7
8
9
10
TIME IN HOURS

Figure 22. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 250µg per ml D-Cycloserine Addition at Four Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 650µg per ml D-cycloserine with a 250µg per ml D-cycloserine addition at four hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 23. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 300µg per ml D-Cycloserine Addition at Four Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 650µg per ml D-cycloserine with a 300µg per ml D-cycloserine addition at four hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

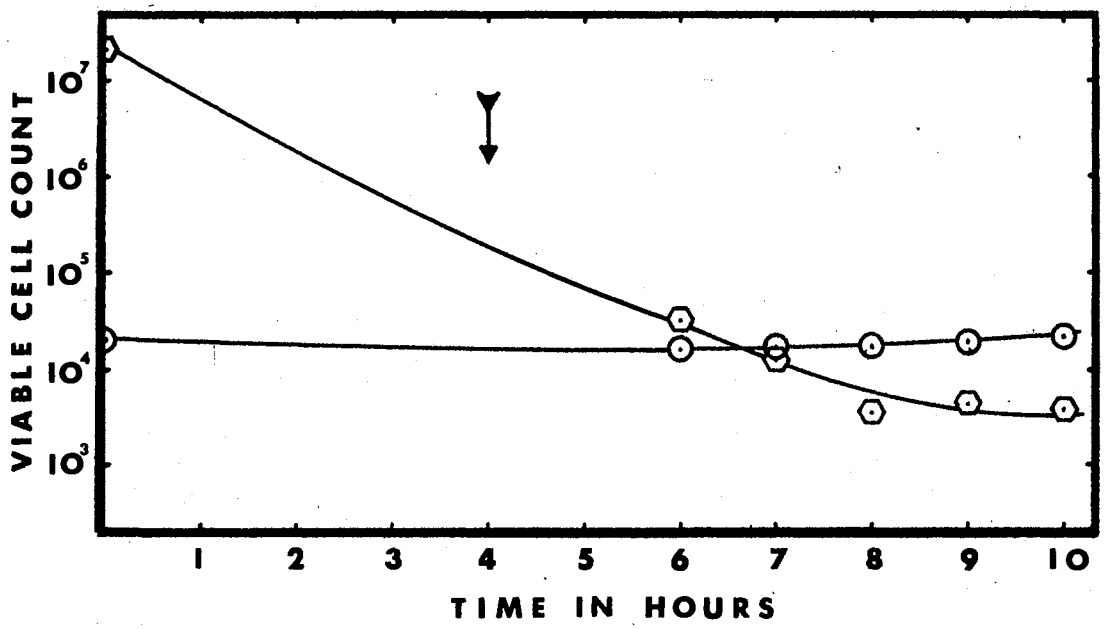
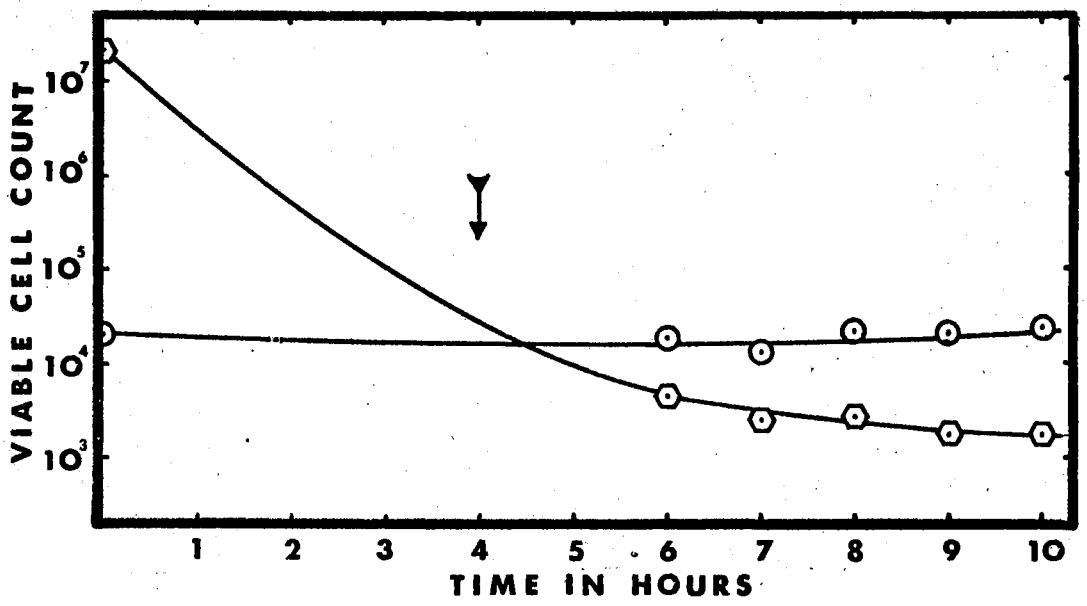

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

Figure 24. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 350µg per ml D-Cycloserine Addition at Four Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 650µg per ml D-cycloserine with a 350µg per ml D-cycloserine addition at four hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

Figure 25. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 650µg per ml D-Cycloserine With a 200µg per ml D-Cycloserine Addition at Three Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal containing 650µg per ml D-cycloserine with a 200µg per ml D-cycloserine addition at three hours was the experimental system. Viable cell counts per ml of enrichment broth at 6, 7, 8, 9, and 10 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

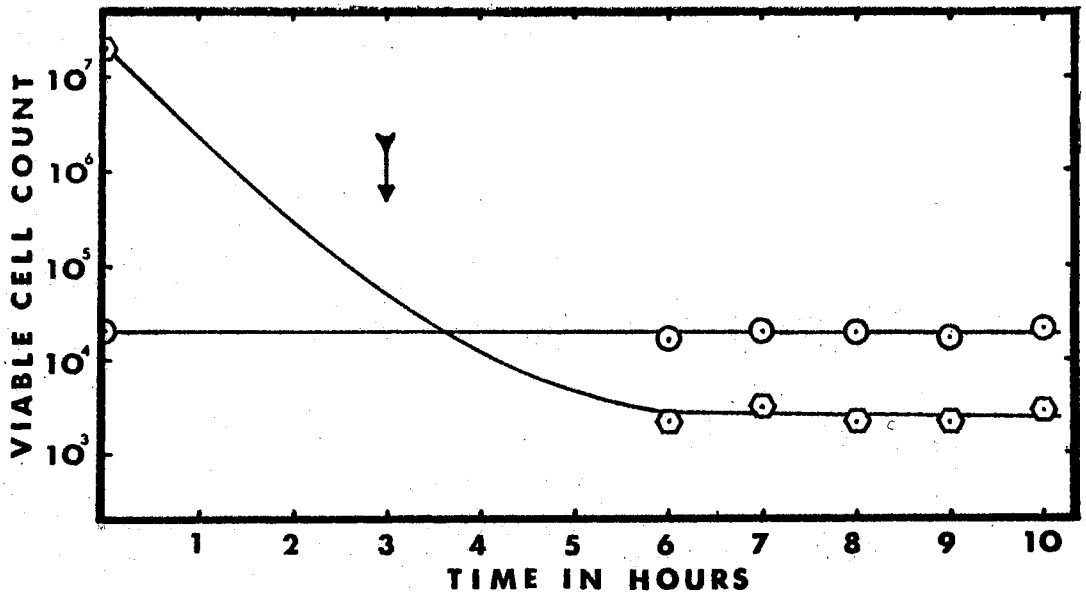

VIABLE CELL COUNT
10⁷
10⁶
10⁵
10⁴
10³
1 2 3 4 5 6 7 8 9 10
TIME IN HOURS

Figure 26. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 660µg per ml D-Cycloserine With a 350µg per ml D-Cycloserine Addition at Two and One-Half Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 660µg per ml D-cycloserine with a 350µg per ml D-cycloserine addition at 2.5 hours was the experimental system. Viable cell counts per ml of enrichment broth at 2, 4, 5, 6, 7, 8, 9, 10, and 11 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).

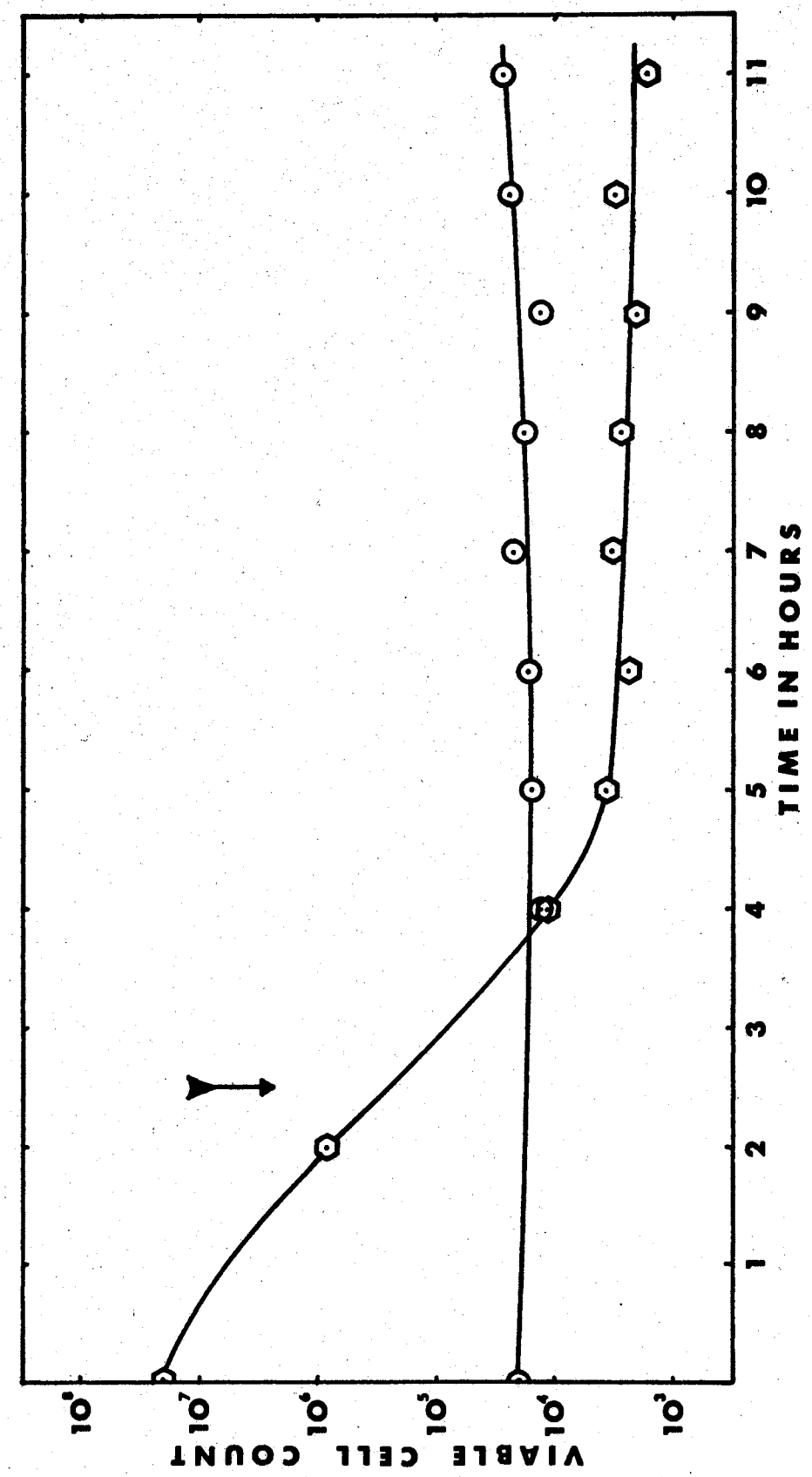
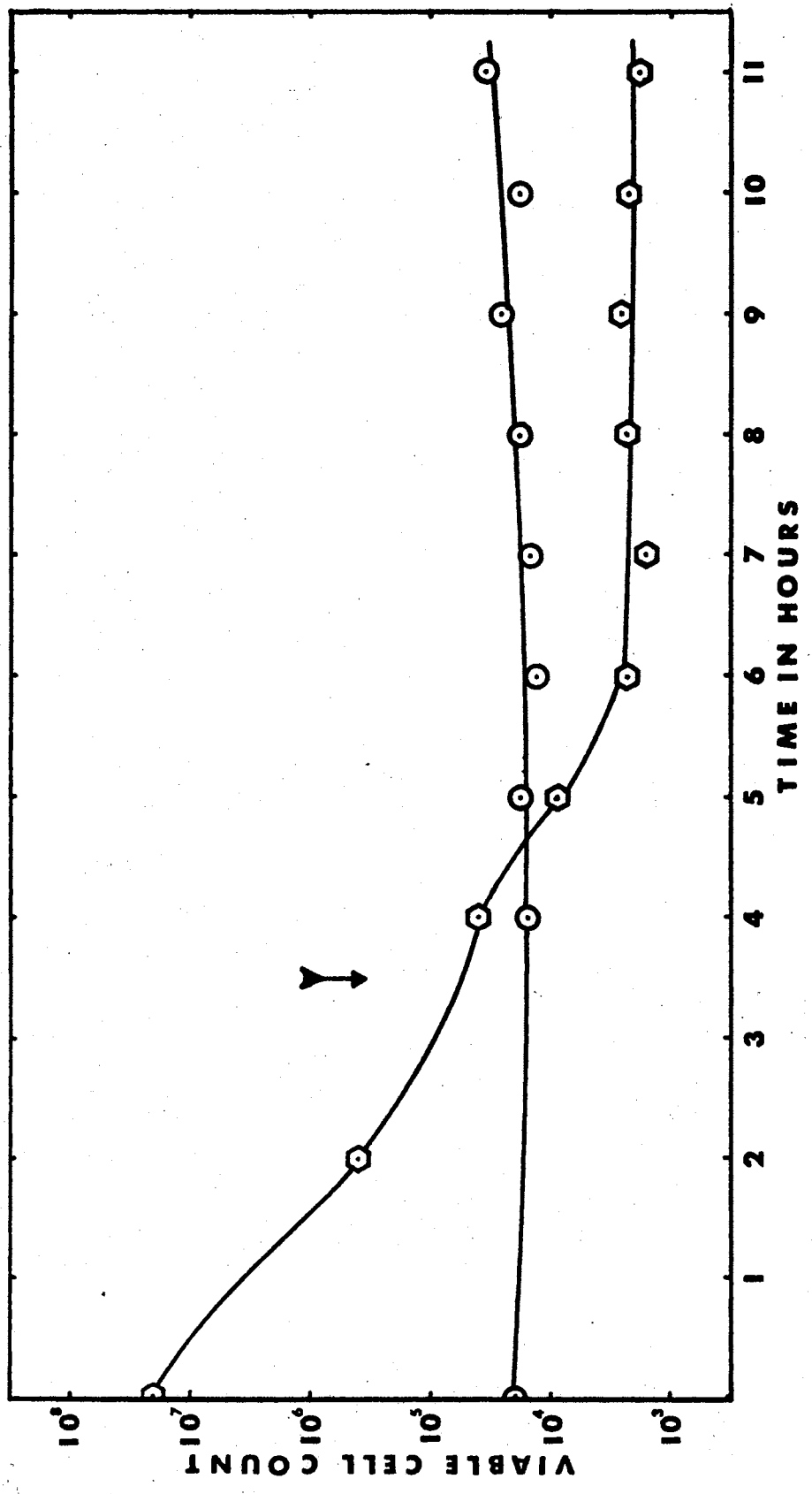

VIABLE CELL COUNT
10^8
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
11
TIME IN HOURS

Figure 27. Survival of Prototroph and Auxotroph Populations in Glucose Minimal Medium Containing 660µg per ml D-Cycloserine With a 350µg per ml D-Cycloserine Addition at Three and One-Half Hours

The bacterial populations were prepared, plated, and counted as indicated in Materials and Methods. Glucose minimal medium containing 660µg per ml D-cycloserine with a 350µg per ml D-cycloserine addition at 3.5 hours was the experimental system. Viable cell counts per ml of enrichment broth at 2, 4, 5, 6, 7, 8, 9, 10, and 11 hours are plotted against time for PA-1 (⊙) and the arginine auxotroph mixture (⊙).


VIABLE CELL COUNT
10^8
10^7
10^6
10^5
10^4
10^3
1
2
3
4
5
6
7
8
9
10
11
TIME IN HOURS

Antagonism of D-Cycloserine by Alanine

A modification of earlier reconstruction experiments was utilized in determining the concentration of alanine necessary to antagonize the effects of the D-cycloserine remaining at eight hours. Eight hours was chosen as the time of sampling since the enrichment techniques yielded the most reproducible data at this point. Utilizing the broth selection technique developed in the previous experimentation, D-alanine, L-alanine, and DL-alanine were added to eight-hour enrichment tubes beginning at 50μg per ml and increasing by 50μg per ml increments to 700μg per ml. The initial cell concentrations were 3.0×10^7 cells per ml for the prototroph population and 3.0×10^5 cells per ml for the auxotroph population. After the alanine addition, the enrichment tubes were reincubated for 30 minutes; then, one-half the experimental volume of 8 ml was discarded and replaced with double strength nutrient broth. The tubes were again incubated until an approximate viable count of 1.0×10^8 cells per ml was reached. Each experimental culture was then diluted to yield approximately 300 to 400 colonies per plate and that dilution was plated on glycerol minimal medium supplemented with 5μg per ml of arginine. The plates for the concentrations of each compound yielding the highest ratio of small colonies to large colonies were then counted and 30 colonies of each morphological type were checked by replica plating from a nutrient agar master plate to which they had first been transferred onto a glucose

minimal and a nutrient agar plate. DL-alanine at a concentration of 500μg per ml, L-alanine at 550μg per ml, and D-alanine at 200μg per ml preserved the highest auxotroph to prototroph ratios during growth in nutrient broth. For DL-alanine and L-alanine, there appeared to be an effective concentration range, but for D-alanine only the 200μg per ml concentration demonstrated any preservative effect. As was shown for plates, rapid growth after an antibiotic enrichment can and usually does decrease the mutant population.

Although these concentrations were being determined so that alanine might be spread on plates with an enrichment sample to determine if any death was occurring once the enriched glycerol minimal plates were spread one must consider these data in attempting cell recycle which will be mentioned in a later section.

When 0.1 ml of a sterile 550μg per ml L-alanine solution was spread along with the 0.1 ml of the diluted enrichment sample, a slight degree of greater stability was observed during the first five hours of an enrichment, but no effect was observed on the sixth hour sample and those following.

An Enrichment Utilizing Two Antibiotics

During the latter stages in the development of the D-cycloserine double addition enrichment, a colleague in the laboratory tried a double antibiotic system which was found to have enrichment value (Heath, 1971). This procedure

utilizes an inhibitory concentration of D-cycloserine (500µg per ml) and an inhibitory concentration of a penicillin G derivative, carbenicillin (100µg per ml).

The D-cycloserine double addition enrichment and this D-cycloserine carbenicillin enrichment were initially compared in two mutant isolation experiments for auxotrophs. Nutrient broth-grown cells were harvested, washed twice, and resuspended in pH 6.0 acetate phosphate medium to which nitrosoguanidine was added to a final concentration of 850µg per ml. The cultures were aerated on a reciprocal shaker at 37°C for 20 minutes, harvested, washed twice and used to inoculate 8 ml of nutrient broth which was incubated for four hours. The two cultures were then harvested, washed twice, and resuspended to a concentration of 3.0×10^9 cells per ml in glucose minimal medium. Each culture was then diluted 1 to 100 into two 20 mm test tubes containing 8 ml glucose minimal medium. One of the tubes contained 660µg per ml of D-cycloserine and the other contained 500µg per ml of D-cycloserine plus 100µg per ml of carbenicillin. The four tubes were aerated on a reciprocal shaker at 37°C with 350µg per ml of D-cycloserine being added to the tubes containing 660µg per ml at 2.5 hours. After 11 hours of incubation, the cultures were diluted and several dilutions were plated on glucose minimal medium enriched with 500 ng per ml of each amino acid in the amino acid mixture (see Materials and Methods). After incubation for 36 hours at 37°C, plates containing 100 to 200 colonies

per plate were replicated onto glucose minimal medium, glucose minimal medium enriched with 60μg per ml acid-hydrolyzed casamino acids plus 2μg per ml tryptophan , and nutrient agar plates. After incubation at 37°C for 24 hours, the replica plates were compared. No colonies were observed on the enriched glucose minimal plates that did not also replicate on the glucose minimal plates. Since the experiment was designed to determine the percentage of colonies, presumed to be auxotrophs, which would grow on the enriched glucose-minimal plates and not on the glucose minimal plates, the experiment was initially considered a failure. However, the nutrient agar plate which was always included as the last plate replicated for a replication control, contained many colonies not present on either of the minimal plates. Several colonies present on the nutrient agar plates but absent on the minimal plates were picked and spotted on nutrient agar plates for replica plating. After overnight incubation, the nutrient agar master plates were replicated onto glucose minimal medium, glucose minimal medium supplemented with 10μg per ml of each of the amino acids in the amino acid mixture, and nutrient agar plates. After incubating for 24 hours, the replica plates were compared and the colonies picked were classified as being auxotrophs, i.e., they grew on the supplemented glucose minimal medium, but not on the glucose minimal medium.

With some of the colonies on the nutrient agar plates having been identified as mutants, colonies were randomly

picked from the nutrient agar plates and used to inoculate nutrient agar master plates. After incubation, the master plates were replicated as before to determine the percentage of colonies present which were mutants. The results of this experiment are shown in Table III.

Microscopic Observations of D-Cycloserine Enrichments

Microscopic observations of enrichments during mutant isolation experiments were included in this study to observe some of the phenomena occurring during an antibiotic treatment with the understanding that much experience is needed to properly interpret observed morphological changes. The progress of the D-cycloserine double addition treatment was observed in glucose minimal medium aerated at 37°C and 30°C and in glycerol minimal medium aerated at 37°C.

The bacterial population was inoculated to 3.0×10^7 cells per ml from a nitrosoguanidine-treated and nutrient broth-grown culture.

One drop of an enrichment sample was placed on a clean slide and covered with a glass cover slip. The enrichment sample was immediately observed by phase contrast microscopy. All observations and photo-micrographs were of 625X diameter magnification. The culture in glucose minimal medium aerated at 37°C was usually observed. During the first four hours of a normal enrichment, a predominance of spheroplasts was observed (Figure 28) with intact cells and

TABLE III

A COMPARISON OF THE EFFICIENCY OF MUTANT SELECTION BY THE D-CYCLOSERINE DOUBLE ADDITION AND THE D-CYCLOSERINE:CARBENICILLIN ENRICHMENT METHODS

Culture	Mutagenized Culture A		Mutagenized Culture B	
Enrichment Method	660:350 D-cycloserine	500 D-cycloserine 100 Carbenicillin	660:350 D-cycloserine	500 D-cycloserine 100 Carbenicillin
Number of Colonies Tested	400	400	400	400
Mutants as a Percentage of the Total Number of Colonies Tested	29.7	32.2	41.0	35.5

660:350 D-cycloserine - 660µg per ml D-cycloserine with a 350µg per ml D-cycloserine addition at 2.5 hours.

500 D-cycloserine:100 carbenicillin - 500µg per ml and 100µg per ml, respectively.

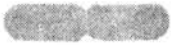
Figure 28. Morphological Forms Predominating in a D-Cycloserine Double Addition Enrichment During the First Four Hours

A culture of Pseudomonas aeruginosa was mutagenized, grown in nutrient broth for two hours and treated in glucose minimal medium containing 660μg per ml D-cycloserine with a 350μg per ml D-cycloserine addition at three hours. Photomicrographs of enrichment culture samples were taken at a 625X magnification during the first four hours of enrichment by phase contrast microscopy. Sketches from photomicrographs of 2.0, 2.25, and 2.5 hour samples depict the bacterial forms which compose approximately 99 per cent of the observed population during the initial four hours of a D-cycloserine double addition enrichment.

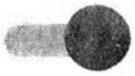
Sketches A, B, and C represent "normal" bacterial forms which are rarely observed after 3.5 hours of antibiotic treatment. The bacterial forms represented in D through M are spheroplasts which predominate in observed samples after one hour of enrichment. The protoplasts represented by N and O represent 1 to 5 per cent of population in 1 to 4 hour samples and occur in greatest percentage in samples taken from a 1.0 to 2.5 hour enrichment culture.

A

B



C



D

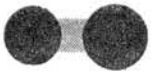
E

F

G

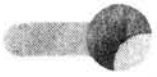
H

I



J

K



L

M

N

O

occasional protoplasts being seen. The protoplasts were observed to lyse on many occasions. This was seen as a quick loss of translucency as the light gray sphere became clearer; this could be induced by placing a drop of distilled water at the cover slip edge. As the distilled water diffused across the slide preparation, a wave front could be seen crossing the field of observation and the protoplasts would lyse when reached by the wave. No spheroplasts were ever observed to lyse; however, their ghosts, which were a much lighter shade, could be found. The spheroplasted portion of a cell was always darker than the cell and light gray to almost transparent areas were often seen (L and M of Figure 28). The bacterial population after four hours was composed mostly of spheroplasted cells and spheroplasts and a normal bacterial cell was rarely observed.

Two phenomena, which were both observed more than once, suggest the possible involvment of a "Bdellovibrio-like" organism in the autolytic phenomenon of P. aeruginosa. During a period of autolysis, some large protoplasts were observed to contain a small "black dot" and in a few instances these dots were moving very rapidly within the membrane-bound environment. Occasionally, a rapidly moving "black comma" approximately one-tenth the size of a bacterial cell was observed in the medium for extended intervals. The dark comma maintained this activity long after all the bacteria had completely ceased movement. Although dark commas were observed, apparently attached to a bacterial

cell by their tails, no comma in motion in the medium was ever observed to attach. Some attached black commas were observed to swing around the point of apparent attachment.

The autolytic phenomenon of P. aeruginosa has been studied since the investigations of Gessard on the blue pus organism in 1882 and Hadley (1924), yet little more is known today (Berk, 1963) than could be learned from the excellent observations of nearly 90 years ago. The experimentation of Vernon (1968) and the observations made in the present study suggest the possibility of a new approach to the investigation of the autolytic phenomenon in P. aeruginosa. The failures of many investigators over many years should suggest a new approach to the problem.

Isolation of Auxotrophs

After completing studies on the survival of prototroph-auxotroph mixtures in enrichment systems, the systems were utilized in a set of four mutant isolations with success (Table III). However, upon attempting to further utilize these enrichment techniques in the isolation of amino acid auxotrophs, difficulty was experienced with the detection of mutants on plates. Several months were spent in trying various combinations of mutagenization procedures and media for plate selection.

The next successful auxotroph isolation was plated on glycerol minimal medium enriched with 500 ng per ml of each amino acid in the amino acid mixture. After incubation at

37° for 36 hours, colonies were picked and tested with no mutants having been isolated. However, the plates were retained for observation. Several days later, many small colonies were found to be present. These were initially suspected to be contaminants, but several of these "contaminants" were picked and inoculated in spots on nutrient agar master plates. After incubation, the plates were replicated onto glucose minimal medium and nutrient agar plates and incubated for 24 hours. The majority of these colonies did not grow on glucose minimal medium, but did grow on the nutrient agar plates. The selection plates which were still being retained for observation were then picked for all the small colonies which had appeared since the original testing and numerous auxotrophs were obtained. In the ten auxotroph isolations to follow, glycerol minimal plates were supplemented with 0, 150, 200, and 300 ng per ml of each amino acid in the amino acid mixture. Although more mutants appeared on slightly enriched plates, it was found that a large number of auxotrophs could be isolated after four to five days of incubation when no nutrients were added. The amino acid auxotroph isolations dropped in efficiency by approximately 95 per cent when no supplementation was added to the plates and a washed agar, Ionagar No. 2 (Bechtle and Scherr, 1958), was used for solidification. Therefore, it seemed apparent that Bacto Agar contains sufficient concentrations of amino acids to allow very slight growth of auxotrophs. Apparently, the nutrients in

the plate slowly diffuse to the more depleted upper portions of the plate and enable the auxotrophs to develop colonies. The concentration of the individual nutrient required will determine whether the colony is invisible, small or large. Future auxotroph isolations for specific pathways should utilize 0.5 per cent glycerol or fructose minimal medium solidified with a washed agar and enriched with a nutrient concentration to be determined for optimal selection, i.e., the growth of small auxotroph colonies. The optimal concentration for most amino acids appears to be in the range of 100 ng to 2μg per ml of minimal medium. The agar depth will influence selective plates of this nature and the agar volume should, therefore, be constant during and after optimization of the nutrient concentration.

Isolation of Catabolic Mutants

The glycerol pathway had been studied in this laboratory for several years (Cowen, 1968; Tsay, 1971). In the many attempts to select glycerol-negative mutants only a few stable mutants had been isolated. In designing a procedure to isolate mutants for the glycerol pathway, many of the procedures used in auxotroph isolations had to be modified. Since plate selection techniques were not available, it was necessary to pick and test each colony. Also, since the growth rate on glycerol is much less than on glucose, the enrichments might not produce the degree of selection observed in survival curves and reconstruction experiments.

Thus, it was decided to recycle the culture by regrowing the remaining cells from an antibiotic selection and submitting the culture to another broth enrichment.

Since cell recycle had not been attempted previously, attention was focused on the results of the alanine antagonism experiments and the problems with death in post-enrichment growth on richer media. After considering these data, fructose was chosen as the carbon and energy source for the minimal medium to be used for growing cells after antibiotic enrichment and as the substrate in the minimal medium for plating, since P. aeruginosa grew comparably on glycerol and fructose (Cowen, 1968) and glycerol had been used successfully in plates for auxotroph selection. It was also considered advantageous to add the 30-minute treatment with 550µg per ml of L-alanine following broth enrichment to antagonize the remaining D-cycloserine. Ornston et al. (1969) had utilized three rounds of recycle in enriching for spontaneous P. putida mutants; therefore, the culture was diluted and plated after the third broth enrichment.

A decision as to length of enrichment and which antibiotic enrichment system to utilize remained. Since the rate of growth of P. aeruginosa on glucose was approximately twice that on glycerol, 14 to 16 hours of selection was considered a minimum time and approximately 24 hours a maximum time. Considering the success with mutant regrowth in the alanine antagonism experiments, it was decided to use the double D-cycloserine addition system in the first two

enrichment cycles adding 550µg of L-alanine at the end of each enrichment for a 30-minute treatment prior to harvesting and washing the culture for regrowth in fructose minimal medium. The D-cycloserine carbenicillin system was chosen for the last broth enrichment since it would introduce a new antibiotic to the system which naturally selects for antibiotic resistance and since the effects of plating from this enrichment onto a slowly metabolized carbon and energy source such as fructose were known. With this experimental protocol, a culture was grown in nutrient broth, harvested, washed, and resuspended in pH 6.0 acetate phosphate buffer to which a 100µg per ml final concentration of nitrosoguanidine was added. The culture was aerated on a reciprocal shaker at 37°C for 15 minutes. The culture was then harvested, washed twice, and grown in nutrient broth for four hours prior to preparation for the first antibiotic treatment. Washed cells were inoculated to a final concentration of 3.0×10^7 cells per ml in 0.5 per cent glycerol minimal medium containing 660µg per ml D-cycloserine. A total enrichment volume of 8.0 ml was aerated at 37°C on a reciprocal shaker for two and one-half hours prior to the addition of 350µg per ml D-cycloserine. The culture was treated with antibiotic for 18 hours prior to the addition of 550µg per ml L-alanine and reaeration for 30 minutes. The culture was harvested and washed by centrifugation at 12,000 rpm at 0°C for 20 minutes and resuspended in 8.0 ml of 0.7 per cent fructose minimal medium. The culture was

aerated on a reciprocal shaker for 51 hours prior to preparation for the second antibiotic treatment. Regrowth for the third antibiotic treatment required 34 hours of aeration at 37°C in fructose minimal medium, at which time the culture was harvested, washed, and resuspended to 3.0×10^7 cells per ml in 8.0 ml of 0.5 per cent glycerol minimal medium containing 500µg per ml D-cycloserine and 100µg per ml carbenicillin. The culture was again treated for 18 hours prior to diluting and plating.

The plates for growth following the third antibiotic treatment contained 0.5 per cent fructose minimal medium solidified with 0.9 per cent Ionagar No. 2. Washed agar was used to suppress the growth of auxotrophs and thereby simplify colony testing which was a random process, i.e., there was no form of plate selection other than the use of pure agar. In testing the colonies formed on the fructose plates, each was picked and spotted on a nutrient agar plate which, after incubation, was used as the master plate and replicated onto glycerol minimal medium, glucose minimal medium, lactate minimal medium, and nutrient agar plates in that order. After incubation, approximately 45 per cent of the colonies tested grew very slowly or not at all on the glycerol minimal plates, all colonies grew on the glucose minimal and nutrient agar plates and all but two colonies grew on the lactate minimal plates. Of these two colonies which would not grow on glycerol or lactate, broth testing revealed one to be leaky and the other stable; they were

thought to be mutants of the upper Embden-Meyerhof-Parnas pathway (Heath, 1971). After testing the non-leaky glycerol-negative strains in glycerol, glucose, and lactate liquid media, twenty-three were selected for a preliminary transduction experiment. These 23 strains were used to produce phage stocks of a transducing phage, F-116; the titers of these phage stocks ranged from 1.3×10^{10} to 5.0×10^{11} plaque forming units per ml. Transduction was performed by the spot plate method using the original phage preparations. After the spots had dried, the plates were inverted and incubated at room temperature overnight prior to 37°C incubation. Colony counts ranging from 400 to 600 colonies per spot were common except for closely linked mutants. The glycerol pathway may be summed as mapping very nicely by transduction in preliminary experiments.

Mutant isolations for glycerol-negative mutants were not again attempted until approximately one year later as this study was primarily concerned with reproducible auxotroph isolations at that time, and the catabolic mutant isolation procedure had proven that catabolic mutants could be isolated by modifying auxotroph isolation procedures. When catabolic mutant isolations were again studied, experimentation centered on developing selective methods to complement the broth enrichment procedures. Since methods had been developed whereby a presumptive auxotroph could be visually selected on a plate bearing 100 to 500 colonies, thus eliminating the necessity of picking and testing the

other 99 to 499 colonies, this degree of efficiency was desired for the plate selection of catabolic mutants.

In contemplating the possibilities for a selective plate system, at least three general methods were considered promising; in each case the substrate for which catabolic mutants were being sought must be included in the minimal medium for plating. The possibilities for a second substrate which utilizes a different degradative pathway were: (1) it might be present in limiting quantities and thereby produce small colonies, (2) it might be added after colonies have formed, utilizing the primary substrate (for which mutants are being sought) and the colonies which then appear may be selected (Lederberg and Tatum, 1946), and (3) it might be very slowly metabolized and retard the development of colonies using it as a sole substrate or it might cause the development of a different colonial morphology.

The plate selection procedure developed for selecting catabolic mutants is a two phase system, based on the third of these three possibilities. The substrate for prototroph growth is added to minimal medium solidified with a pure agar at a minimal concentration adequate for the development of colonies of normal size. Three to five drops of geraniol are placed in the lid and the plates incubated. Mutants utilize the geraniol vapor as a substrate, forming very thin flat colonies. Prototrophs utilize the substrate in the minimal agar, forming convex colonies. An added advantage of this system is that leaky mutants will usually form

raised flat colonies, flat colonies with a convex centers or convex colonies, depending on their quality, i.e., degree of leakiness. The efficiency of this system is comparable to that used for auxotroph selection; a glycerol mutant can be visually selected on a plate bearing 500 or more prototrophic colonies. Selective plates of this nature are efficient enough to eliminate the necessity of cell recycle and are normally spread after the first antibiotic treatment.

Geraniol is an irritant and should be washed from skin surfaces after contact; prolonged contact with skin surfaces, i.e., hours, may result in a rash or tissue swelling depending on skin sensitivity. Geraniol plates are incubated at 37°C for five to eight days prior to visual mutant selection. All plates spread for mutant selection should be spread with a clean glass rod; the residue which accumulates may be removed by scraping with a blade. Some mutant colonies may not be observed unless light is reflected on the agar surface, in these instances a smooth agar surface is necessary.

A Preliminary Methionine Transduction Study

Using the methods developed in this study, a number of mutants had been isolated for the aromatic pathways, the leucine, isoleucine, and valine family, and the serine, proline, histidine, arginine, threonine, and methionine biosynthetic pathways. Although the mutants which had been

isolated behaved well in minimal broth tests of 150 hours duration and did not revert when 0.1 ml of a 1.0 ml slant wash was spread on glucose minimal medium, no data had been obtained to indicate their true value in transduction studies. The methionine pathway was selected for preliminary transduction studies for several reasons. The intermediates of this pathway were available and the pathway in P. aeruginosa had been partially studied in this laboratory although a number of previously isolated methionine auxotrophs could not be mapped by transduction because they were also phage-resistant (Mose, 1970). This pathway had also been studied by Calhoun and Feary (1969).

Twenty-seven methionine auxotrophs were used in preparing transducing phage stocks. The phage stocks were not titered as the study was to be strictly a preliminary investigation which would hopefully give some indication as to the value of these mutants in terms of mapping potential.

The mutants were mapped by the spot plate transduction method (Swanstrom and Adams, 1951). A 0.1 ml sample of a cell suspension prepared by washing a 14-hour slant with 1.1 ml of minimal medium was spread on 0.5 per cent glucose minimal medium supplemented with 0.5 per cent sodium chloride and solidified with 0.9 per cent Ionagar No. 2. The plates were then spotted, five spots per plate, with one drop per spot, using each of the 27 phage stocks prepared on methionine auxotrophs plus one spot of a phage stock prepared on PA-1 as the 28th spot. All phage spots were one

drop from a 1.0 ml pipette held in the horizontal position. The plates were dried, inverted and incubated at 37°C for 72 hours. Upon counting the colonies in each spot, a transduction table with colony counts from 0 to 200 colonies per spot was obtained. This was considered rather poor as most of the spots yielded colony counts below 100 and in a preliminary glycerol transduction study, colony counts had been five times as large.

Thus, a second preliminary transduction experiment was run in a similar manner except that 0.5 per cent glycerol was substituted for glucose as substrate and the plates were incubated at room temperature instead of at 37°C. The colony counts per spot were approximately two to three times higher with these modifications (Table IV).

New phage stocks were prepared for mutants AJ-3, AC-53, AH-67, AH-69, and AN-4 prior to the second preliminary transduction, utilizing the original phage stocks as broth for soaking the fresh phage plates. Mutant AH-75 did not grow on glucose minimal medium or on glucose minimal medium plus methionine, but grew when methionine and threonine or methionine, threonine, and lysine were added to glucose minimal medium. Mutant AC-44 became very leaky upon storage and mutant AN-2 was found to be phage-resistant. Mutant AN-64 was temperature-sensitive and could not be transduced unless it was growing. Therefore, these three mutants were not included in the transduction studies. Phage stocks of mutants AN-6, AC-40, and AJ-1 functioned normally as donors

TABLE IV

A PRELIMINARY TRANSDUCTION OF METHIONINE AUXOTROPHS

Recipients	Donors																				
	AA-1	AC-51	AH-69	AN-4	AH-75	AC-53	AH-67	AC-38	AC-49	AH-70	AN-1	AH-72	AH-71	AJ-3	AN-3	AN-5	AN-11	AJ-2	AC-40	AJ-1	PA-1
AA-1	0	7	0	0	0	25	68	121	99	317	69	194	113	194	28	473	159	207	260	120	162
AC-51	1	0	11	0	2	49	138	153	166	374	236	167	262	249	81	621	388	567	318	137	287
AH-69	0	0	0	0	0	108	164	41	55	120	23	62	85	127	15	265	155	187	302	117	70
AN-4	3	2	1	0	1	44	46	214	209	230	51	134	108	179	20	381	192	189	200	88	174
AH-75	0	1	2	1	0	55	79	148	112	266	81	96	209	195	44	500	286	283	331	153	181
AC-53	166	132	273	128	253	0	5	302	312	282	160	121	113	210	54	504	302	349	480	210	365
AH-67	199	218	220	157	210	2	0	208	248	333	182	95	217	182	61	552	294	398	543	244	215
AC-38	288	353	361	474	329	174	232	0	80	58	22	201	238	173	189	589	361	492	581	270	450
AC-49	229	209	261	145	243	210	198	14	0	6	7	371	177	185	51	439	325	367	402	201	380
AH-70	292	407	352	234	150	217	276	30	22	0	11	407	319	186	82	692	291	391	572	224	548
AN-1	341	454	409	210	277	152	156	11	20	13	0	352	171	196	45	775	311	394	610	322	514
AH-72	175	116	133	64	126	12	47	78	122	253	103	1	0	0	0	4	244	94	545	69	134
AH-71	362	415	279	241	311	82	167	312	439	604	276	0	0	0	1	5	378	277	418	456	445
AJ-3	200	249	161	105	232	37	78	120	126	436	67	2	1	0	0	0	380	257	354	178	301
AN-3	367	242	363	130	199	122	187	348	314	820	190	1	5	0	0	8	429	352	630	308	288
AN-5	280	236	231	122	225	32	117	207	128	455	123	2	1	0	0	0	251	208	545	226	172
AN-11	278	270	211	266	281	67	119	275	233	234	143	90	191	138	90	417	0	317	243	418	301
AJ-2	435	405	282	465	387	72	126	189	307	529	162	255	452	279	193	321	267	1	246	197	789
AC-40	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	2	298	TNTC
AJ-1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	2	135	TNTC

Note: TNTC = Too Numerous to Count.

but these mutants as recipients yielded greater than 1000 colonies per spot when spotted with F-116 prepared from other mutant strains or from PA-1; mutant AN-6 although not mapping with AC-40 and AJ-1 yielded greater than 1000 colonies per spot, even when transduced with homologous phage. Mutant AN-6, however, performed well as a donor strain, mapping with AH-71, AH-72, AJ-3, AN-3, and AN-5. Mutants AC-40 and AJ-1 mapped together and it is interesting to note that two strains from one locus of this nature (high rate of transduction as recipient) were also reported by Calhoun and Feary (1969) and by Smith and Childs (1966) for methionine auxotrophs of one genetic group in S. typhimurium. When AC-40 was treated with homologous phage, two colonies grew in the spot; mutant AJ-1 with its own transducing phage preparation yielded 135 colonies. Each of the 38 phage preparations was spotted on nutrient agar and on glycerol minimal medium as controls for cell contamination of phage stocks and no colonies appeared. The background of each plate spotted with transducing phage served as a control for reversion; however, no strain reverted.

Although no cross-feeding experiments were performed, the strains were incubated in glucose minimal medium supplemented with 1μg per ml of homoserine, cystathionine, homocysteine, or vitamin B_{12}. The results of these intermediate feedings are presented in Table V and the pathway for methionine biosynthesis is shown in Figure 29.

Calhoun and Feary (1969) reported the isolation of 71

TABLE V

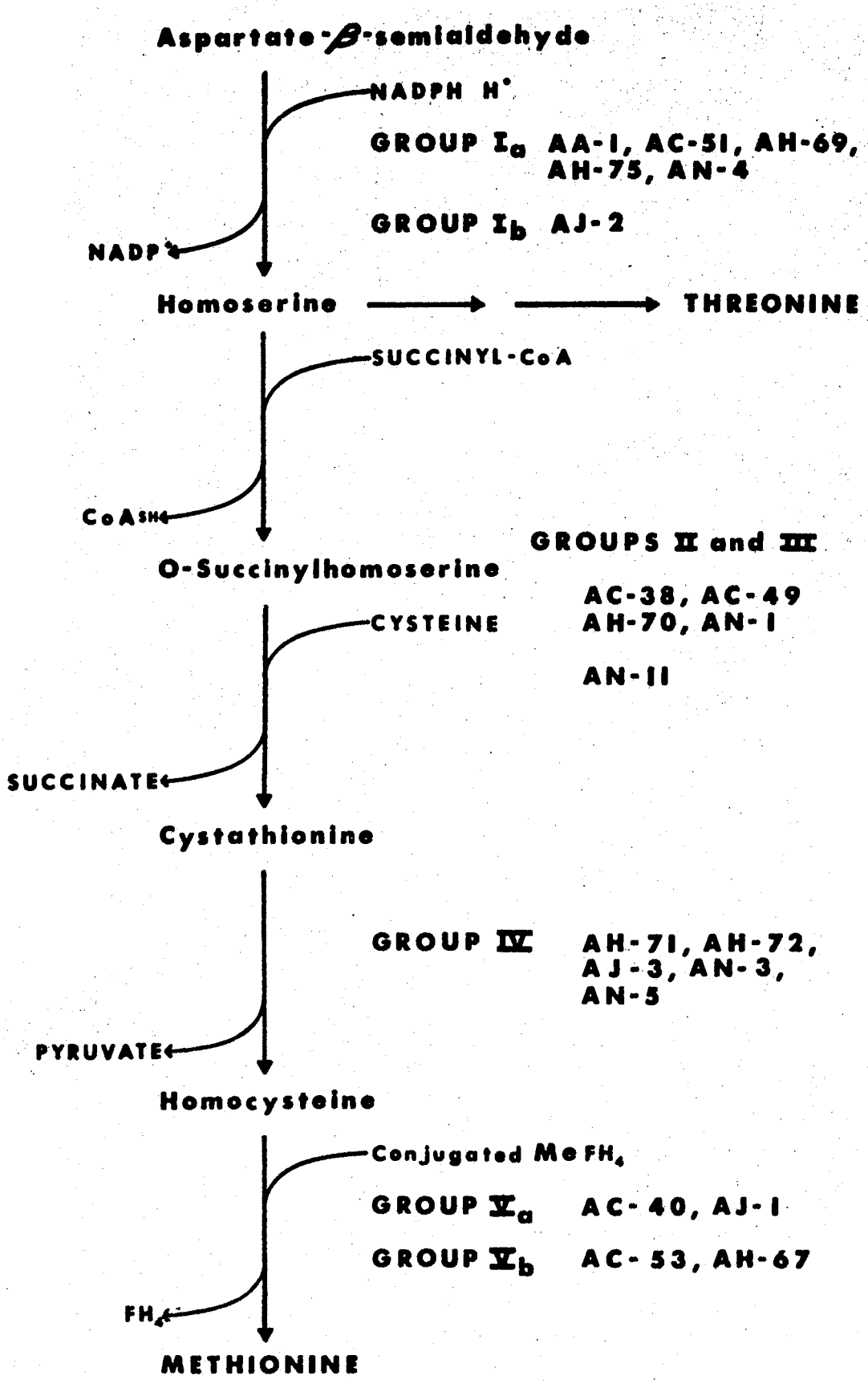
GROWTH OF METHIONINE AUXOTROPHS IN MINIMAL MEDIUM UTILIZING INTERMEDIATES OF THE METHIONINE BIOSYNTHETIC PATHWAY AS NUTRIENTS

	HS	CT	HC	B_{12}	Meth
Group Ia: AH-69, AA-1, AC-51, AN-4	+	+	+	+	+
Group Ib: AJ-2	+	+	+	+	+
Groups II and III: AC-38, AC-49, AH-70, AN-1	-	+	+	+	+
AN-11	-	+	+	-	+
Group IV: AH-71, AJ-3, AH-72, AN-3, AN-5	-	-	+	-	+
Group Va: AC-40, AJ-1	-	-	-	-	+
Group Vb: AC-53, AH-67	-	-	-	-	+

HS - Homoserine
CT - Cystathionine
HC - Homocysteine
B_{12} - Vitamin B_{12}
Meth - Methionine

Figure 29. The Methionine Biosynthetic Pathway With Mutant Grouping as Established by Transduction and Intermediate Feeding

Homoserine dehydrogenase reduces aspartate-β-semialdehyde to homoserine. Two unlinked genetic loci were established with blocks at this enzymatic step. Homoserine O-succinyltransferase catalyzes the synthesis of O-succinylhomoserine from homoserine and cystathionine is synthesized from O-succinylhomoserine by cystathionine-γ-synthase. Two unlinked genetic groups were blocked between homoserine and cystathionine. Homocysteine is synthesized from cystathionine by cystathionase II and one genetic locus was established for this enzymatic reaction. Homocysteine methyltransferase catalyzes the synthesis of methionine from homocysteine; two unlinked genetic loci were found to be blocked at this enzymatic step.


Aspartate-β-semialdehyde
NADPH H⁺
GROUP Ia AA-1, AC-51, AH-69, AH-75, AN-4
GROUP Ib AJ-2
NADP⁺
Homoserine
THREONINE
SUCCINYL-CoA
CoASH
GROUPS II and III
AC-38, AC-49
AH-70, AN-1
AN-11
O-Succinylhomoserine
CYSTEINE
SUCCINATE
Cystathionine
GROUP IV AH-71, AH-72, AJ-3, AN-3, AN-5
PYRUVATE
Homocysteine
Conjugated Me FH4
GROUP Va AC-40, AJ-1
GROUP Vb AC-53, AH-67
FH4
METHIONINE

methionine auxotrophs. They presented data on growth requirements and cross-feeding for 46 of these and mapped 15 of the mutants. A number of differences appear in this preliminary analysis of 27 methionine auxotrophs, of which 24 were mapped, and the study of Calhoun and Feary. Their data detected five mutant groups biochemically and four groups genetically with two biochemical groups mapping together in transductional analysis. The present study detected seven genetic groups with no indication that any two groups are closely linked. The groups reported linked by Calhoun and Feary correspond to I and IV in the present study; however, only one mutant was available in group I in their study. This lack of sufficient mutants may be found in any reported genetic study using P. aeruginosa as the experimental organism. It does appear that the quality of the mutants used for the present study is equal to or better than those in other reported studies, since 24 of 27 methionine auxotrophs in this study were mapped and in the study reported by Calhoun and Feary two of three mutants from one group apparently could not be mapped. Their study had one group with two mutants and two groups with one mutant each which were mapped. This preliminary study also shows the same weakness in having two groups with one mutant each and two groups with two mutants each.

The recipient characteristics of group Va, and a mutant of group IV (AN-6) which was not included, are not understood but have previously been reported for mutants blocked

between homocysteine and methionine, as mentioned above. Mutant AN-6 crossed with itself producing greater than 1000 colonies per spot.

The previously mentioned growth requirement of mutant AH-75, which differs from others in group Ia, can be partially explained since threonine is also synthesized from homoserine. Mutant AH-75 may be a member of group Ia or it could be a mutant of an adjacent locus. One could speculate on the existence of two isoenzymes and three or more loci involved in the synthesis of homoserine from aspartate-β-semialdehyde.

The differences in this preliminary study and the previously reported study of methionine biosynthesis, and the questions arising from the data of this study should serve as a warning to the geneticist considering reporting a study for which insufficient mutants are available. This preliminary investigation lacks the firm genetic and biochemical data necessary for a good report and yet is very much like the reports available. It was included in the present investigation only to demonstrate the quality of mutants which may be obtained by the methods developed for mutant isolation.

The standards initially set in this study for a transduction map are five quality mutants for each mappable or biochemically distinct group (locus). Mutants should not revert on minimal plates or grow during 100 hours aeration on a reciprocal shaker at 37°C in glucose minimal broth.

Mutants should demonstrate a growth rate comparable to prototrophic growth when their nutrient requirement is provided.

CHAPTER IV

DISCUSSION

An introduction to the field of bacterial genetics may leave the impression that one can investigate the genetic aspects of a bacterium by simply applying the existing techniques to species which have not been thoroughly studied. One cannot fully appreciate the difficulties which arise in attempting to investigate a new bacterial genetic system until the techniques which do not exist are needed and one attempts to adapt the genetic techniques for E. coli to another organism. It was not until after six months had been spent in attempting to adapt techniques for E. coli to genetic studies of P. aeruginosa that the present study was initiated.

It was not long after the initial studies on biochemical synthesis in Neurospora crassa utilizing hereditary anomalies (Beadle and Tatum, 1941; Beadle, 1945a; Beadle, 1945b; Tatum and Beadle, 1945) that the potential for their use in studying bacterial physiology and metabolism was realized and initially investigated (Tatum, 1945; Tatum, 1946). Soon methods for the detection of bacterial mutants (Lederberg and Tatum, 1946) and their identification by a modification of the "auxanographic method" of Beijerinck

(Lederberg, 1946) were available. Since methods for inducing mutations in a culture of a microorganism by a variety of agents, including X-irradiation, ultraviolet light and nitrogen mustard gas, were available (Gray and Tatum, 1944; Roepke et al., 1944; Tatum, 1946), an investigator interested in bacterial genetics had a foundation on which to rely in studies utilizing this approach.

The plate detection technique of Lederberg and Tatum (1946) is interesting in that it demonstrates the ingenuity needed in developing genetic methodology. A minimal agar plate was poured in three layers; the basal agar layer was essentially supportive. A molten minimal agar layer was inoculated with 50 to 400 cells and poured over the basal layer. After the second agar layer solidified, a thin minimal agar layer was poured over the cell agar layer to insure uniform depth for all the colonies, to prevent spreading growth which is more apt to occur at a glass or air interface, and to prevent spreading of colonies when another layer was poured. The plates were then incubated until the prototrophic cells developed into colonies in the minimal medium plates; then a layer of nutrient agar medium was poured on the surface layer. Small colonies could be detected in 6 to 24 hours as the "growth factors" diffused through the agar to the cell agar layer during incubation. Thus, very early in the development of E. coli genetics, methods for mutagenization and the detection of mutants were available.

Within a few years, the genetics of E. coli was tremendously advanced by the independent development of two identical methods for the broth selection (enrichment) of mutants prior to plate selection (Davis, 1948; Lederberg and Zinder, 1948). A mutagenized culture of E. coli was washed and resuspended in a synthetic minimal medium containing 300 units per ml of benzyl-penicillin (penicillin G). The culture was aerated by shaking prior to diluting and plating. The penicillin was found to kill growing cells while not affecting resting populations, thus selecting the mutants which could not grow and increasing the mutant to prototroph ratio by two to four logs prior to plate selection.

Since plate methods were efficient enough to allow the selection of one mutant colony in a population of 100 or more prototroph colonies on a plate and broth selection techniques could selectively enrich the mutant to prototroph ratio by up to 10,000 times, the bacterial geneticist using E. coli could normally select one cell from a population of one million cells by simply applying the available techniques. Herein lies much of the strength which has made E. coli genetics, followed by the genetics of S. typhimurium, nearly synonymous with the term "bacterial genetics".

Approximately ten years after the great "gold rush" in genetic studies with E. coli, the genetic investigation of P. aeruginosa was underway and yet in over fifteen years the P. aeruginosa genticist has not developed the technical abilities reliably used by the E. coli geneticist within

five years. Thus, P. aeruginosa genetics, beginning ten years behind, is now only approximately twenty years behind.

During the initial development of E. coli genetics, an antibiotic, penicillin, was available which exerted little killing effect except during an active growth phase (Hobby et al., 1942; Chain and Duthie, 1945); its properties were noted and utilized by E. coli geneticists to develop powerful selective techniques for efficiently isolating mutants. Although P. aeruginosa is extremely resistant to penicillin (Bruce, 1965), an antibiotic, which would effectively kill only growing cells, D-cycloserine, was available during the initial development of P. aeruginosa genetics (Clucker et al., 1955; Ciak and Hahn, 1959); yet in contrast to the development of E. coli genetics, the properties of D-cycloserine were not utilized for establishing selective techniques for the efficient isolation of P. aeruginosa mutants.

An understanding of why efficient broth selection techniques and more appropriate plate selection techniques have not previously been developed can only be obtained by knowing the many problems such a versatile microorganism as P. aeruginosa can present. Thus, unlike the development of the penicillin broth enrichment for E. coli and other penicillin-sensitive microorganisms, mutants could not be consistently isolated via broth enrichments and attempts were made for six months prior to their use in reproducible mutant isolations. During this six-month interval, a

mutagenized and nutrient-broth grown culture was divided and enriched (prior to plating) in both broth systems, i.e., the D-cycloserine double addition and D-cycloserine-carbenicillin systems, an average of once each week. While numerous plate and mutagenization variations were attempted, the two broth enrichments were considered sound and, therefore, were not altered.

It was during this interval when no mutants were isolated that the autolytic phenomenon of P. aeruginosa could not be ignored and seemingly played some role in hampering broth enrichments. An interest in the microscopic phenomena occurring during a normal broth enrichment initiated a short study designed to observe and perhaps photograph the cellular changes occurring during the death phase for prototrophs.

Autolysis was initially observed on plates in areas of high cell density and confluent colony growth. Minimal medium and 2.0 per cent tryptone plates seemed to demonstrate the autolytic phenomenon better than did richer nutrient plates. As the degree of autolysis increased, the central portions of all colonies would lyse and the well-documented irridescent effect could be observed (Hadley, 1924; Berk, 1963). Although colonies from 24-hour plates which did not appear to be autolytic were picked and restreaked each day for 40 transfers, the autolytic phenomenon persisted. Five strains transferred from cold room stocks initially demonstrated slight to mild autolysis, but demonstrated lysis comparable to PA-1 after two to four

transfers and incubation at room temperature. The autolytic phenomenon subsided during stock transfers.

A fresh isolate from a forehead wound was utilized to study the autolytic phenomenon by Hadley (1924). The phenomenon began to diminish after the twenty-eighth transfer and had disappeared by the thirty-sixth slant transfer. In contrast to the fresh isolate used by Hadley, PA-1 appears as a flat whitish slant growth during an autolytic period and changes to a raised glistening green growth on slants during nonautolytic periods. For the PA-1 strain utilized in this laboratory, the appearance of the slant growth is thus far one of the best indicators for the beginning and end of autolytic periods.

The autolytic phenomenon would not be of great interest to a geneticist studying P. aeruginosa if it were not for the effect it has on broth enrichments. The broth enrichments begin to lose efficiency as autolysis increases and simply cannot be utilized during periods of marked autolysis. When autolysis is at its peak, the cell concentrations per ml of broth enrichment medium after eight hours of selection have been observed to be two logs lower than during nonautolytic periods.

Since autolysis appears to occur in old or stationary phase cultures, i.e., heavily populated areas of growth and the central portions of colonies, it seems logical that the mutant population of a broth enrichment might be adversely affected under the starvation conditions prevalent during

the prolonged incubations of broth selections. Although many attempts have been made, broth enrichments in this laboratory during periods of autolysis have yielded few to no mutants and the geneticist using P. aeruginosa should observe his strain with this possibility in mind.

Although it has not been attempted in this study, a modification of the ingenious enrichment used by Lessie and Whiteley (1969) for selecting mutants of P. multivorans could possibly bypass the autolytic phenomenon and/or prolonged broth enrichment. In principle, mutagenized, nutrient-broth grown and washed cells are inoculated into enrichment medium and allowed to double. A sub-inhibitory concentration of D-cycloserine is added and the culture incubated until it again doubles. The culture is then washed twice with distilled water to lyse the osmotically fragile cells and remove the antibiotic. The culture may then be diluted and plated. The advantage this procedure offers, regardless of the degree of selection, is the reduction in length of the mutant starvation period. The D-cycloserine concentration range of 200μg per ml to 300μg per ml should allow growth and yet sufficiently affect wild type cells so that they become osmotically fragile. If short procedures of this nature do not bypass this problem, the geneticist will have to resort to direct plating methods to isolate mutants of P. aeruginosa during autolytic periods. It might also be noted that strains failing to demonstrate autolysis appear during autolytic periods in

plating mutant enrichments. These strains might possibly be utilized after being thoroughly tested for efficiency of plating with transducing phage, antibiotic sensitivity, carbon and energy source utilization, rate of growth, etc. Other treatments have reportedly altered the expression of the autolytic phenomenon (Holloway, 1969).

The enrichment process was slowed for microscopic observation by utilizing glycerol instead of glucose as the carbon and energy source and/or incubating on a reciprocal shaker at 30°C instead of the normal 37°C enrichment temperature. The viable culture was observed by phase contrast microscopy and occasionally photographed. No attempts were made to do more than observe samples of a slowed broth enrichment following mutagenization by nitrosoguanidine. Spheroplasts, rods with spheroplasted centers, sides, and ends, and protoplasts were observed along with some bacterial ghosts. Some protoplasts were observed to lyse; this lysis was observed as the light gray sphere quickly lightened to a ghost which could easily be overlooked if not carefully observed in scanning a field or slide. This lysis was also observed by placing a drop of distilled water at the edge of the cover slip and observing a protoplast. As the convection "wave" was observed to cross the field of observation, the protoplast would quickly lyse upon meeting the "wave". After a normal slide had been observed for five to twenty minutes, no protoplasts could be found upon scanning. Although no spheroplasts were ever observed to

lyse their ghosts could be found without difficulty. These appeared as much lighter then neighboring spheroplasts which were quite dark.

As the autolytic phenomenon increased in magnitude, the normal enrichment observations also changed. The protoplasts and spheroplasts began to appear larger and on occasion a rapidly moving "black dot" could be observed within one of these. In many instances, stationary "black dots" were observed mainly within capped spheroplasts and occasionally within protoplasts. During this period, a rapidly moving "comma" was found in the medium on several occasions and was observed for extended lengths of time in the hope that one would attach to a cell. These comma-shaped organisms appeared to be approximately one-tenth to one twentieth the size of the bacterial cells and were never observed to attach to a cell. They were never photographed as one was never observed to cease movement and similar stationary particles could have been debris. Prior to the end of microscopic observations exceptionally large capped and banded spheroplasts were observed. Except for the outer edge and the dark band or cap, these appeared transparent and were never observed to change shape in any way. Protoplasts were not found in these enrichment samples.

These microscopic observations were a rather interesting experience and it is recommended that one observe an actual enrichment by taking samples in the one to four hour period of enrichment and mounting a drop on a slide for

phase contrast microscopy. One should have no problems in observing a variety of morphological types and spheroplast shapes; however, a little patience may be required to observe lysis of protoplasts. The majority of the protoplasts lyse soon after the slide is prepared and haste is necessary if these observations are intended.

Once the autolytic phenomenon had subsided, auxotrophs were selected on enriched glycerol minimal medium solidified with 2.0 per cent Difco-agar. Although many auxotrophs appeared as small colonies on glycerol minimal medium solidified with some unwashed agars, the numbers of small colonies increased if 150 ng per ml of each amino acid in the amino acid mixture were added.

No attempts were made to adjust the concentration of individual nutrients and thereby optimize the plate selection for these auxotrophs; however, this will be necessary when only one or a few pathways are being investigated. The plate selections for catabolic mutants of a particular pathway were more efficient when a washed agar, Ionagar No. 2, was used for medium solidification. A more important reason for using a washed agar in specific auxotroph plate selections is the variation in nutrient contamination which occurs in different batches of agar. If several different batches of unwashed agar are soaked with distilled water, the supernatant fluid observed after settling of the agar may vary from the appearance of nutrient broth to an almost clear fluid. With the nutrient concentration varying in the

agar being used, it would be extremely difficult to determine the nutrient concentration necessary to produce an auxotroph colony of a certain size. Another advantage of washed agars is that plates do not tend to crack upon prolonged incubation.

The auxotroph enrichments plated on glycerol minimal medium with nutrient supplements in nanogram amounts appears to offer an advantage in that the small colonies will appear two to three days after the prototroph colonies have grown, thus allowing leaky mutants adequate time to produce larger colonies which are not selected and eliminating many problems in distinguishing what colony size to select. Fructose has been satisfactorily substituted for glycerol in occasional plate selections.

If the nutrient requirement for auxotroph selection is optimized to any extent and a slowly metabolized source of carbon and energy is used as the selective plate substrate, an auxotroph can be visually selected on a plate bearing 300 or more colonies. This means that in the time required for selection of 10 stable mutants by random picking and testing of 1000 colonies, one can utilize the methods developed in the present study to select, test, and prepare transducing phage for 40 stable mutants.

The selection of catabolic mutants presented a unique challenge in that a plate system with two sources of carbon and energy must be utilized if random colony testing was to be avoided. One of the substrates had to be a carbon and

energy source which utilizes the catabolic pathway being investigated and the second a substrate which is degraded by another pathway. The objective was to produce a colony of different size, shape, or color when the second substrate was utilized as a sole source of carbon and energy. Although various combinations and concentrations of glycerol and another substrate were tested in plates, none were found to offer a selective advantage over random colony testing.

The first and only successful selective plate system used in this study for selecting catabolic mutants is the two phase plate selection. In these plates, the substrate for the degradative pathway under investigation is added to the minimal salts agar (washed agar for solidification) at a concentration minimal for supporting normal colony growth (size). Leaky mutants and prototrophs will produce colonies of normal morphology on this substrate. The second substrate phase consists of geraniol vapor originating from several drops of geraniol placed in the petri dish lid. One rather unique property of P. aeruginosa is its ability to utilize geraniol, a hydrophobic rose oil terpene, as a sole source of carbon and energy (Stanier et al., 1966). If a culture of P. aeruginosa is streaked on minimal salts agar devoid of substrate and several drops of geraniol are placed in the petri dish lid, very thin spreading colonies may be observed after two to four days of incubation at 37°C. Since the catabolic mutants will utilize only the geraniol as a substrate, they will tend to form very flat, thin

colonies which are readily distinguished in the two phase system among the normal convex colonies. An added advantage of this system is the ability of leaky mutants to utilize enough of the substrate in the agar to form a convex area in the center of their colonies or to form a thick flat colony such that these are also easily distinguished and can be avoided. It has been possible to visually select one glycerol negative mutant from a plate bearing approximately 500 colonies. Again, washed agar is recommended for plate solidification.

Plates for this method of selection are normally incubated five to eight days at 37°C prior to colony selection and in previous glycerol enrichments, 30 plates have been scanned and picked onto nutrient agar in less than one and one-half hours with almost 100% efficiency of selection. In order to pick enough colonies at random from other types of enrichments and to obtain the same number of glycerol-negative strains, it would require an estimated four days of constant colony picking.

Since auxotroph isolations in this study concentrated more on reproducible amino acid auxotroph selection and no attempts were made to optimize the selection for a particular pathway, the mutants isolated have been from a variety of pathways and a brief discussion of the variety of mutants isolated might be helpful for future use.

Lysine, methionine, and threonine were placed in a family since they are all synthesized from aspartic acid via

aspartate-ß-semialdehyde. The lysine and threonine-methionine pathways diverge after aspartate-ß-semialdehyde by synthesizing dihydrodipicolinate and homoserine, respectively. Likewise, the threonine and methionine pathways diverge after homoserine by forming the intermediates homoserine-O-phosphate and O-succinylhomoserine, respectively. Thus, in this family, five mutant groups, as characterized by amino acid supplementation, are possible and mutants were isolated which required methionine; threonine; lysine; methionine and threonine; and lysine, methionine and threonine.

The lysine pathway will be difficult to study as the last intermediate, meso-diaminopimelic acid, is a cell wall constituent (Collins, 1963; Clarke et al., 1967) and its absence during cellular metabolism initiates a phase of lysis very similar to thymineless death (McQuillen, 1958). The cell thus requires lysine for protein synthesis and diaminopimelic acid for cell wall synthesis and a metabolic block in the lysine pathway prior to the last enzymatic step would in effect lyse the cell in the same manner as if D-cycloserine were added (Figure 2). Only five non-leaky lysine auxotrophs have been isolated; however, these did not appear to be as stable as most auxotrophs and all became leaky. All other lysine auxotrophs isolated were initially leaky. Investigation of this pathway may require development of additional techniques for isolation of stable mutants.

Stable auxotrophs for methionine and threonine have been isolated and a mutant requiring both methionine and threonine was isolated. The methionine pathway has been studied by transduction (Calhoun and Feary, 1969) and methionine auxotrophs were also transduced in this study in a preliminary investigation. Calhoun and Feary found five groups in the methionine pathway, four of which were unlinked; only fifteen of the 71 methionine auxotrophs isolated were mapped in their study. Data obtained in the present study by transduction of 27 methionine-requiring mutants indicate at least six and possibly seven loci for the methionine biosynthetic pathway in P. aeruginosa.

A point should be clarified regarding transduction in P. aeruginosa. Since the methionine investigation of Calhoun and Feary is one of the best genetic studies reported for P. aeruginosa, a number of questions must be answered. Why were only 15 of 71 auxotrophs requiring methionine transduced? Since five genetic groups were reported and 15 mutants were transduced, it would be logical to assume that three mutants from each group were transduced. However, this is not the case and the mutants mapped from each group were: Group I - one mutant; Group II - four mutants; Group III - six mutants; Group IV - one mutant; and Group V - two mutants. As one can plainly see, two-thirds of the mutants mapped were in two of the five groups. Although this point will not be discussed for each pathway, it appears to be a general rule in published studies of transduction in

P. aeruginosa that an investigator apparently cannot map approximately 80 per cent of the mutants isolated and most do not report actual colony counts in the form of a transduction table. The one exception to date is the excellent transductional analysis of arginine auxotrophs (Feary et al., 1969). In contrast to the pathways reported for P. aeruginosa, of the 27 mutants transduced in the present study, 24 provided adequate transduction counts, one strain began to leak, one was resistant to phage F-116, and three gave numbers greater than 1000 colonies per phage spot with any phage preparation other than homologous phage. One of these three did cross with itself, yielding greater than 1000 colonies in the spot and grouped separately from the other two. The other two mutants which behaved strangely as recipients crossed with each other and "selfed", yielding 2, 2, 135, and 298 colonies per spot, the underlined counts being self crosses. Comparison of the two studies of the methionine pathway would seem to indicate that pathways in P. aeruginosa can be mapped by fewer investigators isolating better mutants in less time if more efficient techniques are utilized. If one analyzes other genetic studies of P. aeruginosa which have been reported, the same problems are apparent and usually to a greater degree. The preliminary transduction of methionine auxotrophs carried out in the present investigation is mentioned with reservation and does not meet the standards considered necessary by the investigator, i.e., that each genetic and biochemical group

contain five mutants which transduce adequately. These standards are probably not unreasonable if one investigates a pathway efficiently.

The mutants listed below for particular pathways represent those mutants remaining at the end of five months of auxotroph selection during which eleven mutant enrichment and selection experiments were run. At the end of this period, all mutants which did not grow during 100 hours of aeration on a reciprocal shaker at 37°C when isolated were rechecked in the same manner and approximately 40 per cent had developed leaky properties during this storage interval and could not be used. The problem of storage must be stressed; an investigator must have a reliable storage method prior to extensive experiments involving mutant selection. A form of suspended animation, e.g., liquid nitrogen or lyophilization, is probably best.

Leucine, isoleucine, and valine were grouped into a family and mutants requiring leucine; isoleucine and valine; and leucine, isoleucine, and valine were isolated. This family composed approximately one-fourth to one-half the stable mutant population in each auxotroph isolation experiment. A portion of this family has been mapped in P. aeruginosa (Marinus and Loutit, 1969; Pearce and Loutit, 1965). An investigator should have few problems in isolating mutants for this entire family and only three enzymes have been investigated.

Forty-four stable mutants requiring only histidine were

isolated. Mee and Lee (1967) mapped 23 of 107 histidine-requiring mutants; however, of the 23 mapped, 13 had an additional requirement, i.e., were double mutants. This is one of the better genetic studies in P. aeruginosa.

The amino acids tryptophan, phenylalanine, and tyrosine were grouped into the aromatic family which was tentatively subdivided into mutants which would grow with tryptophan as supplement and those which would grow when all three amino acids were added. Twenty tryptophan and 26 aromatic group auxotrophs were isolated.

Serine, glycine, and cysteine composed the serine family. Eight serine auxotrophs were isolated; however, no mutants were isolated with a cysteine or glycine requirement, although Calhoun and Feary (1969) reported the isolation of a number of cysteine auxotrophs.

Twenty proline mutants were isolated and six became leaky within eight months of slant storage.

Only six arginine auxotrophs were isolated in the eleven mutant enrichment and selection experiments. Arginine auxotrophs from previous isolations required 20 to 50μg per ml of arginine supplementation for adequate growth; therefore, the 150 ng per ml arginine supplementation of the enriched glycerol minimal medium used for auxotroph selection was probably far too low to allow arginine auxotrophs to develop small colonies. Feary et al. (1969) isolated 51 arginine auxotrophs of P. aeruginosa strain 1C and 52 arginine auxotrophs of P. aeruginosa strain 78 and reported

transduction data which are probably the best available for each strain to date.

Mutants with glutamine and/or asparagine requirements were not isolated in this series of experiments, but were previously isolated by the direct plating method. The plates used for the last eleven auxotroph isolations were not supplemented with glutamine or asparagine.

Two mutants which were inhibited by methionine but would grow on glucose minimal medium were also isolated by the direct plating method. One mutant which would grow on methionine and methionine-threonine supplemented plates, but not on lysine, methionine and threonine supplemented plates or on glucose minimal medium, was isolated in the tenth auxotroph isolation experiment. It mapped in a well-defined methionine group by transduction.

Although only amino acid auxotrophs were selected, a number of unknown auxotrophs were isolated. One was found to lack triose isomerase and would grow on lactate or glucose minimal medium if glycerol was added, but would not utilize glycerol as a source of carbon and energy. Some of the other unidentified mutants could have been double mutants or may have possessed a requirement which was not selected for or tested.

The efficiency of the small colony selection method on the enriched glycerol minimal medium prompted the testing of colonies which did not replica plate as auxotrophs, i.e., those which grew on each amino acid-supplemented glucose

minimal plate. Twenty-six of these colonies were tested for the ability to utilize glycerol as a substrate and eight were found to be good glycerol-negative mutants. Eight were also found to be leaky glycerol mutants and the other nine were prototrophs. These eight glycerol-negative mutants and the triose isomerase mutant were the results of testing all small colonies picked for which amino acid requirements could not be determined and bear the prefix AH-1 through 9. The other undefined mutants were not tested further.

Presumptive mutants, i.e., small colonies, were "pin point"-spotted on nutrient agar plates and incubated overnight at room temperature. The colonies were then streaked in rectangles on nutrient agar master plates and incubated at 37°C for four to six hours. They were then replica-plated in order mentioned onto glucose minimal medium supplemented with (1) the aromatic amino acids plus histidine, (2) lysine, methionine, and threonine, (3) proline, arginine, glycine, serine, and cysteine, (4) leucine, isoleucine, and valine, and finally onto a nutrient agar plate. Plates of this nature present a much better indication of nutrient requirement if a washed agar is used for solidification, and an indication of which strains revert can be obtained if the plates are dry and good quality replica plating pads are used. If the agar is wet, some colonies may scatter a few cells as the pad is removed from the agar surface. If absorbent replica plating pads are utilized, a clear replica plating is obtained with dry

plates. Replica plating of colonies from the original selective plates is not recommended, but may be successful in certain instances.

Mutant storage cannot be over-stressed as hundreds of mutants have been lost during the course of this study for lack of an adequate method of storage. If slant storage is necessary, minimum transfers at two to three month intervals might be preservative when stocks are stored at 4 to 5°C. These cold room stocks should never be utilized as working cultures and strains should be restreaked every six to eight months for single colony isolations.

Genetic studies of catabolic pathways in P. aeruginosa have been rare; however, glycerol mutants of suitable quality for such studies have been isolated in this investigation by three methods. Small colonies on enriched glycerol minimal medium solidified with unwashed agar have been found to be glycerol-negative mutants. Colonies of normal size on fructose minimal medium solidified with washed agar have been found to be approximately 50% glycerol mutants after three cycles of enrichment, i.e., after cycles of antibiotic treatment in glycerol and regrowth in fructose minimal medium. Flat colonies on glycerol minimal medium with geraniol in the lid have been isolated after one antibiotic treatment and found to be glycerol-negative mutants of good quality.

Higher spot plate transduction counts for amino acid auxotrophs have been obtained by utilizing glycerol instead

of glucose as a substrate and overnight room temperature incubation prior to 37°C incubation.

Although the genticist studying P. aeruginosa has a number of problems not found in similar studies with other organisms, it is concluded that genetic studies with this organism can be done and done efficiently.

A comparison of Figures 8 and 9 suggests the possibility of utilizing penicillin G as the enrichment antibiotic. If a penicillin G addition of 30,000 to 50,000 units per ml is made after eight to nine hours of treatment with 50,000 units of penicillin G, it may be possible to enrich the M:P ratio approximately 3.5 to 4.0 logs with a less toxic antibiotic.

CHAPTER V

SUMMARY AND CONCLUSIONS

Methods of monitoring viable cell counts of mutant and prototroph populations during antibiotic treatment were developed. Cell death was observed when antibiotic-treated bacterial populations were grown on nutrient medium or glucose minimal medium, but could not be detected when cells were grown on minimal media containing a more slowly metabolized substrate, e.g., glycerol or fructose.

Methods of enriching P. aeruginosa mutants by antibiotic selection were thoroughly investigated and an enrichment method utilizing two D-cycloserine additions was developed. Survival curves of the mutant and prototroph populations during D-cycloserine double addition enrichments reproducibly demonstrated an increase in the mutant: prototroph ratio of 3.5 to 4.0 logs while no mutant death was detected. A mutant enrichment method utilizing two antibiotics, D-cycloserine and carbenicillin, was compared to the D-cycloserine double addition method; no difference in the efficiency of mutant selection by the D-cycloserine double addition method and the D-cycloserine:carbenicillin method could be detected.

Mutant and prototroph survival curves during treatment

with penicillin G indicated the selective potential of a benzylpenicillin double addition method. Penicillin G-treated populations of P. aeruginosa demonstrated little, if any, cell death due to post-treatment growth on nutrient agar. Mutant death was observed when cells were vigorously aerated during antibiotic treatment.

Dihydrostreptomycin was thoroughly investigated as an antibiotic for use in the selective enrichment of mutants of P. aeruginosa. The mutant:prototroph ratio was increased two logs during dihydrostreptomycin treatment in glycerol minimal medium; however, the method was found to be restrictive and inferior to all other antibiotic enrichments studied. Mutant death was always observed when more rapidly metabolized substrates were utilized in dihydrostreptomycin treatments.

Although no attempts to optimize the conditions of mutagensis were made, numerous mutagenic treatments were utilized during various phases of this study. N-Methyl-N'-nitro-N-nitrosoguanidine was successfully used as a mutagen in acetate buffer and acetate phosphate buffer. The mutant:prototroph ratio appeared to increase as mutagenized cultures were grown in nutrient broth for longer than two hours. More mutants were isolated from MNNG-treated cultures grown in nutrient broth for four hours than from identically treated cultures grown in nutrient broth for two hours.

Auxotrophs were isolated as small colonies on glycerol

or fructose minimal medium supplemented with nanogram quantities of amino acids. After spreading with appropriate dilutions of treated cultures, plates for isolating auxotrophs were routinely incubated at 37°C for four to five days prior to selecting small to minute colonies for testing. Bacto-agar was found to contain quantities of many amino acids which were adequate for the formation of small auxotroph colonies. Most auxotrophs isolated by this method would not grow in glucose minimal medium aerated at 37°C for 100 hours.

Glycerol-negative mutants were also isolated from the glycerol minimal medium utilized for auxotroph selection; these mutants were selected in glucose minimal broth by the D-cycloserine double addition method and formed small colonies on glycerol minimal plates.

Glycerol-negative mutants were isolated by treating mutagenized cultures of P. aeruginosa in glycerol minimal broth with one of the mutant enrichment methods described above. Glycerol-negative mutants were visually selected after one antibiotic treatment as flat colonies on glycerol minimal plates with geraniol in the lid. Mutants unable to catabolize glycerol were also isolated on fructose minimal medium after three antibiotic treatments; cultures were recycled after each antibiotic treatment by regrowth in fructose minimal broth.

Mutagenized cultures of P. aeruginosa were grown in nutrient broth for two hours prior to selection in glucose

minimal medium by the D-cycloserine double addition method and sampling for microscopic observation. Samples of these enrichment cultures were observed by phase contrast microscopy at a magnification of 625X. "Bdellovibrio-like" organisms were observed in cultures of PA-1 and Soil-2 which demonstrated the autolytic phenomenon when plated on tryptone medium or on minimal medium. Mutants could not be isolated during periods of extreme autolysis and the efficiency of mutant selection via a broth selection method always decreased in cultures demonstrating autolysis. The autolytic phenomenon was rarely observed in any auxotrophic strain or in glycerol-negative mutants.

Preliminary spot plate transduction studies of the glycerol catabolic pathway and the methionine biosynthetic pathway were performed. The number of methionine-positive transductants obtained on glycerol minimal medium incubated at room temperature was greater than on glucose minimal medium incubated at 37°C for any given heterologous cross. The number of glycerol-positive transductants per spot obtained on glycerol minimal medium incubated at room temperature for 12 hours prior to 37°C incubation was greater than those reported in the preliminary methionine transduction study for which data were presented.

Twenty-four methionine auxotrophs were transduced on glycerol minimal transduction agar. Spot plate transduction studies of these mutants on glucose minimal transduction agar yielded lower numbers in spots where two unlinked loci

were crossed. Seven genetic loci were found by transductional analysis and four biochemically distinct groups could be distinguished by intermediate feeding. No genetic linkage was observed between any two methionine loci by transduction.

All mutant strains were difficult to maintain by periodic slant transfer. Mutants of P. aeruginosa cannot reliably be maintained on nutrient slants by periodic transfer for more than one year.

CHAPTER VI

RECOMMENDATIONS FOR FUTURE WORK

In order to conduct efficient genetic research on any given bacterial specie, a number of techniques must be available. An adequate mutagenization procedure, reliable broth enrichment and plate selection techniques, and adequate storage methods must exist to allow efficient isolation and maintenance of mutant stocks of adequate size and quality for genetic mapping by transduction and conjugation. Most of these technqiues do not exist for many of the bacterial species currently being investigated biochemically and genetically. A consideration of the research time wasted in attempts to isolate mutants by inefficient methods in comparison to the information which could be obtained if efficient techniques were applied should prompt the geneticist to initiate research contributing some technical improvement, however small, to the genetics of "his" organism. It is sincerely hoped that these techniques or a modification thereof may benefit studies of the genetics of P. aeruginosa.

The following techniques would be used to initiate further genetic research with P. aeruginosa:

1. Mutagenization - A nutrient broth culture in log phase growth would be harvested, washed, and

resuspended in 6.0 to 8.0 ml of acetate-phosphate buffer, pH 6.0, containing 100 to 850μg per ml N-methyl-N'-nitro-N-nitrosoguanidine. The culture would be aerated on a reciprocal shaker at 37°C for 20 minutes prior to harvesting, washing, and resuspending in nutrient broth, which would then be aerated at 37°C for six to nine hours.

2. Broth Selection:

   a. Auxotroph Isolations - Auxotrophs would be selected in glucose minimal medium containing either (1) 660μg per ml of D-cycloserine to which 350μg per ml of D-cycloserine is to be added in the interval 2.5 to 3.5 hr, or (2) 500μg per ml of D-cycloserine plus 100μg per ml of carbenicillin. The mutagenized nutrient-broth grown culture would be inoculated to a final concentration of 3.0×10^7 to 5.0×10^7 cells per ml after harvesting, washing, and resuspending in minimal medium.

   b. Catabolic Mutant Isolations - Catabolic mutants would be selected by resuspending the mutagenized nutrient-broth grown culture to 3.0×10^7 to 5.0×10^7 cells per ml in minimal medium containing the substrate for which mutants were being selected and aerating at 37°C until the culture began

to grow. Antibiotic system (1) or (2) would then be added and the culture reaerated for a length of time determined by a death curve for prototrophs utilizing the substrate for which mutants were being selected.

c. Cell Recycle - The survivors of an antibiotic-treated culture might be recycled by (1) harvesting, washing, resuspending in either glycerol or fructose minimal medium and aerating until grown; (2) treating with 550µg per ml of L-alanine or 500µg per ml of DL-alanine for 30 minutes, harvesting, washing, resuspending in either glycerol or fructose minimal medium and aerating until grown. Cell recycle for auxotroph isolations requires the growth factor in excess during regrowth of the culture.

3. Plate Selection:

a. Auxotroph Plate Selection - The nutrient for which auxotrophs are desired would be added at a concentration producing small auxotroph colonies on glycerol or fructose minimal medium after approximately four days incubation at 37°C. The plates would be solidified with a washed agar to eliminate the appearance of most of the auxotrophs with other requirements and a constant plate

volume would be used.

b. Catabolic Mutant Plate Selection - The minimal concentration of the substrate, for which mutants are to be selected, which allows normal colony growth would be used in preparing minimal medium plates solidified with a washed agar. After an antibiotic-treated cell population was spread on these plates, three to four drops of geraniol would be placed in the lid and the plates incubated at 37°C for six to eight days. Flat thin colonies would then be selected for testing.

4. Broth Testing of Mutants - Mutants would be inoculated into 8.0 ml of an appropriate minimal broth to slight visual turbidity. The tubes would be aerated on a reciprocal shaker for four to five days, at which time cultures with little or no turbidity would be selected for future study.

5. Mutant Storage - Mutants would be maintained on nutrient agar slants in duplicate, stored at 4 to 5°C and transferred at two to three month intervals. The cultures would be streaked on nutrient agar plates every third transfer and a single colony isolate selected for slant inoculation. These colonies would also be used to

inoculate nutrient agar master plates which would be replicated to test these colonies. A better method for mutant storage would be sought by inquiry and experimentation.

Mutagenization, transduction, and mutant storage would be investigated and hopefully improved. Mutant isolations during autolytic periods and the cause of autolysis would be investigated since it is only through continued improvement of techniques that the geneticist can develop a system for P. aeruginosa which may be compared to the known systems and thereby contribute to better understanding of the living cell.

SELECTED BIBLIOGRAPHY

Abraham, E. P., E. Chain, C. M. Fletcher, H. W. Florey, A. D. Gardner, N. G. Heatley, and M. A. Jennings.

1941 Further observations on penicillin. Lancet. 2: 177-189.

Acred, P., D. M. Brown, E. T. Knudsen, G. N. Rolinson, and R. Sutherland.

1967 New semi-synthetic penicillin active against Pseudomonas pyocyanea. Nature. 215:25-30.

Adelberg, E. A., and J. W. Myers.

1953 Modification of the penicillin technique for the selection of auxotrophic bacteria. J. Bacteriol. 65:348-353.

Adelberg, E. A.

1958 Selection of bacterial mutants which excrete antagonists of antimetabolites. J. Bacteriol. 76:326.

Adelberg, E. A., M. Mandel, and G. C. C. Chen.

1965 Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichа coli K-12. Biochem. Biophys. Res. Commun. 18:788-795.

Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger.

1965 Lipid-phosphoacetyl muramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide. Presumed membrane transport intermediates in bacterial cell wall synthesis. Proc. Natl. Acad. Sci. (US). 53:881-889.

Anderson, J. S., P. M. Meddow, M. A. Haskin, and J. L. Strominger.

1966 Biosynthesis of the peptidoglycan of bacterial cell walls. 1. Utilization of uridine diphosphate acetylmuramyl pentapeptide and uridine diphosphate acetylglucosamine for peptidoglycan synthesis by particulate enzymes from Staphylococcus aureus and Micrococcus lysodeikticus. Arch. Biochem. Biophys. 116: 487-515.

Araki, Y., R. Shimai, A. Shimada, N. Ishimoto, and E. Ito.

1966 Enzymatic synthesis of cell wall mucopeptide in a particulate preparation of Escherichia coli. Biochem. Biophys. Res. Commun. 23:466-472.

Araki, Y., A. Shimada, and E. Ito.

1966 Effect of penicillin on cell wall mucopeptide synthesis in a Escherichia coli particulate system. Biochem. Biophys. Res. Commun. 23:518-525.

Barker, R. and I. Tessman.

1968 Different mutagenic specificities in phages S13 and T4: "in vivo" treatment with N-methyl-N'-nitro-N-nitrosoguanidine. J. Mol. Biol. 35: 439-448.

Beadle, G. W.

1945a Genetics and metabolism in Neurospora. Physiol. Rev. 25:643-663.

1945b Biochemical genetics. Chem. Rev. 37:15-96.

Beadle, G. W., and E. L. Tatum.

1941 Genetic control of biochemical reactions in Neurospora. Proc. Natl. Acad. Sci. U. S. 27: 499-506.

1945 Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Bot. 32:678.

Bechtle, R. M., and G. H. Scherr.

1958 A new agar for in vitro antimicrobial sensitivity testing. Antibio. and Chemother. 8:599-606.

Bell, S. M. and D. D. Smith

1969 Resistance of Pseudomonas aeruginosa to carbenicillin. Lancet. 1:753-754.

Berk, R. S.

1963 Nutritional studies on the auto-plaque phenomenon in Pseudomonas aeruginosa. J. Bacteriology. 86: 728-734.

Bolt, K. F. and D. G. Lundgren.

1962 Auxotrophic mutant production utilizing staphcillin. Can. J. Microbiol. 8:281-283.

Bondi, A., J. Kornblum, and C. Forte.

1957 Inhibition of antibacterial activity of cycloserine by alpha-alanine. Proc. Soc. Exptl. Biol. Med. 96:270-272.

Bruce, B. B.

1965 Genetic studies of allantoin metabolism in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Brumfitt, W., A. Percival, and D. A. Leigh.

1967 Clinical and laboratory studies with carbenicillin. Lancet. 1:1289-1293.

Buogo, A., A. diMarco, M. Ghione, A. Migliacci, and A. Sanfilippo.

1958 Antagonism of D- and L-alanine of the enantiomorphic forms of cycloserine. Chem. Abstr. 54: 17538-17539.

Buttin, G.

1963 Mécanismes régulateurs dans la biosynthèse des enzymes du métabolisme du galactose chez Escherichia coli K 12. J. Mol. Biol. 7:183-205.

Calhoun, D. H. and T. W. Feary.

1969 Transductional analysis of Pseudomonas aeruginosa methionineless auxotrophs. J. Bacteriol. 97:210-216.

Cerdá-Olmedo, E., P. C. Hanawalt, and N. Guerola.

1968 Mutagenesis of the replication point by nitrosoguanidine: Map and pattern of replication of the Escherichia coli chromosome. J. Mol. Biol. 33:705-719.

Chain, E., H. W. Florey, A. D. Gardner, N. G. Heatley, M. A. Jennings, J. Orr-Ewing, and A. G. Sanders.

1940 Penicillin as a chemotherapeutic agent. Lancet. 239:226-228.

Chain, E. and E. S. Duthie.

1945 Bactericidal and bacteriolytic action of penicillin on the staphylococcus. Lancet. 248: 652-657.

Chatterjee, A. N. and J. T. Park

1964 Biosynthesis of cell wall mucopeptide by a particulate fraction from Staphylococcus aureus. Proc. Natl. Acad. Sci. U. S. 51:9-16.

Chutterbuck, P. W., R. Lovell, and H. Raistrick.

1932 The formation from glucose by a member of the Penicillium chrysogenum series of a pigment, an alkali soluble protein and penicillin - the antibacterial substance of Fleming. Biochem. J. 26:1907-1921.

Ciak, J., and F. E. Hahn.

1959 Mechanisms of action of antibiotics. II. Studies on the modes of action of cycloserine and its L-stereoisomer. Antibiotics and Chemotherapy. 9:47-54.

Clarke, K., G. W. Gray, and D. A. Reaveley.

1967 The cell walls of Pseudomonas aeruginosa. General composition. J. Biochem. 105:749-754.

Cohen, G. N.

1965 Regulation of enzymatic activity in microorganisms. Ann. Rev. Microbiol. 19:105-126.

Collins, F. M.

1963 Composition of cell walls of ageing Pseudomonas aeruginosa and Salmonella bethesda. J. Gen. Microbiol. 34:379-388.

Cowen, C. M.

1968 Catabolic pathways and metabolic controls in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Cuckler, A. C., B. M. Frost, L. McClelland, and M. Solotorovsky.

1955 The antimicrobial evaluation of oxamycin (D-4-amino-3-isoxazolidone), a new broad-spectrum, antibiotic. Antibiot. and Chemother. 5:191-197.

Davis, B. D.

1948 Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc. 70: 4267.

1949a Isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Natl. Acad. Sci. 35:1-10.

1949b Isolation of biochemically deficient mutants of bacteria by limited enrichment. Arch. Biochem. 20:166-167.

1950 Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. Experientia. 6:41-50.

Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr.

1967 Microbiology. New York, Evanston, and London: Harper and Row Publishers.

deLay, J.

1964 Pseudomonas and related genera. Ann. Rev. Microbiol. 18:17-41.

Dietrich, C. P., M. Matsuhashi, and J. L. Strominger.

1965 Glycerol diphosphate disaccharide-pentapeptide: A functional group of the lipid intermediate in cell wall glycopeptide synthesis. Biochem. Biophys. Res. Commun. 21:619-623.

Dietrich, C. P., M. Matsuhashi, W. Katz, G. Siewert, P. Thomas, and J. S. Anderson.

1966 Studies of the lipid intermediates in cell wall glycopeptide synthesis. Federation Proc. Abstracts. 25:588.

Drake, J. W.

1969 Mutagenic mechanisms. Annual Review of Genetics. 3:247-268.

Evans, D. H.

1966 Use of kanamycin for the isolation of auxotrophic mutants of Actinobacillus mallei. Can. J. Microbiol. 12:641-652.

Fargie, B. and B. W. Holloway.

1965 Absence of clustering of functionally related genes in Pseudomonas aeruginosa. Genet. Res. 6:284-299.

Feary, T. W., B. Williams, D. H. Calhoun, and T. A. Walker.

1969 An analysis of arginine requiring mutants in Pseudomonas aeruginosa. Genetics. 62:673-686.

Fishbein, L., W. G. Flamm, and H. L. Falk.

1970 Chemical mutagens. New York and London: Academic Press.

Fleming, A.

1929 On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenzae. Brit. J. Exptl. Pathol. 10:226-236.

Fowden, L., D. Lewis, and H. Tristram.

1967 Toxic Amino Acids: Their action as antimetabolites. Advances in Enzymology. 29:89-164.

Gardner, A. D.

1940 Morphological effects of penicillin on bacteria. Nature. 146: 837-838.

Ginsburg, V.

1964 Sugar nucleotides and the synthesis of carbohydrates. Advanc Enzymol. 26:35-88.

Glauert, A. M., and M. J. Thornley.

1969 The topography of the bacterial cell wall. Annual Reviews of Microbiology. 23:159-198.

Gorini, L. and H. Kaufman.

1960 Selecting bacterial mutants by the penicillin method. Science. 131:604-605.

Gray, C. H., and E. L. Tatum.

1944 X ray induced growth factor requirements in bacteria. Proc. Natl. Acad. Sci. U.S. 30:404-410.

Green, C. C.

1969 Genetic transfer in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Hadley, P.

1924 Transmissible lysis of Bacillus pyocyaneus. Journal of Infectious Diseases. 34:260-304.

Harned, R. L., P. H. Hidy, and E. K. laBaw.

1955 Cycloserine. I. A preliminary report. Antibiol. and Chemother. 5:204-205.

Harris, D. A., M. Ruger, M. A. Reagan, F. J. Wolf, R. L. Peck, H. Wallick, and H. B. Woodruff.

1955 Discovery, development, and antimicrobial properties of D-4-amino-3-isoxazolidone (oxamycin), a new antibiotic produced by Streptomyces garyphalus. Antibiot. and Chemother. 5:183-190.

Heath, H. E.

1971 Glucose metabolism and catabolite repression in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Hidy, P. H., E. B. Hodge, V. V. Young, R. L. Harned, G. A. Brewer, W. F. Phillips, W. F. Runge, H. E. Staveley, A. Pohaland, H. Boaz, and H. R. Sullivan.

1955 Structures and reactions of cycloserine. J. Am. Chem. Soc. 77:2345-2346.

Higashi, Y., J. L. Strominger, and C. C. Sweeley.

1967 Structure of a lipid intermediate in cell wall peptidoglycan synthesis: A derivative of a C_{55} isoprenoid alcohol. Proc. Natl. Acad. Sci. U.S. 57:1878-1884.

Hobby, G. L., K. Meyer, and E. Chaffee.

1942. Observations on the mechanism of action of penicillin. Proc. Soc. Exptl. Biol. Med. 50: 281-285.

Hoeprich, P. D.

1963 Alanine:cycloserine antagonism. III. Quantitative aspects and relation to heating of culture media. J. Lab. and Clin. Med. 62:657-662.

Holliday, R.

1956 A new method for the identification of biochemical mutants of microorganisms. Nature (London). 178:987.

Holloway, B. W.

1969 Genetics of Pseudomonas. Bacteriological Reviews. 33:419-443.

Holloway, B. W., M. Monk, L. Hodgins, and B. Fargie.

1962 Effects of radiation on transduction in Pseudomonas aeruginosa. Virology. 18:80-94.

Holloway, B. W., L. Hodgins, and B. Fargie.

1963 Unlinked loci affecting related biosynthetic steps in Pseudomonas aeruginosa. Nature. 199: 926-927.

Holmes, K. K., H. Clark, F. Silverblatt, and M. Turck.

1970 Emergence of resistance in Pseudomonas during carbenicillin therapy. Antimicrobial Agents and Chemotherapy. 1969:391-397.

Horecker, B. L.

1966 The biosynthesis of bacterial polysacoharides. Annual Review of Microbiology. 20:253-290.

Horiuchi, T., J. Tomizawa, and A. Novic.

1962 Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochem. Biophys. Acta. 55:152-163.

Howe, W. B., G. L. Melson, C. H. Meridith, J. R. Morrison, M. H. Platt, and J. L. Strominger.

1964 Stepwise development of resistance to D-cycloserine in Staphylococcus aureus. J. Pharmacol. Exptl. Therap. 143:282-284.

Hungate, R. E.

1960 Symposium: Selected topics in microbial ecology. I. Microbial ecology of the rumen. Bacteriol. Reviews. 24:353-364.

Ikawa, M., and E. E. Snell.

1960 Cell wall composition of lactic acid bacteria. J. Biol. Chem. 235:1376-1382.

Isaac, J. H., and B. W. Holloway.

1968 Control of pyrimidine biosynthesis in Pseudomonas aeruginosa. J. Bacteriol. 96:1732-1741.

Ishida, T., S. Seto, and T. Osawa.

1966 Use of dihydrostreptomycin for the isolation of auxotrophic mutants of Pseudomonas aeruginosa. J. Bacteriol. 91:1387.

Ito, F., T. Aiko, M. Yamamoto, M. Yuasa, H. Mizobata, and K. Tone.

1958 The mode of action of cycloserine. Med. J. Osaka Univ. 9:23-31.

Izaki, K., M. Matsuhashi, and J. L. Strominger.

1966 Penicillin-sensitive enzymatic reactions. Proc. Natl. Acad. Sci. U.S. 55:656-663.

Jacob, F., and J. Monod.

1961 Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

Karlsson, J. L., and H. A. Barker.

1948 Induced biochemical mutants of Azotobacter agilis. J. Bacteriol. 56:671-677.

Kessel, D., and M. Lubin.

1965 Stability of α-hydrogen of amino acids during active transport. Biochemistry. 4:561-565.

Kuehl, F. A., F. J. Wolf, N. R. Trenner, R. L. Peck, E. Howe, B. D. Hunnewell, G. Downing, E. Newstead, R. P. Buhs, I. Putter, R. Ormond, J. E. Lyons, L. Chalet, and K. Folkers.

1955 D-4-Amino-3-isoxazolidone, a new antibiotic. J. Am. Chem. Soc. 77:2344-2345.

Kurihara, T., and K. Chiba.

1956 Orientomycin, a new antibiotic. Ann. Rept. Tohoku. coll. Pharm. 3:83-87.

Lark, C., and R. Schichtel.

1962 Comparison of spheroplast induction in Alcaligenes faecalis by three different agents. J. Bacteriol. 84:1241-1244.

Lawrence, P. J., and J. L. Strominger.

1970 Biosynthesis of the peptidoglycan of bacterial cell walls. XV. The binding of radioactive penicillin to the particulate enzyme prepartion of Bacillus subtilis and its reversal with hydroxylamine or thiols. J. Biol. Chem. 245: 3653-3659.

Leach, F. R., and E. E. Snell.

1960 The absorption of glycine and alanine and their peptides by Lactobacillus casei. J. Biol. Chem. 235:3523-3531.

Lederberg, J.

1946 Studies in bacterial genetics. J. Bacteriol. 52:503.

Lederberg, J.

1948 Problems in microbial genetics. Heredity. 2:145-198.

1950 Isolation and characterization of biochemical mutants of bacteria. Methods in Medical Research. 3:5-22.

1956 Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. U.S. 42:574-577.

Lederberg, J., and E. M. Lederberg.

1952 Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-406.

Lederberg, J., and E. L. Tatum.

1946 Detection of biochemical mutants of microorganisms. J. Biol. Chem. 165:381-382.

Lederberg, J., and N. Zinder.

1948 Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc. 70:4267-4268.

Lessie, T. G., and H. R. Whiteley.

1969 Properties of threonine deaminase from a bacterium able to use threonine as sole source of carbon. J. Bacteriology. 100:878-889.

Lillick, L., R. Strange, L. J. Boyd, M. Schwimmer, and M. G. Mulinos.

1956 Cycloserine in the treatment of nontuberculosis infections. Antibiot. Ann. 1955/1956:158. New York Medical Encyclopedia, Inc.

Lowbury, E. J. L., A. Kidson, H. A. Lilly, G. A. J. Ayliffe, and R. J. Jones.

1969 Sensitivity of Pseudomonas aeruginosa to antibiotics. Emergence of strains highly resistant to carbenicillin. Lancet. 2:448-452.

McQuillen, K.

1958 Lysis resulting from metabolic disturbance. J. Gen. Microbiol. 18:498-512.

Mandel, J., and J. Greenberg.

1960 A new chemical mutagen for bacteria, 1-methyl-3-nitro-1-nitrosoquanidine. Biochem. Biophys. Res. Commun. 3, 575-577.

Marinus, M. G., and J. S. Loutit.

1969 Regulation of isoleucine plus valine biosynthesis in Pseudomonas aeruginosa. I. Characterisation and mapping of mutants. Genetics. 63:547-556.

Martin, H. H.

1964a Composition of the mucopolymer in cell walls of the unstable and stable L-form in Proteus mirabilis. J. Gen. Microbiol. 36:441-450.

1964b Chemical composition of cell wall mucopolymer from penicillin spheroplasts and normal cells of Proteus mirabilis. Abstracts, Sixth Internatl. Congr. of Biochem. 3:518.

Mee, B. J., and B. T. O. Lee.

1967 An analysis of histidine requiring mutants in Pseudomonas aeruginosa. Genetics. 55:709-722.

Mora, J., and E. E. Snell.

1963 The uptake of amino acids by cells and protoplasts of Streptococcus faecalis. Biochemistry. 2: 136-141.

Mose, L. J.

1970 Metabolic defects and phage resistance in Pseudomonas aeruginosa. M. S. Thesis, Oklahoma State University, Stillwater.

Moyed, H. S.

1960 False feedback inhibition: Inhibition of tryptophan biosynthesis by 5-methyltryptophan. J. Biol. Chem. 235:1098-1102.

Müller-Hill, B., H. V. Rickenberg, and K. Wallenfels.

1964 Specificity of the induction of the enzymes of the lac operon in Escherichia coli. J. Mol. Biol. 10:303-318.

Neidhardt, F. C.

1960 Mutant of Aerobacter aerogenes lacking glucose repression. J. Bacteriol. 80:536-543.

Neuhaus, F. C., and J. L. Lynch.

1964 The enzymatic synthesis of D-alanyl-D-alanine. III. On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. Biochemistry. 3:471-480.

Newcombe, H. B.

1948 Delayed phenotypic expression of spontaneous mutations in Escherichia coli. Genetics. 33: 447-476.

Nickerson, W. I.

1964 Composition and function of microbial cell walls. In: Cellular membranes in development (M. Locke, ed.). New York and London: Acad. Press, 1964, pp. 281-297.

Novick, A., and T. Horiuchi.

1961 Hyper-production of β-galactosidase by Escherichia coli bacteria. Cold Spring Harbor Symp. Quant. Biol. 26:239-245.

Oakberg, E. F., and S. E. Luria.

1947 Mutations to sulfonamide resistance in Staphylococcus aureus. Genetics. 32:249-261.

Ornston, L. N., M. K. Ornston, and G. Chou.

1969 Isolation of spontaneous mutant strains of Pseudomonas putida. Biochem. Biophys. Res. Commun. 36:179-184.

Park, J. T.

1952a Uridine-5-pyrophosphate derivatives. I. Isolation from Staphylococcus aureus. J. Biol. Chem. 194:877-884.

1952b Uridine-5-pyrophosphate derivatives. II. A structure common to three derivatives. J. Biol. Chem. 194:885-896.

Park, J. T.

1952c Uridine-5-pyrophosphate compounds. III. Amino acid-containing derivatives. J. Biol. Chem. 194: 897-909.

1958a Inhibition of cell-wall synthesis in Staphylococcus aureus by chemicals which cause accumulation of wall precursors. Biochem. J. 70:2P.

1958b Selective inhibition of bacterial cell-wall synthesis. Its possible applications in chemotherapy. Symp. Soc. Gen. Microbiol. 8:49-51.

1960 Inhibition of synthesis of bacterial mucopeptide by certain antibiotics and its possible significance for microbiology and medicine. Antimicrobial Agents Ann. 1959/60:338-339.

Park, J. T., and J. L. Strominger.

1957 Mode of action of penicillin. Biochemical basis for the mechanism of action penicillin and for its selective toxicity. Science. 125:99-101.

Pearce, L. E., and J. S. Loutit.

1965 Biochemical and genetic grouping of isoleucine-valine mutants of Pseudomonas aeruginosa. J. Bacteriol. 89:58-63.

Pittillo, R. F., and J. W. Foster.

1954 Potentiation of inhibitor action through determination of reversing metabolites. J. Bacteriol. 67:53-57.

Reitz, R. H., H. D. Slade, and F. C. Neuhaus.

1967 The biochemical mechanism of resistance by Streptococci to the antibiotic D-cycloserine and O-carbamyl-D-serine. Biochemistry. 6:2561-2570.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten.

1957 Studies of biosynthesis in Escherichia coli., 2nd printing. Carnegie Inst. Wash. Pub. 607:5.

Roepke, R. R., R. L. Libby, and M. H. Small.

1944 Mutation or variation of Escherichia coli with respect to growth requirements. J. Bacteriol. 48:401-412.

Rolinson, G. N., and R. Sutherland.

1968 Carbenicillin, a new semisynthetic penicillin active against Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy. 1967: 609-613.

Roze, U.

1964 The non-enzymatic reaction between cycloserine and pyridoxal phosphate. Ph.D. Thesis. Washington University, St. Louis, Missouri.

Roze, U., and J. L. Strominger.

1963 The non-enzymatic reaction between D-cycloserine and pyridoxal phosphate. Federation Proc. Abstracts. 22:423.

Ryan, F. J.

1948 On the stability of nutritional mutants of bacteria. Proc. Natl. Acad. Sci. U. S. 34:425-435.

Salton, M. R. J.

1960 Surface layers of the bacterial cell. The Bacteria. 1:97-151

1961 The anatomy of the bacterial surface. Bacteriol. Rev. 25:77-99.

1964 The bacterial cell wall. Amsterdam: Elsevier Publ. Co.

Schlegel, H. G., and H. W. Jannasch.

1967 Enrichment cultures. Annual Reviews of Microbiology. 21:49-70.

Shockman, G. D.

1959 Reversal of cycloserine inhibition by D-alanine. Proc. Soc. Exptl. Biol. Med. 101:693-695.

Shockman, G. D., and J. O. Lampen.

1962 Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bacteriol. 84: 508-512.

Shull, G. M., and J. L. Sardinas.

1955 PA-94, an antibiotic identical with D-4-amino-3-isoxazolidinone (cycloserine, oxamycin). Antibiotics and Chemotherapy. 5:398-399.

Siewart, G., and J. L. Strominger.

1967 Bacitracin. An inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. Proc. Natl. Acad. Sci. (US). 57:767-773.

Smith, D. A., and J. E. Childs.

1966 Methionine genes and enzymes of Salmonella typhimurium. Heredity. 21:265-286.

Stammer, C. H., A. N. Wilson, F. W. Holly, and K. Folkers.

1955 Synthesis of D-4-amino-3-isoxazolidone. J. Am. Chem. Soc. 77:2346-2347.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.

1966 The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159-271.

Stephenson, J. P. B.

1969 Resistance of Pseudomonas aeruginosa. Lancet. 1:1098-1099.

Strominger, J. L.

1962 Biosynthesis of bacterial cell walls. Federation Proc. 21:134-143.

Strominger, J. L., R. H. Threnn, and S. S. Scott.

1959 Oxamycin, a competitive antagonist of the incorporation of D-alanine into a uridine nucleotide in Staphylococcus aureus. J. Am. Chem. Soc. 81:3803-3804.

Strominger, J. L., E. Ito, and R. H. Threnn.

1960 Competitive inhibition of enzymatic reactions by oxamycin. J. Am. Chem. Soc. 82:998-999.

Strominger, J. L., and D. L. Tipper.

1965 Bacterial cell wall synthesis and structure in relation to the mechanism of action of penicillins and other antibacterial agents. Am. J. Med. 39: 708-721.

Swanstrom, M. and M. H. Adams.

1951 Agar layer method for production of high titer phage stocks. Proc. Soc. Exptl. Biol. Med. 78: 372-375.

Tatum, E. L.

1945 X-ray induced mutant strains of Escherichia coli. Proc. Natl. Acad. Sci. U. S. 31:215-219.

1946 Induced biochemical mutations in bacteria. Cold Spring Harbor Symposia Quant. Biol. 11:278-284.

Tatum, E. L., and G. W. Beadle.

1945 Biochemical genetics of Neurospora. Ann. Missouri Botan Garden. 32:125-129.

Tipper, D. J., and J. L. Strominger.

1965 Mechanism of action of penicillins. A proposal based on their structural similarity to acyl-D-alanyl-D-alahine. Proc. Natl. Acad. Sci. U. S. 54:1133-1141.

Toennies, G., and G. D. Shockman.

1959 Growth chemistry of Streptococcus faecalis. Proceedings of the fourth internat. Cong. of Biochem. 13:365-394. London, New York, Paris, Los Angeles: Pergamon Press.

Torriani, A., and F. Rothman.

1961 Mutants of Escherichia coli constitutive for alkaline phosphatase. J. Bacteriol. 81:835-836.

Tsay, S. S.

1971 Transport and catabolism of glycerol in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

van Niel, C. B.

1949 The "delft school" and the rise of general microbiology. Bacteriol Rev. 13:161-174.

1955 Natural selection in the microbial world. The second Marjory Stephenson memorial lecture. J. Gen. Microbiol. 13:201-217.

Varon, M.

1968 Interaction of Bdellovibrio with its hosts. Ph.D. Thesis, Hebrew University, Jerusalem.

Wargel, R. J., C. A. Shadur, and F. C. Neuhaus.

1970 Mechanism of D-cycloserine action. Transport systems for D-alanine, D-cycloserine, L-alanine, and glycine. J. Bacteriol. 103:778-788.

Weidel, W., and H. Pelzer.

1964 Bag-shaped macromolecules - a new outlook on bacterial cell walls. Advanc. Enzymol. 26:193-233.

Witkin, E. M.

1950 Bacterial Mutations Involving Resistance to Destructive Agents. Methods in Medical Research. 3:23-36.

Wood, W. A., and I. C. Gunsalus.

1951 D-Alanine formation. A racemase in Streptococcus faecalis. J. Biol. Chem. 190:403-416.

Woods, D. D.

1940 The relation of p-aminobenzoic acid to the mechanism of the action of sulfanilamide. Brit. J. Exptl. Pathol. 21:74-89.

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