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AN INVESTIGATION OF THE CHANGES IN METABOLISM AND CHEMICAL COMPOSITION OF TWO GRAM NEGATIVE ORGANISMS.

by

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A thesis submitted for the degree of Doctor of Philosophy,

University of Adelaide.

June, 1961.

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This thesis contains no material previously submitted for a degree in any University either by the candidate or by any other person, except where due reference is made in the text of the thesis.

Signed.

June 1961.

ABSTRACT

The work in this thesis is divided into two sections. The first part is concerned with the changes in cell viability and the onset of cellular lysis when _Ps. aeruginosa_ is incubated under anaerobic conditions in the absence of nitrate. The behaviour of the _Ps. aeruginosa_ cultures contrasted sharply with that observed in the corresponding _S. bethesda_ cultures. The rapid decline in viability could be prevented by the presence of nitrate or oxygen in the medium. The prolonged anaerobic incubation of _Ps. aeruginosa_ in the absence of nitrate affected the normal glucose metabolism of the cells.

Both aerobic and anaerobic cultures of _Ps. aeruginosa_ were found to undergo extensive cell lysis during the late decline phase and this contrasted with the behaviour of the _S. bethesda_ cells. A lysis resistant mutant strain of _Ps. aeruginosa_ which was still sensitive to the lethal action of prolonged anaerobic incubation was isolated. The mutation did not render the cell wall resistant to the attack of the autolytic enzymes produced by the parent organism but it apparently prevented the release of the lytic enzymes into the culture medium. The autolytic enzymes of the parent strain were concentrated and shown to contain both mucinase and lethicinase activity but no nucleases were present.

In the second part of the thesis, consideration was given to the possible effects of ageing on the chemical composition of whole cells and cell walls of _Ps. aeruginosa_ and _S. bethesda_. No significant changes in the composition of the whole cells could be correlated with the decline in viability or with the onset of cellular lysis. The decline in cellular RNA was largely compensated by an increase in the protein content. The accumulation of DNA, and, to a lesser extent of

RNA in the culture medium was shown to be a consequence of the massive cellular lysis, together with the absence of nuclease activity in the culture medium. Since cell lysis was presumably due to a hydrolytic attack on the cell wall, chemical and immuno-chemical investigations were made to determine the nature of these changes. Purified cell wall preparations from the three bacterial strains were examined. The chemical composition of the three cell walls varied considerably, particularly with regard to their carbohydrate content. Ageing of the plaque strain Ps. aeruginosa cells coincided with cell wall changes which seemed to involve a progressive removal of a complex of glucose and glucosamine, together with a peptide of alanine lysine serine and DAP. The lysis resistant mutant of Ps. aeruginosa and the S. bethesda cell walls did not show any analogous changes as they aged. Incubation of the cell walls of young Ps. aeruginosa cells in the presence of the crude autolysin concentrate resulted in the complete removal of glucose, glucosamine and DAP, together with some of the muramic acid, lysine and alanine from the walls. The ageing Ps. aeruginosa cell walls also contained progressively less complex lipid compared with the other two strains. The lipopolysaccharide content of the three organisms was not affected by ageing. Thus, the autolysis of the ageing Ps. aeruginosa cells was possibly due to the release by some of the cells of a mucinase and an esterase which then attacked the cell walls of the surrounding bacteria, ultimately resulting in the destruction of the majority of the cells present.

CHAPTER 1.

A GENERAL SURVEY OF THE LITERATURE RELEVANT TO THE GROWTH AND DEATH OF BACTERIA IN LIQUID CULTURES

The problems associated with the internal organisation of a growing bacterial cell are complex and involve far more than the simple absorption of food materials from the surrounding medium and its synthesis into new protoplasm. Despite intensive investigations of growth and cell replication, we are still far from a complete understanding of the processes involved. The small size of the bacterial cells, with their apparent lack of the complicating diffusion factors found in most higher forms of life, make these cells of special value in growth studies. The extremely rapid growth rates and the simple nutritional requirements of many bacteria enable us to follow them through many generations in chemically defined media. Early studies were concerned with little more than tabulating the number of cells present at increasing time intervals, but later the effect of variations in environmental and nutritional factors were considered.

From these studies there developed biochemical investigations into the changes in cellular composition, macromolecular organisation, and enzyme syntheses which occur during the growth of the cells. The development of new techniques such as the synchronisation of growth and the use of continuous cultures has contributed to our rapidly increasing knowledge of growth, and as a result, a clearer picture of the intracellular organisation responsible for cell growth and reproduction is now emerging.

The importance of the studies of the early growth phases cannot be denied, but this emphasis on the lag and logarithmic phases has

led to an almost complete neglect of later phases. Except for some of the more practical aspects of senescence, relatively little is known of the cellular changes associated with the ageing and death of bacterial cultures. The action of various disinfectant processes on cellular activities has been extensively examined both in vivo and in vitro but all too often the effect of the agent has been recorded without any concern as to the exact nature of the cellular changes responsible for death. The metabolic changes responsible for the progressive death of cells suspended in a nutrient medium are necessarily complex, but they have not received the detailed study that they deserve. The present study was carried out, therefore, as a contribution to the available information concerning the cellular changes responsible for the decline of a culture. Since any consideration of the process of ageing and the subsequent death of the cell will require the earlier growth phases as a point of reference, a brief survey of the literature relevant to the growth curve as a whole will first be considered. Following this, the literature dealing with the death and dissolution of cells will be considered.

1. The Growth of Bacteria in Liquid Media.

(a) The lag and logarithmic phases

The well known changes in cell numbers which follow the introduction of a bacterial cell into a liquid medium have been described many times, and the relevant literature has been reviewed by Hinshelwood (1946), Monod (1949) and Gunsalus (1951). The concept of early metabolic activity without cell division followed from the work of Henrici (1923), who demonstrated a continuous increase in cell size without any corresponding increase in cell numbers. This has been amply confirmed

by subsequent workers, (Wade, 1952; Corbett, 1957; Collins, 1960). The increased enzyme activity and stimulated adaptive capacity demonstrated during the lag and early logarithmic phases were too extensive to be explained merely in terms of increased cell volume (Wooldridge, _et.al_., 1936; Hegarty, 1939).

At the same time, considerable increases in the nucleotide content of growing cells of _B. cereus_ have been reported to accompany the increasing cell volume (Malmgren and Heden, 1947). The investigations of Levy, _et.al._, (1949), Mitchell and Moyle (1951) and Countryman and Volkin (1959) into the RNA to DNA ratios in growing cells showed a sharp increase in cellular RNA as compared with the DNA content during the lag phase. The RNA content quickly dropped once cell division began, but the DNA content of the cells remained remarkably constant throughout the life cycle. These workers suggested that the RNA was of importance in the synthesis of protein so that the RNA level reached a peak just prior to the commencement of cell division. This hypothesis has been further substantiated by the studies of Harrington (1958) and Billen (1959) into the nucleic acid and protein content of cells incubated in the presence of chloramphenicol. The RNA to protein ratio then steadily decreased as the logarithmic phase progressed (Mitchell and Moyle, 1951). Despite the extensive changes which accompanied the initiation of cellular division, the actual chemical factors which initiate cell division remain unknown although they have been extensively discussed on theoretical grounds by Hinshelwood (1946).

The physical environment has been shown to affect bacterial growth extensively. The influence of the temperature of incubation

on the growth rate has been studied many times since the classical investigations of Barber (1908). Dorn and Rahn (1939) and Jordan and Jacobs (1947) distinguished between the optimum temperature for cell division and the temperature which gave rise to a maximum population when they showed that the latter was usually about 10°C. lower. Monod (1942) claimed, however, that temperature variation had little effect on the total cell yield, unless the incubation temperature was over 40°C., when impairment of the synthetic abilities of the cells resulted in a reduced total population due to the lowered efficiency in energy utilisation. Hegarty and Weeks (1940) pointed out another temperature effect when they showed an increased susceptibility to cold shock for logarithmic phase cells compared with those in other growth phases. Similar findings for sensitivity to heat shock and to disinfectants have been reported by Sherman and Albus (1923). The reasons for this heightened sensitivity to the lethal effect of various environmental factors are still unknown.

One other environmental factor of prime importance to aerobic bacteria is the degree of aeration of the medium. Rahn and Richardson (1942) and Dagley, _et.al._, (1953) clearly demonstrated the extremely high oxygen demand of actively growing bacterial cultures. They further demonstrated that the degree of aeration affected the length of the logarithmic phase, the mean generation time and the final yield of cells. Dagley, _et.al._, (1951) reported that the density of growth of _A. aerogenes_ in liquid media depended only on the oxygen supply, provided that the food supply was not also a limiting factor. On the other hand, Sherman and Naylor

(1942) and Anderson and Meanwell (1936) were unable to demonstrate any such relationship for the Gram positive cocci, but the exact significance of this disparity was uncertain.

A number of physico-chemical changes were known to occur as the bacterial cells passed through the lag and logarithmic phases. Moyer (1936) showed that logarithmically growing cells have a greater electrophoretic mobility than could be demonstrated during the lag phase. At the same time, the cells became less susceptible to non-specific acid agglutination (Sherman and Albus, 1923). These changes merely reflected a number of unspecified variations in the surface charge on the cell walls. The fact that many cells became progressively less smooth as they aged, suggested that a change or loss in one or more of the surface components of the cell wall was responsible for these changes. However, very little information on these important points is at present available (Topley and Wilson, 1955).

The logarithmic phase carried with it the concept of continuous rapid growth in which all the cells present were actively dividing. In the presence of an adequate food supply, the viability of such a culture should be nearly 100%. The finding by Wilson (1922) that only 75% of logarithmic phase cultures of S. cholerae-suis were viable has been debated ever since the report was first made. While subsequent workers have agreed that viability is seldom 100%, few have found such a large disparity between the viable and the total counts (Jordan and Jacobs, 1944; Thimann, 1955). The assumption that each viable cell would develop into a visible colony was a dangerous one, as was shown by the findings of Curran and Evans (1937), Nelson (1943) and Straka and Stokes (1957) when they demonstrated that some organisms

were unable to grow as well on some agar media as on others. Similarly, Williams and Reed (1942) obtained better recoveries at certain incubation temperatures than at others. Investigation of irradiated cells indicated that better recoveries were obtained where the cells were grown on a partially deficient medium rather than on a complete one. The complex medium resulted in so called "unbalanced growth", which led to the death of many of the cells. The slower growth on the deficient medium permitted the cells to repair the damage before active division occurred (Alper and Gillies,1958; Billen,1959). However, the claim by Heinmets, et.al., (1954) that reactivation of apparently dead cells was possible by the use of a number of organic acids has not been confirmed (Chambers, et.al., 1957). Thus the criteria at present employed to define a viable cell are not considered altogether satisfactory, but for want of better, have been used in this study.

(b) The Stationary and Decline Phases.

These phases have received relatively little study compared with that given to the earlier ones. Many of the existing studies have been limited to those factors resulting in the transition from the logarithmic to the stationary phase. The relative importance of the various possible factors was often controversial and probably indicated the interaction of several factors at once. Hinshelwood (1946) and Monod (1942) have both shown that food or growth factor depletion was important only in basal media. Previously, Penfold (1914) and Graham-Smith (1921) had shown that nutrient broth could support two or three successive populations without depletion of the nutrients. Thus, starvation was not usually an important hazard for the bacterial

cell. Of greater importance for aerobic bacteria was an oxygen shortage arising from the growing demands of the culture which ultimately surpassed the rate at which the oxygen could diffuse into the medium from the atmosphere. Forced aeration of a stationary phase culture was shown by White and Munns (1951) to result in further logarithmic growth resulting in an increased final yield of cells. The influence of toxic end products resulting from the utilisation of the food stuff in the medium may be important, and the acid pH shift following sugar fermentation can frequently be sufficient to limit or prevent further cell multiplication (Topley and Wilson,1955). Where aeration and the supply of nutrients were adequate for maximum cell growth, the accumulation of metabolic end products appeared to be one of the most important lethal factors (Hinshelwood,1946). However, in most cultures toxic products were merely another contributing factor in the transition from the logarithmic to the stationary phase.

The stationary phase could last from several hours to several days, depending on the medium, the organism and the environmental conditions (Porter, 1946; Topley and Wilson, 1955). After this time the death rate increased still further and the culture passed into the decline phase. The rapidity of the decline varied considerably with the organism and the environmental conditions. Steinhaus and Birkeland (1939) followed the viability of ageing broth cultures of Ps. aeruginosa over a two year period and reported that the viability never fell completely to zero. The decline was not logarithmic and the frequent but limited increases in viable numbers were due to the utilization of the dead cells by the survivors, the process being referred to as

"cannabalism" (see also Topley and Wilson, 1955). *Ps. aeruginosa* was also shown to grow luxuriantly on agar containing dead *Ps. aeruginosa* or *S. lutea* cells as the sole source of carbon. *Staph. aureus* cells were not utilised in this way, presumably because *Ps. aeruginosa* did not produce an enzyme capable of effectively destroying the Staphylococcal cell walls. Similar specificity of action by the lytic enzymes produced by various bacteria has been noted by other workers (Nomura and Hosoda, 1956a).

The slow decline noted above confirmed a similar finding by Heap and Cadness (1929). On the other hand, Wilson (1922) reported rapid death of *S. suipestifer* during this phase. The anomaly of these varying results was probably due to differences in the aeration of the culture and also to the variability in the oxygen demands of the different strains. Rahn and Richardson (1941), and Allen, *et.al.*, (1952) noted that rapid death followed the storage of strict aerobes under anaerobic conditions. However, care must be used in interpreting some of these results, since aqueous and saline suspensions were frequently used and the bacteriocidal action of both these diluents has been frequently noticed in the past (Gunter, 1954; Straka and Stokes, 1957; Demain, 1958). Winslow and Falk (1923) showed that the lethal effect of distilled water was enhanced when the reaction was strongly acid or alkaline and it would appear that the pH of the suspending fluid may also have an important effect on the viability of cell suspensions. Death during the decline phase was seldom complete as the curve usually flattened out after a time. However, the resistance to the lethal factors operating during the decline phase was probably a statistical illusion, since attempts to obtain resistant

strains by re-peated training have been uniformly unsuccessful (Jordan, et.al., 1947).

This summary concludes the brief survey of literature concerning the growth and decline of bacteria in liquid cultures. As can be seen, the main emphasis in the past has been on the growth of bacteria rather than the decline and death of the culture. Very few growth studies have been carried out with bacteria grown under strictly anaerobic conditions, but it was thought that the factors that had proved important with the aerobes would prove equally important with the anaerobes. The ability of Ps. aeruginosa to grow under anaerobic conditions when nitrate or nitrite was present in the medium was well known. The cells rapidly destroyed the nitrate, subsequent incubation occurring in complete anaerobiosis. This organism therefore offered an excellent opportunity to study the intra-and extra-cellular changes which followed the cessation of cell division in a nutrient medium. The cells went into a rapid decline under controlled and fairly reproducible conditions thus permitting a study of the cells at various stages of decay. The literature relevant to some of these latter changes will now be considered.

2. Lethal Factors Resulting in the Death of Cells in Liquid Cultures.

The examination of various lethal factors has obvious and practical applications in Microbiology, and the applied aspects of the subject have been very thoroughly investigated. The effects of the physical environment on cell death have therefore been very extensively reviewed. The importance of heat, purely as a sterilising agent is outside the scope of the present work, and has, in any case, been fully reviewed by McCullock (1945), Reddish (1954)

and others. However, the temperature of incubation of the culture can be an important factor, since it markedly influences the rate of growth of the cell. Once the temperature for optimal growth has been passed, quite small increases in temperature lead to extensive death of the cells in the culture (Barber, 1908). Conversely, cold shock and prolonged storage at near freezing temperatures can lead to rapid and extensive cell death (Hegarty and Weeks, 1940; Kock, 1959). The reason for this last effect was uncertain, and was further complicated by the fact that greatest sensitivity was noted in young cells (Meynell, 1958). The relative insensitivity of old cells to heat and cold shock, ultra violet irradiation and various chemical agents has been demonstrated many times, but its significance is still largely unknown (Sherman and Albus, 1922; Hinshelwood, 1946).

The pH of the medium has an important influence on the viability of bacterial cultures. How much of this effect was directly due to the pH of the medium was uncertain, since Vogler, et.al., (1942) have demonstrated that the intracellular pH may be largely independent of that of the medium. Adjustment of the pH beyond the tolerated range was assumed to damage the osmotic barrier of the cell, resulting in death. Such irreparable damage was assumed to occur largely because of leakage of intact macromolecules such as RNA from the cells into the medium (Dubos, 1937; Mitchell, 1951; Stephenson and Moyle, 1949). Gale and Epps (1942) have described an ingenious intracellular pH regulating mechanism involving the adaptive formation of amino acid deaminases and decarboxylases. The disappearance of free amino acids from resting or ageing cells has been advanced

as a possible cause of lethal intracellular pH shifts (Mitchell, 1951). However, within physiological limits, extensive and rapid pH changes have very little effect per se on cell viability (Sherman and Cameron, 1934).

Another important environmental factor was the E_h of the medium. Hewitt (1948) emphasised that although the E_h of the medium may be determined easily, this value did not necessarily bear any relation to the redox potential which occurred within the cell itself. Extrapolation of the E_h of the medium to the intracellular environment was extremely dangerous, especially when interpreting possible lethal effects. Nevertheless, evidence has been brought forward that cell death (often followed by lysis) occurred when strict aerobes were incubated under strictly anaerobic conditions (Rahn and Richardson, 1941; Kaufmann and Bauer, 1958). Apparently the aerobes were unable to metabolise the energy yielding materials under such conditions and death was thought to be due to energy starvation rather than to the low E_h of the medium. It would appear therefore, that the presence or absence of oxygen affected not only the growth rate (Clifton, 1957) but also the viability of certain organisms.

The subject of chemical disinfection of bacterial cultures has been dealt with exhaustively by many authors (McCullock, 1945), but the action of toxic chemicals on bacteria has little applicability to the present problem, except from the point of view of the dynamics of the disinfection process (Hinshelwood, 1946; Topley and Wilson, 1955). Furthermore, most of these studies have been carried out on non-growing or resting cell suspensions and have little direct

bearing on the present problem.

In general, we know little about the actual cellular changes which result in the inability of a cell to replicate itself. Efforts to solve this problem may well have to await fundamental research into the means by which cell growth itself is initiated. This present discussion will therefore turn to another process which frequently follows cell death, namely the dissolution of the cell wall, with the release of part or all of the cytoplasmic contents into the medium.

3. The Lysis of Bacterial Cells.

The destruction of a part or even the whole of a bacterial culture frequently occurs as the cells age. Such changes can be conveniently detected either by total counts or by changes in the opacity of the culture (Salton, 1957). Under normal circumstances almost 100% of the cells present at the end of the logarithmic phase were viable and this frequently continued into a prolonged stationary phase. Thus Jordan and Jacobs (1944) demonstrated that the total population showed only a slow increase in numbers with age. However, the total population may decrease rapidly during the decline phase, the process being referred to as autolysis. The actual rate and extent of lysis will vary with the organism and the environmental conditions. The process of cell lysis is thought to follow various changes in the cell wall composition, induced by a number of unrelated mechanisms operating within or without the cell. Studies of the action of external agents such as fat solvents or detergents suggests that lipid extraction removed an inhibitor of the proteolytic enzymes which were always present within the cell

(Pethica, 1958). The removal of cell wall lipid possibly allowed access of the autolytic enzymes to the cytoplasmic membrane, where they attacked the previously protected structural proteins of the cell wall (Salton, 1957; Murohashi and Seki, 1958). The normal intracellular organisation of the growing cell was thought to specially localise the proteolytic enzymes so that they were unable to attack the structural components of the cell.

The living cell requires a constant supply of energy for maintenance of the complex macromolecular structure of the growing cell wall, and any process which interferes with the production, transportation or utilisation of energy by the cell may indirectly induce autolysis (Welsch, 1958). The absence of autolytic changes within a bacterial culture may indicate the absence of a suitable autolytic enzyme in the cell, or, more probably, the presence of an inhibitor which inactivates the existing enzymes.

Lysis may also occur "from without" due to external factors such as enzymes which were released into the medium by previously lysed cells. Such cell-free lysins may attack free cell walls or protoplasts or both (Mitchell and Moyle, 1957; Work, 1959). They may attack several different species, or they may be specific for the cell wall of only one strain (Strange, 1959). The characteristic properties and methods for the isolation and purification of a number of these enzymes have been the subject of several recent reviews (Salton, 1957; Strange, 1959). Several of these enzymes have been partially purified, and have proved capable of completely destroying the cell walls of susceptible cells (Nomura and Hosoda, 1956 a and b; Strange and Dark, 1957; Richmond, 1959). Some of

the enzymes have been shown to be mixtures of proteinases (Farrer, 1956; Richmond, 1958) while McCarty (1952) described a polysaccharase which was produced by Streptomyces albus cultures. Jeener (1959) reported that high concentrations of RNase were lytic for some cells while lower concentrations were lethal. This report is interesting since earlier workers have frequently used this enzyme to remove RNA from bacterial cells without noting any serious decrease in cell viability or in the total counts. These conflicting reports may be due to differences in the enzyme concentrations used, and so may be more apparent than real.

Studies of the autolytic systems of Gram negative bacteria have not been extensive since the cells seemed to be more resistant to this type of change than many of the Gram positive organisms. A number of autolytic enzymes isolated from various sporing Bacilli have been shown to be effective for some of the Gram negative bacteria (Mitchell and Moyle, 1957), but the relative lytic activity was usually much less than with the Gram positive cells. Similar findings were reported for lysozyme (Salton, 1958). Stacey (1949) reported that a change in Gram reaction always preceded the autolysis of Gram positive cells. Criticism has been levelled at this finding on the grounds that loss of RNA from ageing cells could explain the change in Gram reaction, and that this process did not necessarily have any direct connection with the cell lysis (Welsch, 1958).

Many Gram negative bacilli were resistant to the action of lysozyme (Salton, 1958) unless the cell wall was first rendered sensitive by heat treatment, acetone extraction (Warren, et.al., 1955), or by the presence of Versene (Repaske, 1958).

The action of the Versene suggested the removal of some metallic inhibitor by the chaelating agent. However, the nature of this metal and the mechanism of its inhibition was unknown. Salton (1958) showed that lysozyme treatment was unable to induce the lysis of several Gram negative bacilli, but analysis of the medium revealed the presence of considerable quantities of muramic acid, glucosamine, and the amino acids DAP, alanine and glutamic acid. Thus it was shown that hydrolysis of the cell wall substance was possible without resultant lysis of the cell. These and similar studies have given a number of clues as to the chemical structure of the cell walls of Gram negative bacteria.

So far we have considered only degradative changes in the cell wall structure which may, or may not, lead to lysis of the dead cell. However, evidence exists that lysis of living cells may occur when cell wall synthesis was partially inhibited under conditions which did not greatly affect the simultaneous synthesis of the protoplasm (Toennies and Shockman, 1953). Exclusion of the cell wall amino DAP from a DAP-requiring mutant of _E. coli_ prevented cell wall synthesis without affecting the growth of the cytoplasm. The naked cells were osmotically fragile and lysed immediately (Meadow, _et.al_., 1957). Similarly, the ability of penicillin to interfere with cell wall synthesis has been used by Lederberg (1956) to produce "protoplasts" of _E. coli_. Examination of the cell wall fragments from penicillin treated cells suggested that penicillin blocked cell wall synthesis in some way (Park, 1952). Thus, certain metabolic blocks may lead to cell lysis when cell wall synthesis was preferentially affected. Lysis of this type resulting from synthetic disabilities within the

cell was distinguishable from the autolysis of old, and usually dead cells in many ways and often the only common feature of the two systems was the final dissolution of the cell.

Cell lysis may also be due to phage action. The lytic patterns produced in surface growth on agar have been described in great detail for a wide variety of micro-organisms. The morphology of the plaques, the host ranges and other criteria useful for the identification of the phage have been the subject of an enormous amount of study. However the mechanism of the actual lytic process has received less attention. Recently, the phenomenon of defective lysogenicity in E. coli K12 provided a useful tool for the study of cell lysis at the enzymic level. Ultra-violet irradiation of the defective cells set up lysis without the release of mature phage particles (Jacob and Fuerst, 1958). The culture filtrate from the irradiated culture yielded an enzyme which could lyse E. coli K12 cells treated with Versene. The so-called "endolysin" closely resembled lysozyme in its action (Repaske, 1956). In the presence of sucrose, the endolysin removed the cell wall and produced "protoplasts". Jacob and Fuerst (1958) reported that E. coli K12 produced the endolysin at the same rate as phage, but the the exact relationship between the two was uncertain. Jacob and Wollman (1956) ascribed the development of the defective strain of E. coli K12 to a prophage mutation which interfered with the normal process of phage production. It is interesting to note that Jacob (1950) reported isolating a defective lysogenic strain of Ps. aeruginosa after treatment of the cells with a temperate phage. Such mutants were unable to produce infectious particles.

A second type of lytic change has been described for *Ps. aeruginosa* cultures. This was a peculiar type of plaque characterised by an "irridescent" or metallic appearance when examined by reflected light. Early workers considered the plaques to be due to the action of a phage, largely because of the circumscribed appearance of the lytic areas. However, Lische (1924) clearly distinguished between the metallic plaques and those produced by a bacteriophage. In common with all subsequent workers, Lische was unable to propagate any phage which could produce the metallic plaques, and he claimed that the two types of lytic phenomena were due to entirely different mechanisms. Hadley (1924) developed a plaque-free strain of *Ps. aeruginosa* from a plaque producer, but all attempts to re-introduce the plaque producing ability into the mutant failed. Subsequent workers have confirmed these findings many times without greatly adding to our knowledge of the process. (Conge, 1948; Schultz, *et.al*., 1948; Don and van den Ende, 1950). Irrespective of the exact mechanism of this unusual lytic process, autolysis of *Ps. aeruginosa* liquid cultures has been explained in terms of the "irridescent" plaque type of lysis (French, 1947), particularly since it may frequently be seen in the surface pellicle of broth cultures of this organism (Warner, 1950b).

This concludes the brief survey of the literature to the growth, death and final lysis of cells. The entire emphasis of the existing data was on the growth of the cells, and most of the studies were concluded once the cells reached the stationary phase. Only a small number of workers have followed the cells into the decline phase, or considered the possible reasons for the increased death rate.

Even the investigations into enzyme activities and adaptation by ageing cells usually continued only into the early stationary phase. Similarly, morphological investigations of this phase have usually been concerned only with the changes associated with the formation of spores. Thus a considerable bias exists in most growth studies, and this has resulted in very slow progress in the understanding of the problems of cell death in nutrient cultures.

The present study stemmed from this relative lack of information on the behaviour of various bacteria in the decline phase, especially when grown under anaerobic conditions. Although strain differences were thought to occur in the sensitivity of various bacteria to prolonged incubation in nutrient culture media, very little experimental proof exists on which to base this impression. Variations certainly do occur in the sensitivity of various cells to autolytic breakdown, but the reasons for this are not always very clear. Several possible lytic mechanisms may be responsible for the dissolution of the ageing cells. However in general the process of lysis by various organisms in ageing cultures has not been as extensively studied with the Gram negative organisms as it has, for instance, with the sporing Bacilli. A survey of the relevant literature shows that not a great deal is known about the characteristics of the so-called autolytic enzymes, nor has the exact site of action of these enzymes on the cell walls of Gram negative organisms been defined.

The Ps. aeruginosa cultures are subject to a very rapid decline under certain conditions, and thus constitute an excellent opportunity for a study of the last phase of the growth cycle.

The sensitivity of the dead cells to autolytic breakdown makes this organism a suitable experimental tool for the study of the chemical changes associated with lysis. By comparison of the Pseudomonad with an organism not susceptible to this rapid anaerobic death and extensive lysis it was hoped that some of the biochemical and micro-chemical changes in cell composition associated with these processes might be detected. Such a study might thus make some contribution to our understanding of the cellular changes associated with ageing, death and lysis. We will turn therefore to a consideration of the literature relevant to the present investigation.

CHAPTER II

SPECIFIC INTRODUCTION

Ps. aeruginosa is a strictly aerobic organism frequently encountered in the environment. It has also proved to be a troublesome wound contaminant. In recent years the resistance of this organism to the common disinfectants and chemotherapeutic agents has led to an increasing amount of attention being paid to it by medical bacteriologists. The organism has been repeatedly shown to be very versatile biochemically, and when this ability is considered along with its rapid growth rate and simple nutritional requirements, one would expect Ps. aeruginosa to be frequently used as a tool for biochemical studies. However, except in the more practical aspects of the identification of the organism or of the therapy of its infections, the quantity of fundamental biochemical study done with this organism compares unfavourably with that done on the coliforms and Salmonellas.

Ps. aeruginosa frequently occurs in soil and sewage where the ability to reduce nitrate to gaseous nitrogen anaerobically led to its early description as a denitrifying bacterium (Waksman, 1931), a potentiality which has been frequently overlooked during later studies. Kluyver and his associates established that, under anaerobic conditions, nitrate was utilised almost exclusively as a hydrogen acceptor. The nitrate could be replaced by nitrite or nitrous oxide, but the organism did not appear to utilise any other substances as alternative hydrogen acceptors (Kluyver and Verhoeven, 1954).

A culture of Ps. aeruginosa grown under strictly anaerobic

conditions in a medium in which the nitrate supply was limiting, was unable to continue growing once the nitrate supply was exhausted. Such a culture afforded an opportunity for a study of the changes which occur within non-proliferating cells held in an otherwise favourable culture medium. Investigations of the viability of such cultures were carried out before chemical investigations of the composition of the ageing cells were attemped. The behaviour of the Ps. aeruginosa cultures contrasted sharply with several other Gram negative organisms, and S. bethesda was finally chosen as a control organism for the present study. The two organisms were therefore examined in an effort to determine some of the reasons for the observed cell death and lysis in the ageing cultures.

Before considering the changes which can be detected in the ageing cells by various micro-chemical techniques we must consider the normal composition of the living cell. Few detailed studies have been made of the chemical composition of Ps. aeruginosa cells, and therefore frequent reference will be made to the composition of other Gram negative organisms. We will consider the various cellular materials which are known to occur in most of the Gram negative bacilli.

In general, the total nitrogen content of the Gram negative bacteria varies from 8% to 15% of the dry weight. A large proportion of this nitrogen has been shown to be protein-nitrogen (Topley and Wilson, 1955). Nicolle and Alilaire (1909) reported that in Ps. aeruginosa the total nitrogen constituted 9.8% of the dry weight of the cell but no estimate of the protein-N was

given. Linton, et.al., (1934) reported total nitrogen figures varying from 12% to 15% for V. cholerae, while Boivin and Mesrobeanu (1934) recorded a figure of 13.7% nitrogen for a strain of Ps. aeruginosa. The latter workers claimed that 80% of the total nitrogen was accounted for as protein and that very few free amino acids were present. Hydrolysis of the bacterial proteins showed that the amino acids were mainly those found in higher cells, although Stokes and Gunness (1946) showed quantitative differences in the proportions of the various acids present. Polson (1948) demonstrated the presence of 17 amino acids in acid hydrolysates of E. coli.

Depending on the age of the cell, the nucleic acid content of many Gram negative organisms may constitute as much as 25% of the dry weight of the cell (Belozersky, 1957; Vendrely and Lipardy, 1946). Sevag, et.al., (1940) reported that as much as 80% of some Gram negative organisms may consist of nucleoprotein but relatively little work has been done with the protein moiety. However, claims have been made that histones and protamines may be combined with the bacterial nucleic acid (Mirsky, 1943). Taylor (1946) determined that about 66% of the total phosphorous of the cell was associated with RNA, 19% with the DNA and only 12% with the phosopholipid fraction. The RNA content of A. aerogenes was shown to vary extensively during the growth cycle whereas the DNA content remained remarkably constant (Caldwell and Hinshelwood, 1950). Mitchell and Moyle (1951) have shown that considerable amounts of free nucleotide can be extracted from young Staph. aureus cells. The amount of RNA present was approximately proportional to the

rate of growth of the cells, and Caldwell, et.al., (1950) concluded that RNA was somehow associated with protein synthesis. However, the reasons for the rapid decrease in RNA during the logarithmic growth phase remain largely speculative. The base proportions of a number of bacterial nucleic acids have also been examined (Chargaff, 1955; Stuy, 1958; Scarletti and Naylor, 1959), and it has been claimed that species differences are distinctive enough to enable them to be used as a basis for bacterial classification (Vendrely, 1958). Much of this work has been carried out with Gram positive bacteria or with E. coli, and there is still a great deal of scope for detailed studies with a number of other Gram negative species, including the Pseudomonads.

Up to 30% of the dry weight of the cell may consist of carbohydrate and the total carbon content may be as high as 50% (Knaysi, 1951). The exact amount of carbohydrate present will often depend on the amount of mucopolysaccharide slime on the outside of the cell. Warren and Gray (1955) and Bonde, et.al., (1957) have both reported the presence of a hyaluronic acid capsule around Ps. aeruginosa cells. Much of the polysaccharide material in the cell wall itself may be combined with protein and lipid fractions. Many of these complexes thus constitute the smooth "O" antigens of the Gram negative organisms (Salton, 1960; Kabat and Meyer, 1948).

The free lipid content of most bacteria is low and seldom exceeds 8% of the dry weight (Knaysi, 1951). Stephenson and Whetham (1923) showed that the free lipid content of starved Myco. phlei cells decreased rapidly, indicating that the lipid

acted as a reserve food supply. Much of the lipid in the cell wall is combined with polysaccharide, being released only after acid hydrolysis (Salton and Horne, 1951b). However, Williams, et.al., (1939) reported that a high proportion of the total lipid of several strains of enteric bacteria consisted of phospholipid.

Boivin and Mesrobeanu (1937) extracted a TCA soluble glycolipid complex from Ps. aeruginosa cells. They referred to this extract as an endotoxin and demonstrated that the free polysaccharide acted as a hapten. The lipopolysaccharide, but not the polysaccharide, reacted with an antiserum prepared with whole cells. Recently, Weidel, et.al., (1954) extracted lipoprotein and lipopolysaccharide complexes from a number of Gram negative bacilli and showed them to be closely associated with the smooth "O" antigens of the cell wall. French (1947) carried out an extensive serological survey of human strains of Ps. aeruginosa. He also extracted a lipoidal complex from a number of strains and observed that the extracts reacted specifically with antisera prepared against the whole cells.

French (1947) concluded that the organisms were antigenically heterogeneous and that no simple serological classification was possible. However, more recently, Habs (1957) demonstrated the presence of a common heat stable "O" antigen in a member of human strains of Ps. aeruginosa and proposed a serological classification of twelve groups distinguished on the basis of other minor "O" antigens. Similar results were reported by Sandvik (1960) using absorbed antisera. The last three workers were mainly concerned with the development of a workable typing system for Ps. aeruginosa strains rather than with a fundamental study of the antigenic groupings in the cell wall.

A great deal more work remains to be done on the complex problems of the antigenic relationships between the various _Ps. aeruginosa_ groups since the existing studies do little more than add to the general confusion.

Studies of the antigenic make-up of _Ps. aeruginosa_ have not been extended on a micro-chemical basis similar to that done for a number of other Gram negative bacilli. Such investigations must take into consideration the structure and composition of the cell wall rather than the intact whole cell, and this has been made possible in recent years by the development of techniques permitting the isolation of pure cell walls. Most of these investigations of cell wall composition have been made with Gram positive cells (Holdsworth, 1952; Salton and Horne, 1951b; Salton, 1952; Work, 1957; Yoshida, _et.al._, 1957). The earliest techniques involved the use of heat as a lytic agent (Salton and Horne, 1951a) but as this method was open to criticism (Welsch, 1958), mechanical shaking (Salton and Horne, 1951b) or ultra-sonics (Salton, 1953a) were substituted.

Recently, attention has been turned to some of the Gram negative organisms but so far only a few genera have been extensively studied. The main differences in composition, compared with that of the Gram positive cells was a higher lipid content and a more varied amino acid composition (Salton, 1960). Brown (1958) briefly investigated the composition of stationary phase cell walls of a strain of Pseudomonas and detected the presence of glucosamine, muramic acid, DAP, alanine and glutamic acid. The composition of the Pseudomonas cell walls was therefore similar to that already reported for _E. coli_ (Salton, 1958). Most workers have commented on the complete absence

of nucleic acid from the purified cell wall (Salton and Horne, 1951b; Salton, 1953b; Shockman et.al., 1958), but traces of RNA have been recovered from some cell wall preparations (Barkulis and Jones, 1957).

A number of physico-chemical changes occur within the cell during the process of ageing. The extensive alterations to the size of the cell and its intensity of staining have been commented upon many times(Malmgren and Heden, 1947) but in addition, extensive changes have been noted in the enzymic constitution and adaptive ability of the growing cell (Hegarty, 1939; Gale, 1940; Pinsky and Stokes, 1952). The cells also showed a varying sensitivity to osmotic, heat and cold shock (Christian and Ingram, 1959, Kook, 1959) but these variations have not as yet been placed on any firm chemical basis. Most of these studies have involved only lag or logarithmic phase cells, and much less is known about the changes which occur during the stationary and decline phases. The extensive morphological changes associated with bacterial senescence have an obvious practical importance and they have therefore been described in extensive detail (Knaysi, 1951), but the underlying chemical changes, and their significance, have only recently become apparent. Thus the swollen distorted cells which are so frequently found in senescent cultures are due to the partial breakdown of the rigid cell wall, possibly due to lytic processes within the cell itself.

Recently, a number of investigations have been made into the chemical composition of the walls of growing cells. Shockman, et.al.,(1958) reported that the cell wall fraction of ageing Str. faecalis increased considerably in total nitrogen, rhamnose and hexosamine when estimated per unit weight of cells. The overall

composition of the ageing cell walls did not vary significantly when estimated per unit weight of cell wall however. The above increases were interpreted as a continuation of the synthesis of cell wall material without any corresponding cytoplasmic growth. The exhaustion of the cell wall amino acid, lysine from the medium prevented further cell wall synthesis without greatly affecting cytoplasmic growth and lysis rapidly ensued. These workers suggested that any process which interfered with cell wall synthesis would result in conditions favouring autolysis. Thus, autolysis was induced in growing Str. faecalis cultures exposed to penicillin. However, Truco and Pardee (1958) were unable to demonstrate inhibition of cell wall synthesis in penicillin-treated cells, nor could they demonstrate any change in the composition of the cell walls. Toenies, et.al., (1959) reported variations in the amino acid and carbohydrate composition of ageing cells, but, except for these isolated reports, very little quantitative data exists on this interesting problem. Thus although a considerable amount of detailed information on the chemical composition of many Gram positive and negative bacilli is now available the composition of Ps. aeruginosa cells has only been sporadically and incompletely investigated in the past. More thorough studies of this organism are therefore more than justified. The results of these studies should be correlated with the growth cycle as far as possible, so that any variations due to ageing, might be made more apparent.

The recent investigations into some of the changes in cell wall composition have given a good deal of information about the actual mechanism of cell lysis. Most of the present information has

accumulated from studies using lytic enzymes, but some phage lysis experiments are also relevant here. Salton and Milhaud (1959) treated E. coli cells with phage and then examined the supernatent fluid for possible lysis products. They isolated a peptide-amino sugar complex consisting of alanine, DAP, glutamic and aspartic acids, together with small amounts of glucosamine, muramic acid and ribitol phosphate. Absorption of the phage onto the cell wall resulted in a partial hydrolysis of the cell wall material, some of which was released into the medium. This resulted in an inability of the cell wall to function as an osmotic barrier, and lysis ultimately occurred. Weidel and Primosigh (1957) isolated an enzyme from E. coli T2 phage which hydrolysed the lipopolysaccharide in the cell wall releasing a complex of DAP, glutamic acid, alanine, glucosamine, muramic acid, glycine and lysine into the medium. The residue left in the cell wall contained lipid and polysaccharide units but no amino acids or amino sugars. As a result of the hydrolysis, the rigidity of the cell wall was reduced so that a progressive bulging occurred until lysis resulted. The bulging of the cell wall, particularly at the ends of the rods, has been visually confirmed with the electron microscope (Salton and Horne, 1951a). Slade and Slamp (1960) treated Str. pyogenes cell walls with an enzyme preparation from a Streptomyces albus culture and showed that considerable quantities of cell wall components were released into the medium. This occurred some time before cell lysis could be detected. Salton (1958) treated the cell walls of E. coli with lysozyme and showed that all the DAP, glucosamine and muramic acid were removed from the wall, after which a complex

of alanine, glutamic acid, DAP and glucosamine, together with a trace of muramic acid could be isolated from the culture medium.

The available information on the action of lytic enzymes on cell walls has become quite extensive although most of the studies refer to the Gram positive sporing Bacilli, and so are not particularly relevant to the present discussion. This work has been extensively reviewed by Strange (1959) and in view of the considerable differences between the cell wall composition of Gram positive and negative organisms, no further reference will be made to these studies. The interpretation of the available results has not been made any easier by the fact that very few of the bacterial enzymes have been purified or adequately characterised. The literature makes it clear that the enzymes vary widely in composition and specificity, and often their only common feature seems to be their ability to cause cell lysis.

Lately, several reports have been made that rapid death followed by extensive lysis occurred when a strict aerobe was incubated under strictly anaerobic conditions (Nomura and Hosoda, 1956a). These workers partially purified a cell-free autolysin and showed that it removed part of the cell wall substance.Kaufman and Bauer (1958) isolated a similar enzyme from a sporing Bacillus which they showed to be active against a number of other Gram positive organisms. Significantly, inhibitors which poisoned the cytochrome oxidase produced a similar effect to anaerobic incubation in these cells. Thus Kaufman and Bauer postulated that the autolysin present in the aerated cell was maintained in an inactive form so long as active respiration continued. Any agent which inhibited this respiration

resulted in the release of active enzyme. On the other hand, Richmond (1958, 1959a) reported that strong aeration of B. subtilis cultures inactivated the bacterial lysozyme, suggesting that the enzyme itself was oxygen sensitive. Although claims have been made that some enzyme preparations have a separate bacteriocidal action as well as a lytic effect, generally speaking there has been very little attempt to differentiate between the two effects. The main exception appears to be with lysozyme which has been shown to attack the cell wall of living bacteria, while the ensuing lysis brought about the death of the cell (Weibull, 1953).

Cells unable to grow or multiply over an extended period of time either because of a nutritional deficiency in the medium or some inhibitory environmental condition, ultimately die (Hinshelwood, 1946; Mitchell, 1957; Gunsalas, 1951). Thus, the anaerobic death of a strict aerobe was not surprising. However, the rapidity of the cell death and the ensuing cell lysis was unexpected and seemed to be dependent on more dynamic factors than a simple cessation of cell growth.

One possible explanation may be that, in the absence of a suitable hydrogen acceptor, the cell cannot produce sufficient energy to maintain certain key enzymes concerned with the repair of the cell wall. Alternatively, certain enzyme inhibitors may be destroyed under anaerobic conditions, allowing the internal enzymes of the cell to attack the cell wall lipoproteins and mucopolysaccharides, resulting in lysis from within (Welsch, 1958). The released enzymes may then initiate the lysis of the adjacent cells. At present we are unable to express these reactions in anything but the most

general terms, although a clearer picture of the enzyme substrates is now emerging from the studies of cell wall chemistry. However a great deal more fruitful research remains to be done.

The autolytic enzymes hydrolyse part of the cell wall releasing the cellular contents into the medium, but some of these products may be difficult to distinguish from the complex slimes or capsular secretions which normally diffuse away from the cell wall. These extracellular secretions are usually mucopolysaccharide in nature and can be detected in cultures of a wide variety of micro-organisms (Wilkinson, 1953). Many of the cell secretions were highly viscous and resulted in a mucoid culture medium in which the cells clumped together as a ropey deposit. The more structurally defined mucoid capsules may be produced by young cells of many different species and the presence of these structures may bear no relationship to the process of ageing or lysis by the cells (Salton, 1960).

Several claims have been made for an extracellular nucleic acid slime layer. Smithies, et.al., (1955) demonstrated the presence of a highly polymerised RNA on the cell walls of M. halodenitrificans grown in media containing less than 0.7 M. sodium chloride. However when P^{32} was used to label the intra - cellular nucleic acid it was shown that death and lysis of the cells always preceded the release of the RNA into the medium. Catlin (1956) and Catlin and Cunningham (1958) detected significant amounts of DNA in the slime layer of a strain of Ps. fluorescens. The DNA did not diffuse into the medium but remained attached to the cells. No suggestions were made as to its means of secretion at the cell wall or of its possible function. Many of the claims

for extra-cellular nucleic acids do not distinguish between possible secretion at the intact cell wall by growing cells and the release of cellular nucleic acids following cell lysis. However, Carrol, *et.al.*, (1952) demonstrated the release of P^{32} labelled compounds from non-proliferating *A. aerogenes* cells at a rate proportional to the death of the cells. Lysis of dead or dying bacteria seemed to be the most likely explanation of the release of high molecular weight polymers into the medium. However, the viable and total counts necessary to demonstrate this point were all too often missing.

Several claims of leakage of portion of the cytoplasmic contents of apparently intact cells into the medium have been made recently. Kock (1959) reported rapid death of chilled *E. coli* cells followed by the release of five per cent of the cell contents into the medium every hour. No leakage was detected in the unchilled control. The results with unchilled cells confirmed the earlier findings of Hershey (1954) and Labaw, *et.al.*, (1950) who were unable to detect any significant release of P^{32} labelled nucleic acid from logarithmic or stationary phase cells. Similar findings were reported for *Ps. aeruginosa* cultures by Halleck and Szafran (1957) and Newton (1953). The close correlation between the death of the cells and slime formation reported by Kock (1959) strongly suggested that cold shock followed by slow lysis of the dead cells was a necessary preliminary to the release of cellular DNA into the medium.

The lysis of the cell may not result in the release of all of the cellular contents into the medium. Trucco and Pardee (1958) and Slade and Slamp (1960) demonstrated swelling of penicillin treated

E. coli cells followed by leakage of about 50% of the cellular contents into the medium. De Lamater, et.al., (1959) reported extensive leakage of RNA breakdown products into the medium when intact B. megatherium cells were stored in buffer. The cellular DNA was conserved, as was to be expected from its obvious importance to the cell (Horiuchi, et.al., 1959, Herbst and Doctor, 1959). Thompson, et.al., (1958) noted the same effect in mammalian cells, and Higuchi and Vemura (1959) in yeasts. Leakage of partial breakdown products of RNA may therefore occur during the life of certain cells kept under appropriate conditions. Nevertheless, the presence of un-degraded nucleic acid in the medium should be taken as an indication that death and lysis of at least a portion of the population has occurred. The accumulation of extensive amounts of nucleic acid in the culture medium may simply be a consequence of the absence or inactivation of the appropriate nucleases (Catlin, 1956; Catlin and Cunningham, 1958). Thus careful consideration should always be given to the experimental details before data is accepted as proof of the excretion of intra-cellular materials through an intact cell wall. This concludes the present survey of the literature concerning the chemical composition of Ps. aeruginosa cells and cell walls together with those variations in composition known to accompany cell growth and ageing.

Conclusions to Chapters I and II

The problems associated with the growth and multiplication of bacterial cells have received a good deal of detailed investigation in the past. The majority of these studies have

been limited to the lag and logarithmic phases. Even the more recent comparisons of cell structure, composition, enzymic constitution and adaptive capabilities have usually only continued into the early stationary phase. Thus, although text books on bacterial physiology state that liquid cultures ultimately pass into a decline, few definite physiological reasons for the progressive death of the culture have been advanced. The existing studies do little more than demonstrate the wide variations which occur in the behaviour of different bacteria during the decline phase.

The variability of the behaviour of different bacteria during ageing may also be illustrated by the sensitivity of some, but not all, to autolytic changes. At present we know much less about the autolytic enzymes of the Gram negative bacteria and of _Ps. aeruginosa_ in particular than we do about the analogous enzymes of the sporing Bacilli. This apparent omission is surprising in view of the probable pathogenic importance of the autolytic breakdown of certain of the Gram negative bacilli. Thus, the release of various pathogenic cell wall components (the so-called endotoxins) has been very well documented, but the properties of the hydrolytic factors have not been so well studied.

This survey also shows a lack of detailed investigation into the chemical composition of _Ps. aeruginosa_ cells and cell walls. This is in sharp contrast to the voluminous literature which deals with the Gram positive and certain of the Gram negative bacteria. Much of the work with the Gram negative organisms was done with coliform bacilli or with one of the Salmonellae, and there is still

need to make further detailed studies with other more neglected genera.

General aims of the present study.

The present investigation began as a study of the process of ageing by non-sporing Gram negative bacteria. This led to a comparison of the growth and death of aerobic and anaerobic cultures of *Ps. aeruginosa* and *S. bethesda*. The rapid and extensive death and lysis of the *Ps. aeruginosa* cells, particularly in anaerobic culture was in sharp contrast with the behaviour of *S. bethesda* under similar conditions. This led to a search for the possible causal factors. These early studies thus comprised a basis for the subsequent chemical investigations. It was hoped in this way to relate at least some of the well defined and reproducible cultural changes observed in the *Ps. aeruginosa* cultures with corresponding changes in the chemical composition of the cells.

At the time of commencing these studies only superficial investigations had been made of the chemical composition of the whole cells or cell walls of *Ps. aeruginosa*. Furthermore, few investigations of the cellular chemistry of the Gram negative bacilli had been extended into the decline phase. Thus the present study is intended as a contribution to the existing knowledge of the chemistry of *Ps. aeruginosa* cells and to the steadily accumulating body of information on the processes of death and lysis in the Gram negative bacteria.

CHAPTER III

MATERIALS AND METHODS

1. Media

a. Digest broth and agar, blood base agar (BBA) and tryptone soya agar (TSA) were prepared from dehydrated Oxoid media (Oxo Ltd., London) made up to the recommended strength in copper-free distilled water.

b. The casein hydrolysate medium (CHT) contained the following nutrients: Oxoid acid casein hydrolysate, 5.0g.; sodium nitrate, 5.0g.; ammonium sulphate, 1.0g.; hydrated magnesium sulphate, 0.1g.; trace elements, 1.0 ml. (Meiklejohn, 1950) and 0.067M. Tris (hydroxy methyl) amino methane buffer (pH 7.0), one litre. Inorganic phosphate was removed from the casein hydrolysate as insoluble magnesium ammonium phosphate before it was added to the medium.

c. The synthetic glucose medium contained the following nutrients: glucose, 10.0g.; sodium nitrate, 5.0g.; ammonium sulphate, 1.0g.; potassium hydrogen phosphate, 3.0g.; disodium hydrogen phosphate, 7.0g.; hydrated magnesium sulphate, 0.1g.; trace elements, 1.0 ml.; distilled water, one litre.

Unless otherwise stated analytical grade reagents were used throughout. The media were sterilised by autoclaving at 115° C. for 15 minutes.

2. Organisms.

a. Ps. aeruginosa N.C.T.C. 6750 was obtained from London and a single cell isolate (Johnstone, 1943) was obtained and immediately dried (Annear, 1958). A stab culture was prepared in Oxoid digest agar and was incubated at 37° C. for 24 hours. The stock was kept

at room temperature and was subcultured on to an agar slope as required. The slope culture was then used as the inoculum for one experiment and discarded. A fresh dried culture was opened after six months.

b. Ps. aeruginosa strains Th5, Th8, Th12 and Th16 were isolated from Adelaide garden soils. Two strains (Hayes and S1) were isolated as laboratory contaminants. Single cell isolates were dried as above after checking the biochemical and cultural characteristics of the strains.

c. Ps. aeruginosa strains Foggo, Jenkins, Wall, Tremwith, Cole and Newman (isolated from wound infections in the Royal Adelaide Hospital) were purified by repeated plating and dried as before.

d. S. bethesda strain Ma II was obtained from the N.H.M.R.C. Salmonella Typing Laboratory, Adelaide. The culture was checked for purity and then dried. Cultivation was similar to "a". The Ballerup-Bethesda subgroup resembled the Salmonellas in many respects so that the organisms were originally placed in the Genus Salmonella (Topley and Wilson, 1955). However, Kauffmann (1954) considered that the differences between the Genus Salmonella and the Bethesda subgroup were sufficient to warrant their separation. Such a distinction had little significance so far as the present work was concerned and so largely for the sake of simplicity, the organism will be referred to as S. bethesda throughout this thesis.

e. M. lysodeikticus N.C.T.C. 2665 was obtained from London. The culture was checked for purity before it was grown on a digest agar stab at 28°C. for three days and stored at room temperature.

3. Cultural Conditions

a. Aerated medium. Liquid media were dispensed in 300 ml. quantities in two litre Erlenmeyer flasks and autoclaved. When cool, a suspension of the organism (prepared by removing a little growth from the dry surface of an 18 hour slope culture into nine ml. of sterile tap water) was pipetted aseptically into the flask. An initial population of approximately 10^4 cells was normally present. The flask was incubated at 37°C. on a constant speed shaking machine (Kantorowitz, 1951). Under these conditions, the cultures were aerated optimally throughout the growth cycle (Collins, 1956).

b. Anaerobic medium. Liquid medium was dispensed into a one litre screw capped pyrex serum bottle and autoclaved. When cool, the medium was inoculated with a tap water suspension of the organism. The cap was screwed down firmly and the bottle inverted to test for possible air leaks. The rubber wad was exposed through two small holes cut in the metal cap. The cap and the exposed rubber was sterilised with 70% alcohol and a sterile gauge 20 hypodermic needle was pushed through the wad. The air was removed with a vacuum pump before cylinder nitrogen was run back into the bottle. A cotton wool plug was used to remove any bacteria from the nitrogen stream. The process was then repeated and cultures of S. bethesda were given a positive gas pressure of 10 p.s.i. to facilitate the later anaerobic collection of samples. The Ps. aeruginosa cultures produced copious amounts of gaseous nitrogen following the reduction of the nitrate and this obviated the need to introduce an initial positive gas pressure into the culture.

With the bottle held in an inverted position samples were

collected periodically by inserting a sterile hypodermic needle through the sterilised wad. The positive gas pressure ensured that air did not leak back into the bottle during the prolonged incubation. A fresh sterile needle was used for each sample and in practice only an occasional culture had to be discarded because of contamination.

4. Counting Methods.

a. Viable counts. Counts were always completed within 15 minutes of the collection of the sample. The sample was diluted in sterile normal saline and the viable cells were estimated by the drop plate method of Miles and Misra (1938). The number of colonies per drop were counted after 18 hour's incubation at 37°C. The S.D. for twelve replicates was usually less than 10%.

b. Total counts. Dense suspensions were diluted 1 in 10 in sterile saline and the cells were embedded in a film of agar (Williams, 1952). The dried agar film was examined under dark ground illumination, using a Leitz Ortholux microscope. A ruled grid was placed in the eyepiece and at least ten fields, containing a minimum of 100 organisms, were counted. The S.D. for the counts was usually 20%.

c. Turbidimetric estimations. The opacity of bacterial suspensions was read with a Spekker photo-electric absorptiometer using an Ilford red filter No. 608 and a 0.5 cm. quartz cell. The probable number of bacteria per ml. was read from a previously prepared standard curve for each organism.

d. Dry weight estimations. A known volume of a distilled water suspension of cells was pipetted onto a weighed stainless steel

planchette which was then heated to 105°C. The dried planchette was cooled and immediately re-weighed. The process was repeated until the preparation reached a constant weight.

5. pH and E_h Estimations.

The pH of each sample was measured with a Jones Model B meter. E_h estimations were made with a platinum electrode using the pH meter as a millivoltmeter (Hewitt, 1950). The meter was standardised at + 0.434 volts (pH 7.0 and 20°C.) against 0.003M potassium ferrocyanide and ferricyanide made up in 0.1M potassium chloride. The anaerobic culture was grown in a sealed 500 ml. Grignard flask under an atmosphere of oxygen-free nitrogen.

6. Viscosity Determinations.

A standard Ostwald viscometer was placed vertically in a 37° ± 0.1°C. waterbath. Ten ml. of culture medium were pipetted into the reservoir, and, after a five minute equilibration period, the time taken for the liquid to pass through the capillary was measured in seconds. An average of three readings was made with an error of 0.5%. The change in viscosity over a 60 minute period was determined after adding one ml. of a series of enzyme solutions to nine ml. of culture medium. The enzymes used were: *V. cholerae* mucinase, one ml. (by courtesy of Dr. E. French of the Walter and Eliza Hall Institute, Melbourne), pancreatic enzyme, 10mg.; *Streptomyces albus* protolytic enzyme preparation, one ml.; testicular hyaluronidase, one mg.; ribonuclease, 5 µg.; and deoxyribonuclease, 5 µg. A control containing one ml. of saline in place of the enzyme preparation was also included.

7. Quantitative Analyses.

a. Nitrate - N was measured colourimetrically by the method of Noll (1945) using a Spekker absorptiometer. A standard nitrate curve was prepared and checked periodically.

b. Nitrite - N was measured colourimetrically by the dimethyl α naphthylamine reagent described by Rider and Mellon (1946). A standard curve was prepared against silver nitrite which was checked periodically.

c. Total nitrogen was estimated by digestion in Micro-kjeldahl flasks (Kabat and Meyer, 1948) followed by steam distillation in a Markham still into saturated boric acid and back titrated with 0.014 N. hydrochloride acid. The method was accurate to within five μg. of nitrogen. The apparatus was periodically checked against standard ammonia solution.

d. Protein was estimated by the Folin-Ciocalteau or the Biuret reagent (Kabat and Meyer, 1948). Standard curves were prepared using purified bovine albumin containing 15.5% total nitrogen. The S.D. for six replicates of the standard was ± two mg.%.

e. Carbohydrate. Glucose was determined by the Somogyi (1945) method. Total carbohydrate estimations were routinely made with the Anthrone reagent (Seifter, et.al.,1950) using a glucose standard. The S.D. for three replicates was 11 mg.%. The total reducing sugar estimations following the hydrolysis of the cell wall with 2N. HCl at 100°C. for two hours were made by the ferricyanide method described by Kabat and Meyer (1948). The error was usually less than five mg.%. A few estimations were made by the Dische and Popper (1926) method, and also using

the Orcinol reagent (Kabat and Meyer, 1948).

f. Phosphorous estimations were made by the Fiske and Subbarow (1925) method described by Kabat and Meyer (1948). The S.D. for four replicates was four mg.%. More reproducible results for very small samples were obtained by the method of Cowgill and Pondee (1957). A standard curve was prepared for each batch of samples tested.

g. Nucleic acids. Cells were washed twice in distilled water and made up to a known concentration in distilled water. The cells were extracted in the cold with three per cent TCA to remove any free nucleotides (Mitchell and Moyle, 1951). The nucleotide content of the extract was estimated from the absorbance at 260 mμ. after removal of the TCA. The cells were washed twice in distilled water before extraction of the intact nucleic acid by one of the following methods.

(1) Schmidt-Thannhauser method. The pH of the suspension was adjusted to 4.0 with glacial acetic acid and an equal volume of absolute alcohol was added. The cells were heated to 100°C. for two minutes to destroy any nucleases present. The cooled cells were washed with five volumes of cold acid alcohol (pH 4.0). Free lipid was removed by two washes with absolute alcohol, followed by one in 50% alcoholic-ether and finally with one in pure ether. The cells were then allowed to air dry at room temperature.

The protein in the cells was dissolved by digestion in 0.3 N. caustic potash at 37°C. for 18 hours (Schmidt and Thannhauser, 1945; Davidson and Smellie, 1952). The pH was adjusted to 8.0

with cold N. perchloric acid (Volkin and Cohn, 1954) after which the potassium chlorate was spun down and washed once. The DNA was precipated with 50% alcohol after the pH had been adjusted to 4.0 with acetic acid. The RNA remained in solution and was concentrated by freeze drying. The DNA was extracted from the precipitate with hot five per cent TCA for 20 minutes and the insoluble protein residue spun off. The TCA was removed by ether extraction and the DNA concentrated by freeze drying. The dry powdered RNA and DNA were taken up in 3.0 ml. of 0.1 N.HCl and stored at - 20°C. The absorbance of the RNA was determined at 260 and 290 mμ. and the DNA at 268 and 290 mμ. with a Shimadzu model QR - 50 spectrophotometer. The nucleic acid content of each solution was estimated by the method used by Cerotti (1955).

(2) Schneider method. The nucleic acids present in the cell were extracted in six ml. of five per cent TCA at 100°C. for 20 minutes (Schneider, 1945, 1957). The cells were spun off and washed with an equal volume of five per cent TCA. The extract and washings were combined and made up to 12 ml. The RNA was estimated colourmetrically by the method used by Militzer (1946). A standard curve was constructed using a standard RNA solution whose absorbance at 260 mμ. was checked immediately before use. The DNA content of the extract was determined by the Webb and Levy (1955) method against a standard solution of thymus DNA. The absorbance of the standard solution at 268 mμ. was checked immediately before use. The nucleic acid content of the culture medium was estimated in a similar manner. The cell-free medium was adjusted to pH 4.0 with acetic acid,

and an equal volume of absolute alcohol was added to precipitate the nucleic acids. The precipitate was spun down at 5000 r.p.m. for 15 minutes, washed twice with acid alcohol (pH 4.0) and after defatting, the RNA and DNA content of the precipitate was determined as before.

h. Determination of the base composition of RNA and DNA from cells or medium.

(1) Hydrolysis. A sample containing approximately one mg. of RNA or DNA was placed in a small test tube and freeze dried. The nucleotides present were hydrolysed in 90% formic acid in a bomb heated to 180°C. for 20 minutes (Wyatt, 1951; Bendich, 1957). The formic acid was removed *in vacuo* over solid caustic potash, and the residue was taken up in 100 µl. of N. HCl.

(2) Paper chromatography. Whatman paper No.1 was washed in 50% methanol in N. HCl for three days; then in water, 0.1% calcium acetate, water and 50% ethanol, each for one day (Ada, 1958; personal communication). Usually 30 µl. of the extract were spotted onto the paper which was run at 37°C. as an ascending chromatogram in isopropanol - HCl (Wyatt, 1951; 1955). After seven hours the papers were dried at room temperature in a stream of air. The spots were identified under ultra-violet light and the chromatogram was printed onto Ilford Reflex Contact Document 50 paper. The areas on the chromatogram corresponding to the spots were carefully cut out and the bases eluted in three ml. of 0.1 N. HCl. Similar sized blank spots were removed as controls. The absorbance of the eluates was determined over the range 220 to 290 mµ. against a 0.1 N. HCl blank (Loring, 1955) using a

one cm. quartz cell in a Shimadzu spectrophotometer. The amount of each base present was estimated from the absorbance (Bendich, 1957) and expressed as molar percentages. The error for the base estimations for six replicates was eight per cent.

i. Gas analysis. The gas evolved from the anaerobic culture was collected over mercury and the volume corrected to S.T.P. The composition of the gas was determined with a Haldane gas analysis apparatus which detected carbon dioxide, hydrogen, methane, nitrous oxide and oxygen. The remaining inert gas was assumed to be nitrogen. The weight of carbon dioxide present in the sample was calculated. Carbon dioxide evolution by a vigourously aerated culture was measured gravimetrically by absorption into a known amount of normal caustic potash. Water vapour was first removed in an alcohol-dry ice trap.

j. Organic acids. Ps. aeruginosa was grown anaerobically in synthetic glucose medium and the cells were spun down. The volatile organic acid content of the medium was estimated by titration of the steam distillate with 0.01 N. sodium hydroxide. The non-volatile acids were absorbed onto acid washed celite, from which they were extracted with freshly distilled ether (Collins, 1960a). The techniques of column and ascending paper chromatography used were those evolved by Ladd and Nossal (1954). The acids were eluted from a silica gel column with a mixture of n-amyl alcohol and tert-amyl alcohol in chloroform saturated with 0.05 N. sulphuric acid (Nossal, 1956; personal communication). The effluent was cut into fractions by an automatic fraction collector operating on a time basis. One minute fractions were

collected for the first 150 minutes, two minute fractions for 100 minutes and five minute fractions for the remainder of the experiment. Each fraction was titrated with 0.001 N.sodium hydroxide, the titres plotted against the corresponding fraction number, and the amount of acid represented by each peak calculated in mg.

8. Lethal Curves for Whole Cultures and Resting Cell Suspensions.

a. Addition of nitrate during growth. Ps. aeruginosa was inoculated into 0.5% nitrate broth and incubated anaerobically at 37°C. Periodically samples were withdrawn for total and viable counts and quantitative nitrate estimations. When the nitrate supply was exhausted, it was returned to its initial level by the addition of sterile 10% sodium nitrate. A calculated volume of concentrated HCl was added to some cultures to correct the alkaline swing in the pH due to the destruction of the nitrate radicle. Excess nitrogen gas was also withdrawn from the bottle at intervals.

b. Killing curves for Ps. aeruginosa and S. bethesda resting suspensions.

The organism was grown in 0.5% nitrate broth for 24 hours. The culture was spun at 3000 r.p.m. for 20 minutes and the cells were washed twice in sterile saline. The cells were suspended in distilled water, normal saline, 0.067 M. phosphate buffer (pH 7.0), glucose basal medium or digest broth at a final concentration of about 10^9 cells per ml. The suspensions were incubated at 37°C. and viable counts were carried out at 15 or 30 minute intervals for two hours. The viable counts were plotted against time.

c. Effect of aeration of the suspension. The washed cells were suspended in 0.067 M. phosphate buffer (pH 7.0) at a final

concentration of about 10^9 cells per ml. The suspension was divided into four aliquots which were treated as follows:-

(1) Shaken in air.

(2) Shaken in air in the presence of 0.5% sodium nitrate.

(3) Shaken under nitrogen.

(4) Shaken under nitrogen in the presence of 0.5% sodium nitrate.

Viable counts were carried out for a 72 hour period and the results were plotted against time.

d. Effect of "staled" medium on viability. The cells from an anaerobic 24 hour 0.5% nitrate broth culture were spun down and washed twice with sterile saline. The cells were divided into two halves, one of which was suspended in fresh broth while the other was made up in cell-free broth from a five day old anaerobic culture. Each suspension was then divided into three aliquots which were treated as follows:-

(1) "Stale" broth shaken in air.

(2) "Stale" broth shaken under nitrogen.

(3) "Stale" broth shaken under nitrogen with 0.5% sodium nitrate.

(4) "Fresh" broth shaken in air.

(5) "Fresh" broth shaken under nitrogen.

(6) "Fresh" broth shaken under nitrogen with 0.5% sodium nitrate. The viable counts made over a 72 hour period were plotted against time.

9. Lysis of Ps. aeruginosa Cultures on Solid Media.

Ps. aeruginosa N.C.T.C. 6750 was inoculated onto the surface of Oxoid digest agar, BBA, TSA and also CNT medium solidified with 2% agar. For five days the growth was examined for the formation of

pigment and for "irridescent" (or metallic) plaques. Similar plates containing nitrate enriched agar were incubated anaerobically in sealed paint tins for seven days, the oxygen in the tins being absorbed with iron wool (Parker, 1955). The aerobic and anaerobic cultures were compared.

a. Attempts to propagate a phage producing the "irridescent" plaques.

Growth from the centre of a plaque was inoculated into digest broth which was incubated at 37°C. for 18 hours. Some of this growth was then inoculated into a sterile five ml. broth together with an equal volume of one of the twelve other strains of *Ps. aeruginosa* available (see section 1). A mixture containing all the strains was also prepared. After six hour's incubation, the cells were killed by heating to 56°C. for 30 minutes and then removed by centrifugation. A loopful of each clear broth sample was carefully spotted onto a separate plate inoculated with one of the twelve strains of *Ps. aeruginosa*. After 18 hour's incubation the growth was examined for lytic areas.

b. Isolation of a non-plaque producing mutant of *Ps. aeruginosa* N.C.T.C. 6750. The organism was first "tagged" with Streptomycin resistance, (see section "9c"), and a single colony was picked off into digest broth. Incubation of the broth culture was continued at 37°C. for three days before the organism was transferred to a fresh culture. After six such transfers the organism failed to produce any green pigment or "irridescent" plaques even after three days incubation on TSA. Attempts to cause the mutant to revert to the wild type by ultra-violet radiation were uniformly unsuccessful. For this test about 10^9 cells were spread over a TSA plate which was then exposed to ultra-

violet irradiation from a Sterilamp (Oliphant Industries, Adelaide) at a distance of 10 cm. for 10 minutes. The plate was incubated in the dark for 24 hours at 37°C. The number of survivors varied between 20 and 200, and up to 20 of these were picked off onto TSA and examined for "irridescent" plaques.

e. Antibiotic resistant mutants were obtained by growing the organism on a gradient TSA plate containing the following quantities of drugs:- penicillin G, 2000 units per ml.; streptomycin, 500 µg. per ml.; chloramphenicol, 500 µg. per ml.; tetracycline, 500 µg. per ml. Inoculation was made across the gradient and the most resistant colonies were selected after 24 to 48 hour's incubation at 37°C. The antibiotic resistance of the culture was determined by tube titration before and after the treatment.

10. Autolysis of Liquid Ps. aeruginosa Cultures.

a. Concentration of the enzyme. Ps. aeruginosa N.C.T.C. 6750 was grown in 0.5% nitrate broth under aerobic or anaerobic conditions for five to eight days. The cells were centrifuged at 5000 r.p.m. for 30 minutes and discarded. The pH of the medium was adjusted to 7.0 with acetic acid and the medium chilled to 0°C. Solid ammonium sulphate was slowly stirred into the medium until 50% saturation was reached. The preparation was allowed to stand at 4°C. for 30 minutes after which the precipitate was spun down in a M.S.E. refrigerated centrifuge at 5000 r.p.m. The addition of more ammonium sulphate precipitated more protein which had very little lytic activity. The 50% saturation precipitate was washed once in 50% aqueous ammonium sulphate and dissolved in cold 0.033 M. phosphate buffer (pH 7.0). The solution was dialysed in the cold against 0.033 M. phosphate

buffer (pH 7.0) until the dialysate no longer contained free ammonia. The dialysate was centifruged at 5000 r.p.m. in the cold for 10 minutes and the deposit discarded. Nucleic acid was removed by treatment with protamine sulphate (Korkes, et.al., 1950). The enzyme was re-precipitated with 50% ammonium sulphate followed by dialysis. After spinning at 5000 r.p.m. for 30 minutes the clear supernatant fluid was diluted with 0.033 M. phosphate buffer (pH 7.0) until the solution contained three to five mg. of protein per ml. The final yield was equivalent to 30 to 50 mg. of protein per litre of original culture. The preparation was stored at - 20°C. until required.

b. Lysis of resting cell suspensions. Ps. aeruginosa N.C.T.C. 6750 was grown aerobically or anaerobically in 0.5% nitrate broth at 37°C. for 16 hours. The cells were spun down and washed twice with saline at room temperature. The cells were re-suspended in sterile 0.067 M. phosphate buffer (pH 7.0) and standardised photometrically to a final concentration of 10^{10} cells per ml. Five ml. of this suspension were pipetted into 6" x ½" test tubes placed in a 45°C. water bath. The enzyme preparation was added so that a final concentration of two mg. protein per ml. was present. A control in which the enzyme preparation was replaced by buffer was always included, along with a tube containing boiled enzyme. The total volume was made up to 10 ml. with buffer. The change in opacity was recorded with a Spekker photo-electric absorptiometer using a red filter No.608. Viable counts were carried out at 30 minute intervals up to 240 minutes. The opacity expressed as Log. (Io/I) was plotted against time while the viable population was expressed as a percentage

of the initial value.

c. Hydrolysis of Ps. aeruginosa (plaque-strain) cell walls by the crude enzyme concentrate. The cell walls(*vide* section 11) were prepared from 24 hour cells grown anaerobically in CNT medium. Two hundred mg. dry weight of cell walls were suspended in five ml. of cold 0.067 M. Tris (hydroxy methyl) amino methane buffer (pH 7.0) in each of three 6" x 5/8" test tubes. The first tube was used as a control and was made up to a final volume of 10 ml. with buffer. The second tube received three ml. of crude enzyme concentrate (three mg. of protein per ml.) while the third tube contained a similar amount of boiled enzyme. In each tube the final volume was made up to 10ml. with buffer, and the three tubes were incubated at 45°C. for 20 hours. The opacity of the three tubes was determined photometrically both before and after incubation. The absence of contaminating bacteria in the cell wall suspensions was confirmed by examining Gram stained smears of the three preparations. The cell walls were centrifuged at 20,000 g. for 30 minutes in a Spinco Model L ultra-centrifuge, after which they were washed once with cold 0.067 M. Tris buffer (pH 7.0), re-suspended in 10 ml. of cold distilled water and then stored at -20°C. until required. The chemical composition of the three preparations was determined by the methods later described in sections 12 a,b,c, and d.

d. Lysis of resting cells in the presence of versene and Tris. The cells were grown and harvested as described in Section "10 b." The suspension was standardised to $2x10^{10}$ cells per ml. The Tris buffer and versene concentrations were those recommended by Repaske (1956) and a final volume of four ml. was employed. The decrease in

opacity was read with a Spekker photo-electric absorptimeter using a red filter No. 608. Controls containing cells or cells plus versene only were included. The tests were read at room temperature over a period of six minutes and the decrease in turbidity [Log. (Io/I)] plotted against time.

e. Lysis of M. lysodeikticus. The organism was grown in digest broth at 28°C. for 72 hours. The cells were washed twice and standardised by opacity to 10^{10} cells per ml. The reaction mixture contained 1 ml. of cells, one ml. of enzyme preparation (three mg. protein per ml.) or lysozyme (50 µg.) and two ml. of 0.067 M. Tris buffer (pH 8.0). The change in opacity [Log. (Io/I)] was plotted against time.

f. "Protoplast" formation. Ps. aeruginosa (N.C.T.C. 6750) was grown aerobically in digest broth at 37°C. for 18 hours. The cells were washed twice with sterile saline and re-suspended in 20% sucrose broth. "Protoplasts" were prepared by the addition of 0.5 M. glycine (Weibull, 1953) or lysozyme (Mahler and Fraser, 1956). Both the glycine and the lysozyme treatments brought about an almost 100% conversion of the cells into swollen, osmotically fragile forms. The "protoplasts" were mixed with an equal volume of Ps. aeruginosa autolysin made up in 10% sucrose broth and the mixture incubated at 37°C. Periodic samples were examined under phase contrast, and the change in turbidity was followed photometrically. A control, to which sucrose broth was added in place of the autolysin was also examined.

g. Activity of the enzyme preparation for known substrates.

(1) Mucinase. An enzyme preparation containing three

mg. protein per ml. was tested against purified ovomucin by the method of Burnet (1948) using an ovomucin and an enzyme preparation control. The end titre was expressed as the highest dilution of the enzyme preparation which was able to completely hydrolyse the ovomucin in 60 minutes at 37°C.

(2) Proteinase. A solution of egg albumin (four mg. per ml.) was made up in 0.067 M. phosphate buffer (pH 7.0). An equal volume of enzyme preparation (one mg. protein per ml.) was added to the first tube and two fold dilutions were then added to the subsequent tubes. A protein and a boiled enzyme control were included. After 60 minute's incubation at 37°C. an equal volume of three per cent sulphosalacylic acid was added, and the precipitated protein was spun off and estimated by the Folin-Ciocalteau method. A trypsin control was always included.

(3) Nucleases. Estimations of RNase and DNase activity of the enzyme concentrate were made by the method of McCarty (1948), using a two mg. per cent solution of RNA or DNA. After 30 and 60 minute's incubation a one ml. sample was added to one ml. of N. HCl and the optical density of the precipitate was read at 425 mμ. with a Shimadzu QR-50 spectrophotometer. Nucleic acid and boiled enzyme controls were included, together with RNase and DNase controls, (the nuclease preparations by courtesy of Mr. G.L. Ada, Walter and Eliza Hall Institute, Melbourne.)

4. Esterases.

(a) Acetyl cholinesterase. Two ml. of enzyme preparation were mixed with an equal volume of saline containing 0.004 acetyl choline and 0.04 magnesium chloride (Nachmansohn

and Wilson, (1955). The mixture was incubated at 37°C. for four hours in the presence of a few drops of phenol red.

(b) Phospholipase.

i. Growing cultures. A medium containing 0.2% ammonium sulphate, inorganic salts and 1.8% washed agar was melted and held at 45°C. Twenty per cent of inactivated human serum (heated at 56°C. for 30 minutes) was added aseptically and the agar poured into a sterile Petrie dish. The plate was dried at 37°C. for 20 minutes and inoculated from 18 hour agar slope cultures of Ps. aeruginosa (plaque and plaque-free strains). The plates were incubated at 37°C. for 48 hours and examined for a white ring of precipitated fatty acid in the agar.

ii. Tube titration of the enzyme preparation. One ml. of enzyme preparation was mixed with 0.5 ml. of inactivated human serum and the volume made up to two ml. with 0.033 M. phosphate buffer (pH 8.0). The test, together with a heated enzyme control were then incubated at 37°C. for 120 minutes. The preparations were neutralised to pH 7.0 and the intact lethicin precipitated with cold three per cent perchloric acid. The lethicin content was determined by the method of Hayaishi and Kornberg (1954).

11. Preparation of Cell Walls of Ps. aeruginosa and S. bethesda.

The cells were suspended in cold distilled water at a concentration of 20 to 30 mg. dry weight per ml. The cells were then exposed to sonic disintegration in a Raytheon sonic disintegrator with an output of 50 W. at nine Kc. After 15 minutes treatment the opacity of the suspension decreased by 70 to 80%. The cells were kept at 0°C. in an ice bath throughout the treatment. The whole

cells were spun down at 3000 r.p.m. for 10 minutes, and the cell wall separated from the intracellular contents by centrifugation at 20,000 g. for 30 minutes in a Spinco Model L ultra-centrifuge. The cell walls were then washed three times with cold 0.067 M. Tris buffer (pH 7.0). The button of cell wall material was carefully emulsified in cold buffer, leaving the small pellet of denser whole cells undisturbed. The washed cell walls stood overnight at 4°C. and were then spun at 3000 r.p.m. for 10 minutes to remove any remaining whole cells. (Munoz, et.al., 1959). The cell walls were concentrated at 20,000 g. for 30 minutes and suspension was freeze dried, weighed and stored in vacuo at room temperature. After shadowing with palladium, electron micrographs of the cell wall preparations were examined with a Philips model E.M. 100 electron microscope at a magnification of 10,000 diameters.

12. Analysis of the Cell Walls.

a. Amino acids. Twenty five mg. dry weight of cell walls were hydrolysed in 6N.HCl at 100°C. for 16 hours (Salton, 1953b). The humin-free residue was dissolved in 200 µl. of distilled water. Two dimentional chromatograms were then run on Whatman No.1 paper. Twenty µl. of the unknown were spotted on the paper and run, first in 80% phenol, and then in a mixture of butanol-water-acetic acid (4:4:1). The papers were dried at room temperature and sprayed with 0.2% ninhydrin. The colour was allowed to develop over 24 hours at room temperature. The spots were cut out carefully and eluted with distilled water, and the amount of amino acid in each was estimated by comparison with the colour obtained with standard spots of known amino acids developed under identical conditions.

The error varied with the degree of separation of the spots but was usually less than 15%.

b. Sugars. Forty mg. dry weight of cell wall were hydrolysed with 2 N. sulphuric acid at 100°C. for two hours (Salton, 1953b). The neutral residue was dried over $P_2 O_5$ before being dissolved in 200 µl. of distilled water. Thirty µl. samples were then spotted onto Whatman No.1 papers. Single dimentional descending chromatograms were run in ethyl acetate-acetic acid-water (3:3:1) for 18 hours. The dried papers were sprayed with silver nitrate-ammonia or aniline phthalate developers. Hexosamines were detected by spraying with acetyl acetone and p-dimethyl amino benzaldehyde (Cramer, 1954). The spots from the phthalate sprayed paper were carefully cut out and eluted in six ml. of glacial acetic acid for 24 hours. The sugar content of the hydrolysates were estimated by the method of Baar (1954).

c. Hexosamine estimations. Twenty to forty mg. of cell wall were hydrolysed in 2 N. sulphuric acid at 100°C. for two hours. The solution was neutralised to pH 4.5 with N. sodium hydroxide. The estimation was made by a modification of the Elson and Morgan (1933) reaction using M. carbonate buffer (pH 10.0) as the solvent for the acetyl acetone (Strange and Kent, 1959). The developed colour was read at 540 mµ. with a Shimadzu model QR-50 spectrophotometer against a freshly prepared glucosamine standard. The error for four replicates was usually less than four per cent.

d. Muramic acid estimation. Muramic acid was separated from other sugars and glucosamine by elution from a Dowex "50" column according to the method of Strange and Kent (1959). Each three ml. fraction was carefully neutralised to pH 4.5 and examined for hexosamine

by the modified Elson and Morgan reaction.

e. The O-acetyl content of the cell walls was determined by the method of Hestrin (1949) using acetyl choline as a standard. The error was usually less than four per cent.

f. Lipopolysaccharide. This was extracted from the cell wall by the phenol/water partition method described by Westphal, et.al., (1952). The lipolysaccharide in the aqueous phase was precipitated overnight with alcohol at 4°C. The precipitated lipopolysaccharide was spun down at 5000 r.p.m. for 20 minutes in a M.S.E. refrigerated centrifuge, dissolved in cold distilled water, and then centrifuged at 78,000 g. for 120 minutes. The pellet was then dissolved in water and the centrifugation repeated. Samples of each fraction obtained during the partition were retained for chemical analysis. The phenol layer and the phenol residue were dialysed in distilled water until the smell of phenol could no longer be detected. Determination of dry weight, as well as of total nitrogen, total protein and total carbohydrate were carried out on the various fractions.

13. Preparation of Antisera.

Each organism was checked for smoothness after one ml. of a dense saline suspension had been injected intraperitioneally into white mice for six consecutive days. When tested with 0.002 M. Trypaflavine, the S. bethesda suspension was completely stable but the Ps. aeruginosa suspensions were only partially so. (Plate 1). Further mouse passages of the Ps. aeruginosa plaque strain were not attempted, as previous efforts to smooth the strain by prolonged animal passage resulted in the selection of a plaque-free strain.

"O" vaccines of the three strains were prepared by steaming dense saline suspensions for 150 minutes, (Kauffmann, 1954). Sterility tests carried out on the vaccines were negative after three days incubation at 37°C. The vaccines were stored at 4°C. until required. The injection programme was carried out as recommended by Kauffmann (1954) for 'O' antisera. The sera were stored at 4°C. after 0.1% merthiolate had been added as a preservative.

14. Preparation of "O" Agglutinating Suspensions.

Ps. aeruginosa (plaque or plaque-free strain) or S. bethesda was inoculated into CNT medium and incubated anaerobically at 37°C. Samples were withdrawn daily for eight days. The cells were spun down, washed twice with saline and made up in 10 ml. of saline. An equal volume of absolute alcohol was added and the suspension was incubated at 37°C. for 18 hours. After standardisation the suspension was stored at 4°C. (Kauffmann, 1954).

The suspensions were diluted to 10^9 cells per ml. in 0.9%, 0.6%, 0.3% and 0.1% saline. Each suspension was incubated at 37°C. for four hours, stored at 4°C. overnight (Mackie and McCartney, 1950) and examined for auto-agglutination. The 0.3% saline was found to stabilise the Ps. aeruginosa suspensions sufficiently to enable agglutination tests to be carried out. The serum was diluted from 1/10 to 1/5120 in 0.3% saline and an equal volume of cells (10^9 per ml.) added. The end titre was determined after four hours at 37°C. and checked again after 18 hours at 4°C.

This completes the survey of the methods used in the experimental sections, and the results of these tests will now be considered.

CHAPTER IV.

METABOLIC CHANGES ASSOCIATED WITH AGEING: GROWTH AND DEATH IN LIQUID CULTURES.

Ps. aeruginosa is a strictly aerobic Gram negative rod-shaped bacillus. As a group, the Pseudomonads are well known for their metabolic versatility and have been used in a variety of biochemical studies. The organisms have the further advantage that they are nutritionally non-exacting and grow well on simple aerated culture media, giving high yields of cells. *Ps. aeruginosa* is also able to reduce nitrate to nitrogen gas by means of adaptive enzymes. However the ability of this organism to produce these adaptive denitrifying enzymes varies as the cells pass through the growth cycle (Collins, 1960b). As part of this study, a number of experiments were carried out to determine the fate of the enzyme "nitrate reductase" as the adapted cells were permitted to age in the absence of the specific substrate. The exact details of these experiments are outside the scope of this present thesis but they resulted in the finding that all denitrifying activity ceased within 24 hours of the removal of the substrate. The ageing suspensions darkened in colour, developed an offensive odour and frequently became highly mucoid or ropey. Viable counts carried out on the suspensions showed a 90 to 100% mortality over this period. On the other hand, the total counts usually only revealed a decrease if incubation was continued for a further 24 hours. The behaviour of the *Ps. aeruginosa* suspensions was in sharp contrast with several other Gram negative bacilli being currently examined. At the time of commencing the present study very little information was available on the behaviour of aerobic bacteria held under

anaerobic conditions. Thus, the small number of detailed studies concerning the decline and death of ageing bacterial cultures was one of the original stimuli for the present study. *Ps. aeruginosa* and *S. bethesda* were selected as the test organisms because they seemed to represent two extremes in their sensitivity to death and subsequent cell lysis as the cultures aged. Consideration will be given to some of the cultural and metabolic changes associated with the ageing of *Ps. aeruginosa* and *S. bethesda* cultures in Chapters IV, V and VI while the studies of the chemical composition of the ageing cells will be detailed in Chapters VII, VIII and IX of this thesis.

1. Growth and Death of *Ps. aeruginosa*.

The effects of variation of the cultural and environmental conditions on the growth and viability of the cells was first determined. The experiments were conducted in complex media in which nutritional factors were unlikely to be growth limiting. Later, these results were compared with growth experiments in chemically defined media.

a. Complex medium (0.5% sodium nitrate broth).

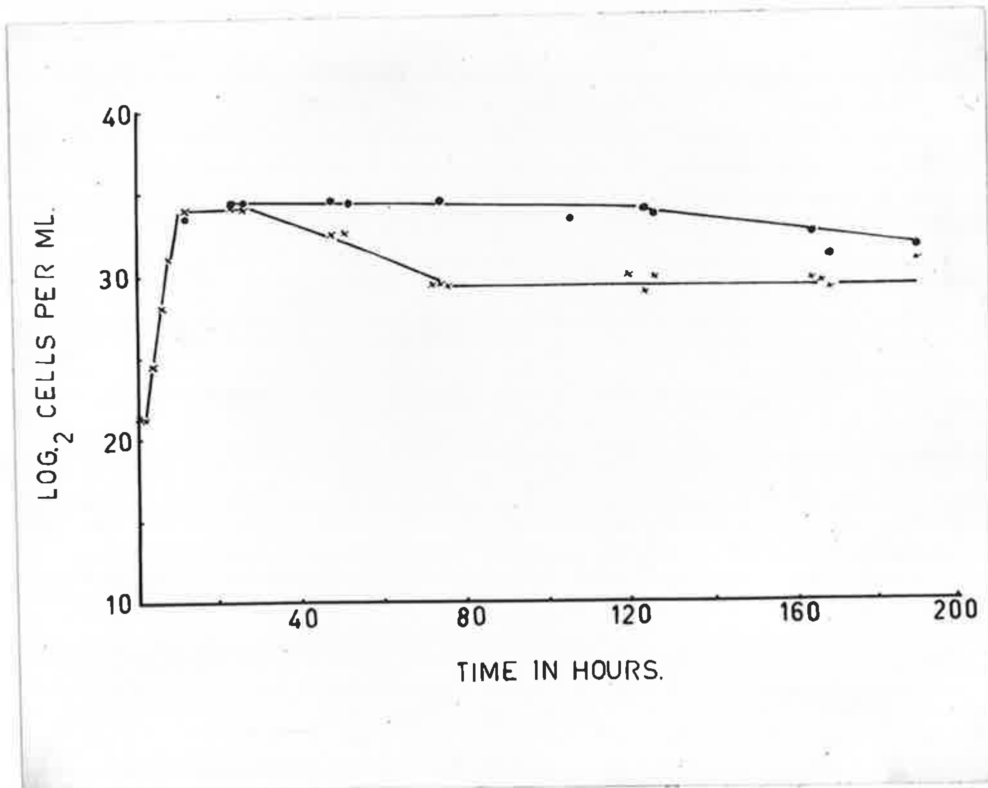
(1) Aerobic culture. *Ps. aeruginosa* grew vigourously in nitrate broth and one of the typical growth curves obtained with this medium was recorded in Fig.1. The two hour lag phase was followed by logarithmic growth (M.G.T. of 30 minutes) which continued until a maximum population of 2×10^{10} cells per ml. was present. The stationary phase lasted for 20 hours, after which a gradual decline in the viable population occurred until only 10% of the maximum could be detected (192 hours). Over the same period, the total population decreased by 25%.

Fig. 1. The total and viable populations in aerobic *Ps. aeruginosa* (plaque strain) cultures grown in the complex medium.

X——X Viable cell counts.
●——● Total cell counts.

Fig. 2. The total and viable populations in anaerobic *Ps. aeruginosa* (plaque strain) cultures grown in the complex medium.

X——X Viable cell counts (nitrate broth).
●——● Total cell counts (nitrate broth).
+——+ Viable cell counts (digest broth).


40
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LOG.2 CELLS PER ML.
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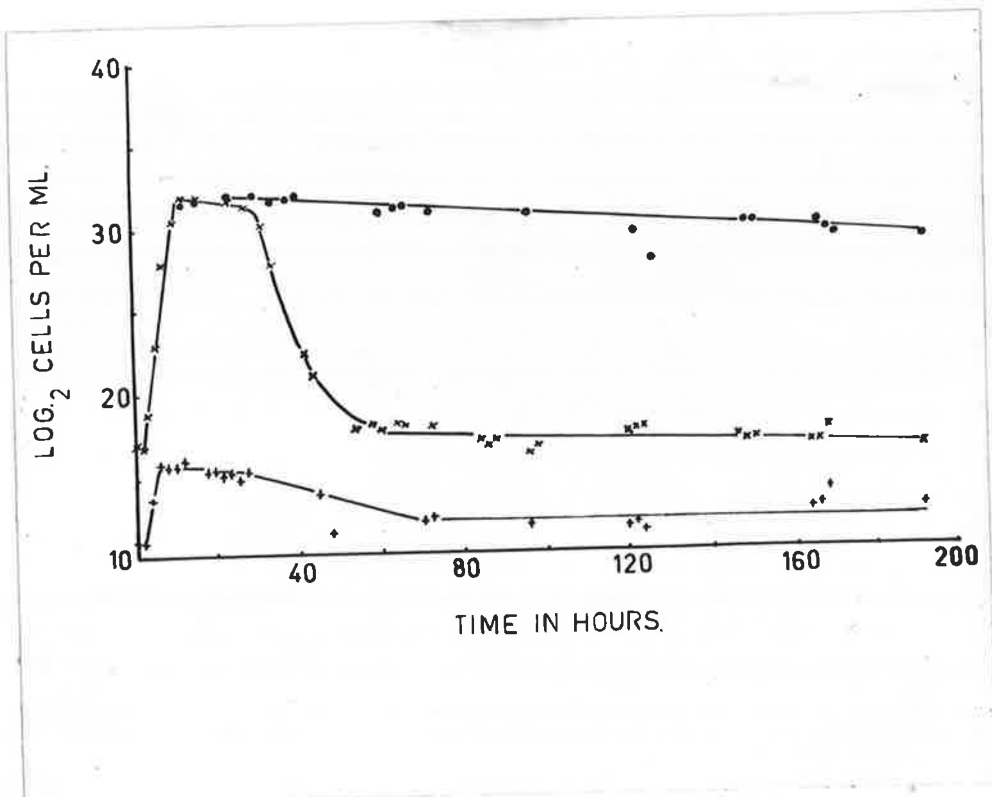
TIME IN HOURS.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LOG.2 CELLS PER ML.
40
80
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TIME IN HOURS.

2. Anaerobic culture. When the air over the culture was replaced with cylinder nitrogen, a similar type of growth curve was recorded until the end of the logarithmic phase (Fig. 2). The two hour lag phase was followed by logarithmic growth (M.G.T. of 31 minutes) which continued until a maximum population of 5.0×10^9 cells per ml. was recorded. The viable population remained stationary for only eight hours after which a rapid, logarithmic decrease was observed, and after 36 hours the viable population was less than 0.1% of the original maximum. The total count declined at a slower rate, but after 192 hours only 20% of the maximum population was still intact. The decrease in total cells appeared to be roughly logarithmic. Neither the viable nor the total count fell to zero, even when incubation was continued for several weeks. All the nitrate was removed from the medium by 14 hours. The pH of the medium rose from 7.4 to 8.4 because of the release of sodium hydroxide as the nitrate radicle was destroyed.

Growth of Ps. aeruginosa in nitrate-free digest broth under aerobic conditions gave an identical curve to that recorded in Fig. 1. On the other hand, less than four cell divisions occurred when Ps. aeruginosa was grown in the absence of nitrate and oxygen (Fig. 2). The length of the lag and the logarithmic growth phases was largely independent of the hydrogen acceptor. However, considerable variations in viability were observed during the late stationary and decline phases. During the late decline phase, the viable population fluctuated between 10^4 and 10^6 cells (Fig. 2). These variations could still be observed after 12 months continuous incubation under anaerobic conditions, and were thought to be a manifestation of the "cannabalism" phenomenon which has been described by earlier workers (Steinhaus and Birkeland,

1939). The small number of resistant cells probably utilised various breakdown products released by the lysis of the dead cells as alternative hydrogen acceptors to oxygen, and were thus able to demonstrate a limited growth. The possibility that the temporary increases in the viable population were due to an accidental introduction of air into the bottle during sampling, could be discounted, as a high positive nitrogen gas pressure was present throughout the experiment.

The rapid death of Ps. aeruginosa cells was duplicated many times, and appeared to be associated with the continued anaerobic incubation of the old nitrate broth cultures. The growth experiments were repeated using a defined medium (referred to as CNT medium) and also a basal glucose medium to determine whether the nutritional complexity of the medium had a significant influence on the rate of growth or death of the Ps. aeruginosa cells. The results of these tests were compared with those recorded above for nitrate broth.

b. Defined media.

(1) CNT medium (vide Chapter III)

a. Aerobic culture. The growth curve in this medium was recorded in Fig. 3 and it will be observed that in all important details it closely resembles the nitrate broth curve.

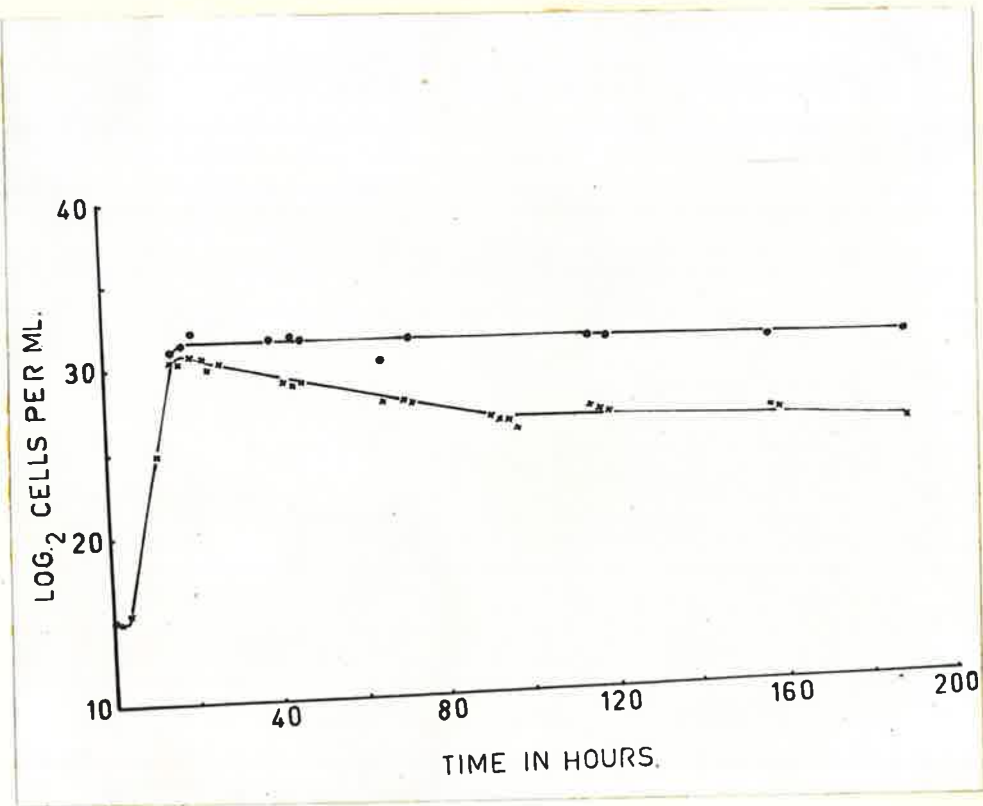
b. Anaerobic culture. The lag and logarithmic phases were very similar to those of the nitrate broth cultures. During the logarithmic phase the cells recorded a M.G.T. of 46 minutes, the slower growth resulting in a longer logarithmic phase (12 to 14 hours) which ended when the viable population reached 3.0×10^9 cells per ml. (Fig.4). The stationary phase lasted until 90 hours, after which the viable population went into a sharp decline, so that by 130 hours

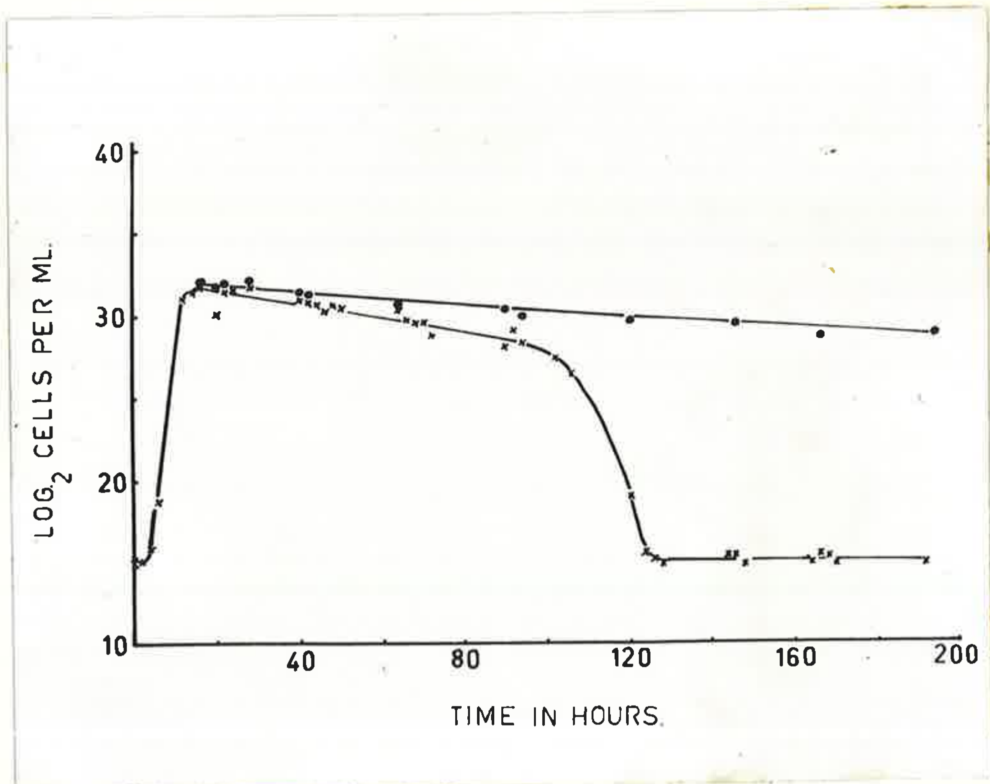
Fig. 3. The total and viable populations in aerobic *Ps. aeruginosa* (plaque strain) cultures grown in the defined medium.

x —— x Viable cell counts.
● —— ● Total cell counts.

Fig. 4. The total and viable populations in anaerobic *Ps. aeruginosa* (plaque strain) cultures grown in the defined medium.

x —— x Viable cell counts.
● —— ● Total cell counts.


40
30
20
10
LOG.2 CELLS PER ML.
40
80
120
160
200
TIME IN HOURS


40
30
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10
LOG.2 CELLS PER ML.
40
80
120
160
200
TIME IN HOURS

less than 0.0004% of the original population was still viable. The curve eventually flattened out and remained constant at 10^4 cells per ml. until the completion of the experiment. During the decline phase the total count decreased slowly until by 192 hours, only 14% of the cells were still intact. The slower growth rate of the Ps. aeruginosa cells in the defined medium, combined with the smaller maximum population compared with the nitrate broth cultures, was paralleled by a slower rate of nitrate utilisation. Exhaustion of the nitrate supply did not occur until after 72 hours incubation, which explained the late viable decline by this culture. The pH of the medium rose to 8.6 within 60 hours, and the strongly alkaline reaction may have been responsible for the slow decline in viable numbers between 40 and 90 hours.

(2) Glucose - nitrate medium.

a. Aerobic cultures rapidly accumulated extensive amounts of organic acid which resulted in autosterilisation of the culture (Fig. 5). Further experiments with aerated cultures in this medium were therefore abandoned.

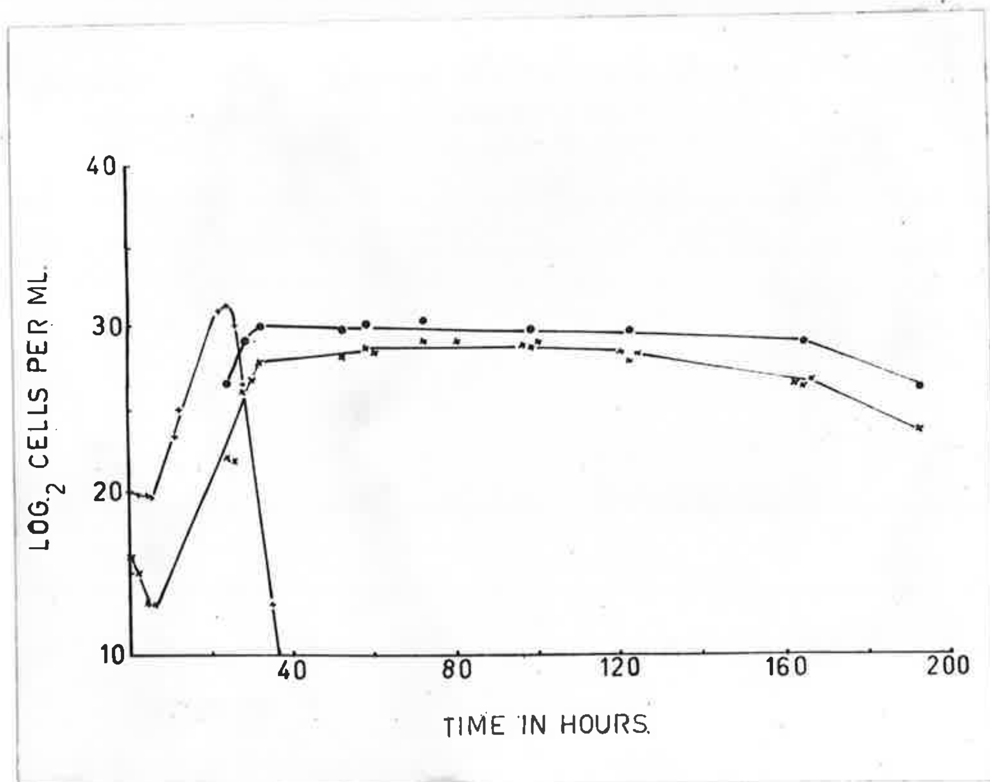
b. Anaerobic culture. The lag phase lasted for five hours, during which time the viable population decreased almost 25 fold (Fig. 5). The addition of 0.1% ammonium sulphate to the medium shortened the lag by two hours, but had no effect on the later phases. The logarithmic phase (M.G.T. of 105 minutes) continued until 2.4×10^9 viable cells per ml. were present. The viable count remained stationary for more than 80 hours before it went into a decline so that less than 10% of the cells were still viable by 192 hours. The nitrate supply was utilised very slowly, and exhaustion did not occur until after 120 hours of incubation. The total counts decreased slowly, so that 42%

Fig. 5. The viable populations in *Ps. aeruginosa* (plaque strain) cultures grown under aerobic and anaerobic conditions in a synthetic glucose-nitrate medium.

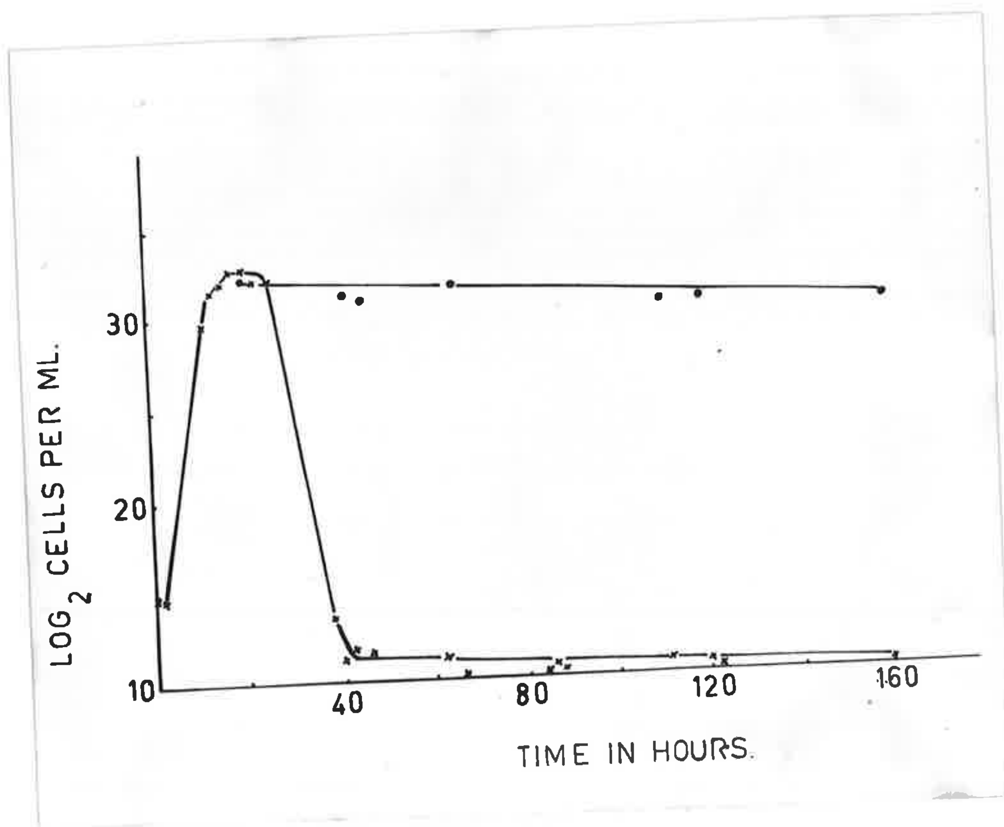
+ —— + Viable cell counts in the aerobic culture.
x —— x Viable cell counts in anaerobic culture.
● —— ● Total cell count in anaerobic culture.

Fig. 6. The total and viable populations in *Ps. aeruginosa* (plaque-free strain) cultures grown anaerobically in the complex medium.

x —— x Viable cell counts.
● —— ● Total cell counts.



40
30
20
10
LOG.2 CELLS PER ML.
40
80
120
160
200
TIME IN HOURS.



30
20
10
LOG2 CELLS PER ML.
40
80
120
160
TIME IN HOURS.

of the maximum population was still present at 192 hours.

Thus alteration of the nutritional complexity of the medium did not greatly affect the growth curves. The duration of the stationary and decline phases, however, varied considerably, depending on the presence or the absence of air or nitrate from the culture. Attempts were made to determine whether other strains of Ps. aeruginosa also exhibited a similar rapid decline in viability in anaerobic culture. Tests on a number of soil strains of Ps. aeruginosa available in this laboratory, confirmed the lethal effect of anaerobic incubation. However, strain TA5 did not show any corresponding decline in total numbers compared with the other strains. The resistance to lysis seemed to be associated with the absence of the "irridescent" lytic phenomenon observed in many strains of Ps. aeruginosa (vide Chapter VI.). Several naturally occurring plaque-free strains of Ps. aeruginosa were available in the laboratory and also a mutant strain of the N.C.T.C. 6750. The growth curves of these organisms were found to be very similar to each other, and the results obtained with the plaque-free mutant of Ps. aeruginosa N.C.T.C. 6750 were typical of the growth of the lysis resistant strains.

2. Growth and Death of Ps. aeruginosa (plaque-free strain).

a. Complex medium. The lag and logarithmic growth phases of this organism grown under aerobic and anaerobic conditions were almost identical to those of the parent strain. The anaerobic decline in viable numbers occurred some 18 hours after the exhaustion of the nitrate supply (Fig. 6), the curve flattening out when the viable population had decreased to about 0.05% of the maximum. However, the total count decreased very slowly, so that by 192 hours more

than 70% of the cells were still intact.

b. <u>Defined medium</u>. The growth curve for the anaerobically grown mutant strain in CNT medium was similar to that of the parent strain until the end of the logarithmic phase (Fig. 7). The rate of nitrate utilisation was slower than with the parent strain, so that the nitrate supply was not exhausted until 140 hours (c.f. 70 to 80 hours for the parent culture) and this was apparently responsible for the very late onset of the decline phase (168 hours). The viable population at 192 hours still represented 10% of the maximum. Similarly the total population remained very high and at least 90% of the cells were still intact after 192 hour's incubation. No definte reason could be given for the slower nitrate utilisation by the mutant in the defined medium, since the rate of growth and the maximum population of both organisms in the anaerobic medium was almost identical.

The growth curves for the two <u>Ps. aeruginosa</u> strains described above made it clear that most of the <u>Ps. aeruginosa</u> cells could not survive for more than 24 hours in the complete absence of air or nitrate. However, as they aged some strains of <u>Ps. aeruginosa</u> were not as extensively autolysed as others, and this phenomenon did not appear to bear much relationship to the death rate.

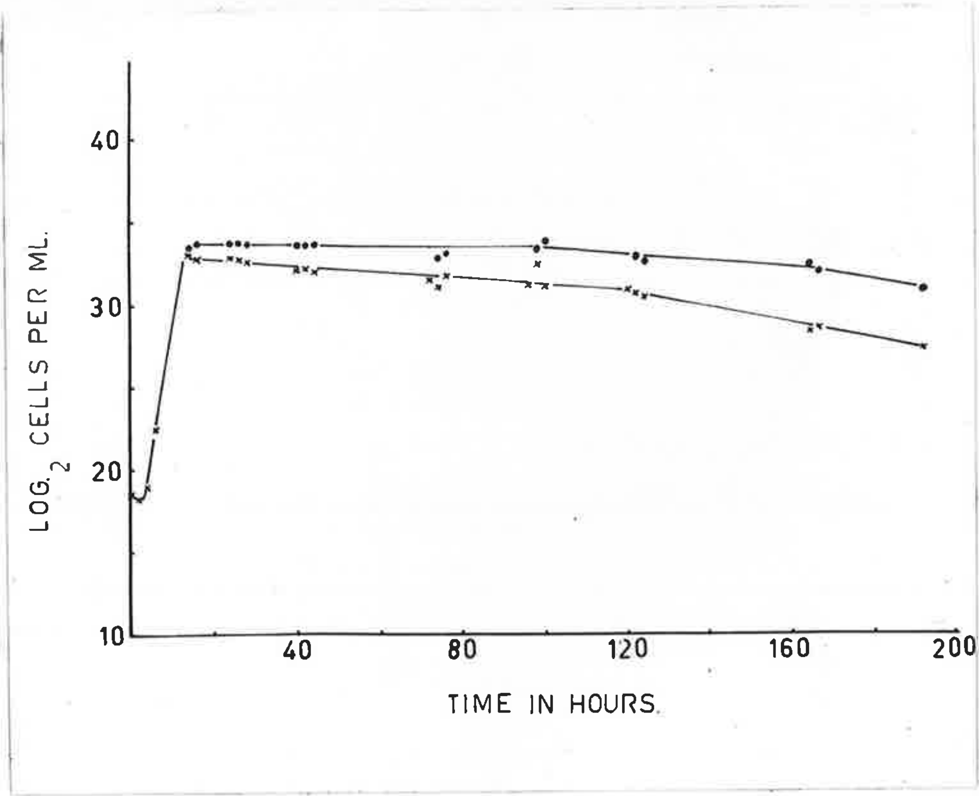
These results were then compared with those of a similar series of experiments conducted with another Gram negative organism, to determine whether the observed death of the anaerobically incubated cells was peculiar to the <u>Ps. aeruginosa</u> strains, or whether this was in fact a more general phenomenon. Preliminary experiments with anaerobically incubated cultures of <u>Alk. faecalis</u>, <u>A. aerogenes</u>, <u>E. coli</u>, <u>S. bethesda</u> and <u>Pr. vulgaris</u> failed to demonstrate the rapid decline

Fig. 7. The total and viable populations in *Ps. aeruginosa* (plaque-free strain) cultures grown anaerobically in the defined medium.

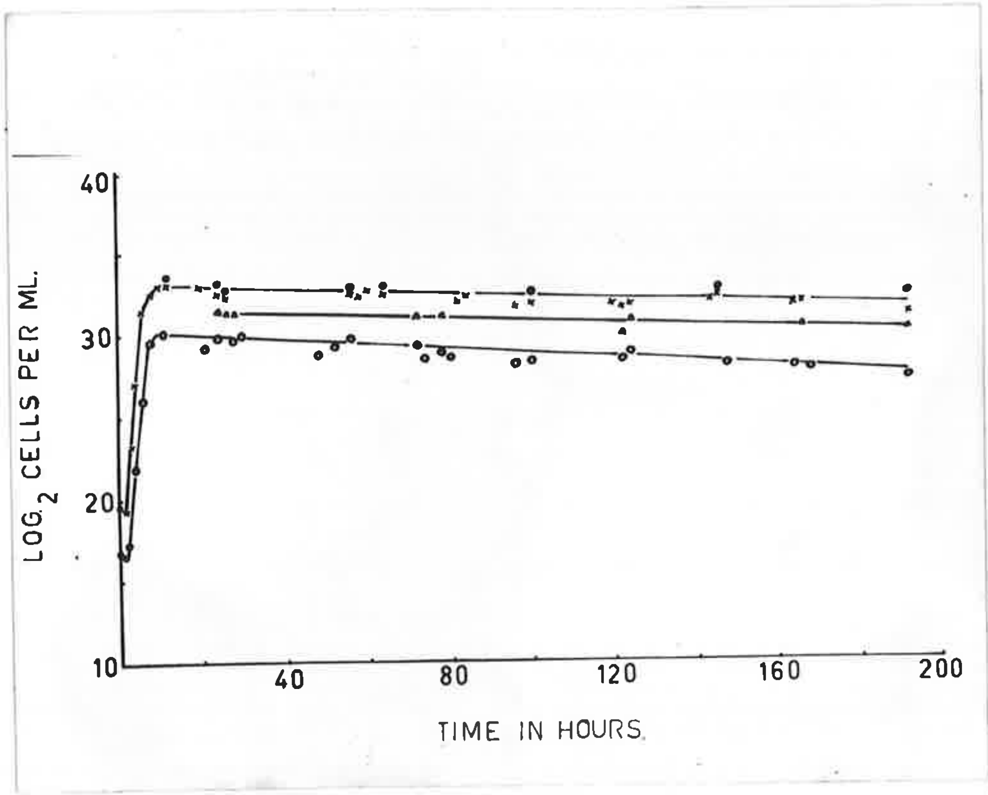
X —— X Viable cell counts.
● —— ● Total cell counts.

Fig. 8. The total and viable populations in *S. bethesda* cultures grown under aerobic and anaerobic conditions in the complex medium.

X —— X Viable cell counts (aerobic medium).
● —— ● Total cell counts (aerobic medium).
⊙ —— ⊙ Viable cell counts (anaerobic medium).
▲ —— ▲ Total cell counts (anaerobic medium).



LOG.2 CELLS PER ML.
40
30
20
10
40
80
120
160
200
TIME IN HOURS.



LOG.2 CELLS PER ML.
40
30
20
10
40
80
120
160
200
TIME IN HOURS.

in viability or in the total counts as the cultures aged. This suggested that Ps. aeruginosa was unusual, both with respect to the early and rapid decline in viability and in the extensive autolysis of the dead cells. S. bethesda strain Ma II. was selected for more detailed experiments, since this organism appeared to contrast most sharply with Ps. aeruginosa. The aerobic and anaerobic growth curves for S. bethesda both in complex and in defined media, will now be considered.

3. Growth and Death of S. bethesda Ma II.

a. Complex medium (0.5% nitrate broth).

(1) Aerobic culture. Logarithmic growth (M.G.T. of 22 minutes) followed a lag of 90 minutes until a maximum population of 12.0×10^9 viable cells per ml. was recorded (Fig. 8). The viable population then decreased gradually, so that by 192 hours 66% of the cells were dead. The total population did not change appreciably throughout the experiment.

(2) Anaerobic culture. The viability of the inoculum decreased by 45% during the 90 minutes lag period. The logarithmic phase (M.G.T. of 27 minutes) then continued until a maximum viable population of 708×10^6 cells per ml. was recorded (Fig. 8). The viable population decreased gradually so that by 192 hours the figure was still 27%. The total population remained at about 2.6×10^9 cells per ml. throughout the experiment, and by 192 hours more than 80% of the cells were still intact.

b. Defined medium incubated under anaerobic conditions.

(1) CNT medium. The growth of S. bethesda cells followed a four hour lag period, during which time the viable population decreased

by 20%. Logarithmic growth (M.G.T. of 45 minutes) continued until a maximum population of 680 x 10^6 cells per ml. was present (Fig. 9). The stationary phase lasted for 60 hours after which the culture went into a very gradual decline, so that by 192 hours, 20% of the cells were still viable. The total count remained high throughout the experiment (1.9 x 10^9 cells per ml. at 20 hours) and more than 60% of the cells were still intact at 192 hours.

c. Glucose-nitrate medium incubated under anaerobic conditions. Inoculation of S. bethesda into this medium was followed by a four hour lag, during which time the viable count decreased by 40%. The logarithmic growth (M.G.T. of 110 minutes) continued until a maximum population of 325 x 10^6 cells per ml. was observed (Fig. 10). The stationary phase continued for 48 hours, when the culture went into a gradual decline until 192 hours, when the viable and total population represented 40% and 75% of their respective maxima.

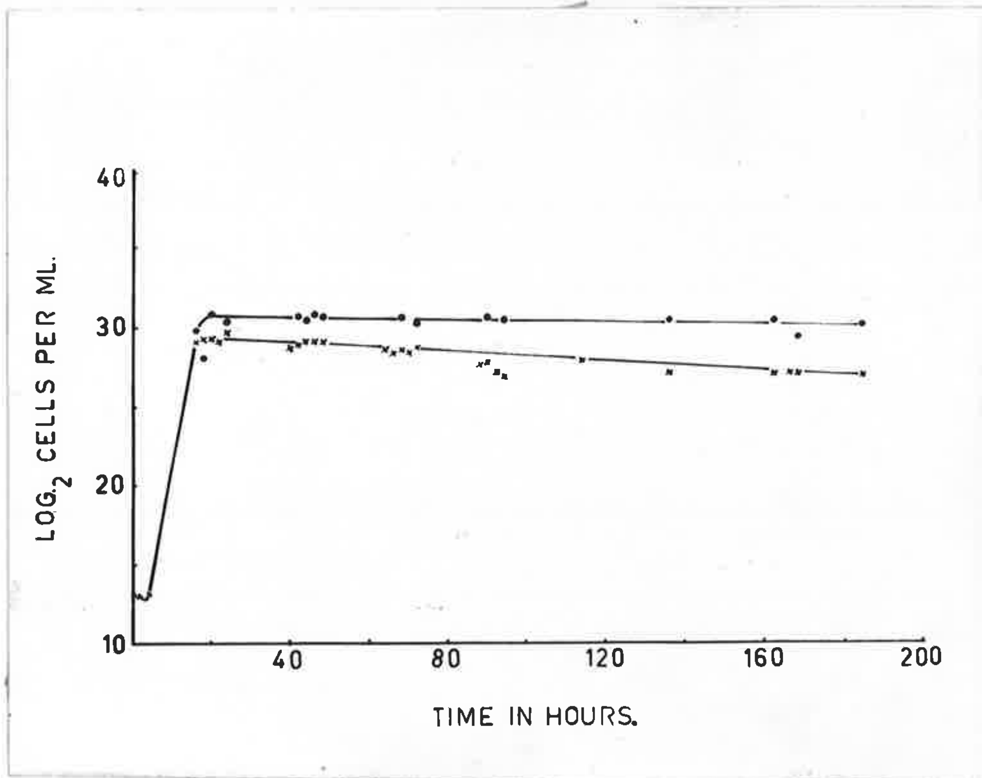
Thus the aerobic and anaerobic growth of the three organisms in the various media behaved in a predictable manner until the active growth phase ended. As expected, the presence of air did not appear to affect the length of the lag phase or the M.G.T. significantly, but it did result in a tenfold increase in the final yield of cells as compared with that of the anaerobic cultures. In general, the early growth of the two organisms under aerobic and anaerobic conditions was more remarkable for the similarities rather than for the differences that could be detected. The sudden decrease in the viable numbers in the anaerobic Ps. aeruginosa cultures seemed to be related to exhaustion of the nitrate supply, although the rapid and extensive alkaline pH swing may also have been involved. A second difference

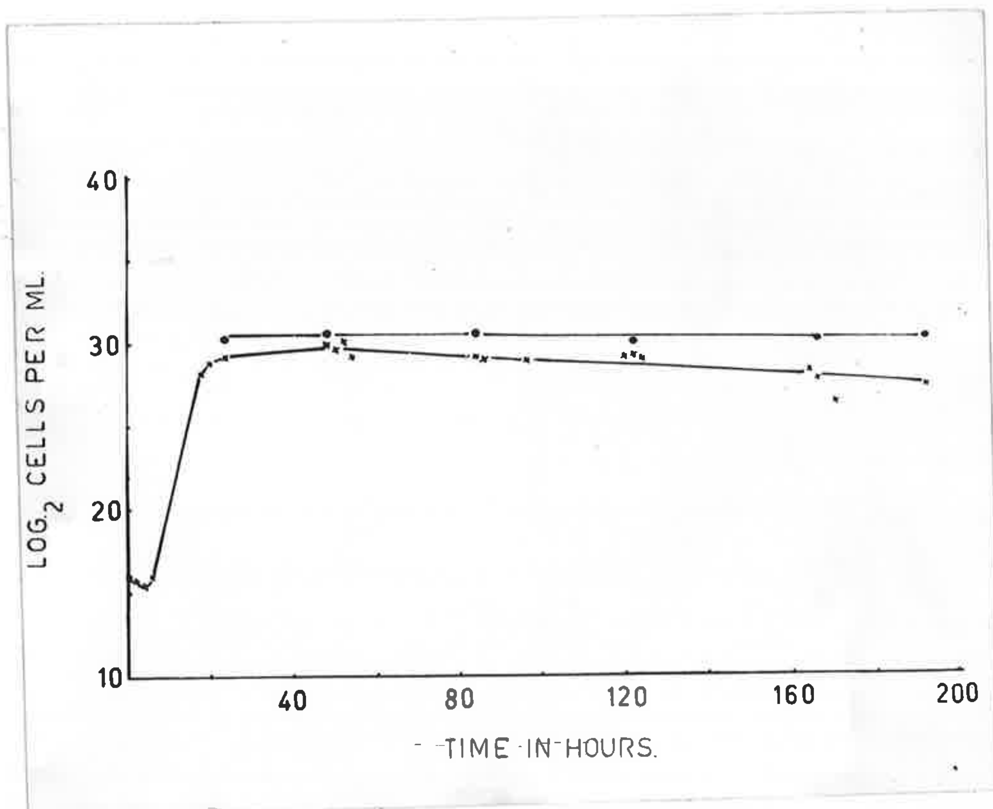
Fig. 9. The total and viable populations in *S. bethesda* cultures grown anaerobically in the defined medium.

X —— X Viable cell counts.
● —— ● Total cell counts.

Fig. 10. The total and viable populations in *S. bethesda* cultures grown anaerobically in the synthetic glucose-nitrate medium.

X —— X Viable cell counts.
● —— ● Total cell counts.


LOG.2 CELLS PER ML.
40
30
20
10
40
80
120
160
200
TIME IN HOURS.


LOG.2 CELLS PER ML.
40
30
20
10
40
80
120
160
200
TIME IN HOURS.

between the strains was the absence of extensive lysis by the ageing *S. bethesda* cells. There were no obvious environmental factors which could have been responsible for the observed extensive cell lysis in the aerobic and anaerobic cultures of *Ps. aeruginosa* since similar conditions appeared to occur in the corresponding suspensions of *S. bethesda*, which, however, remained intact. The rapid death of most of the cells, followed by lysis appeared to be a property of the Pseudomonas cells themselves and further experiments were carried out to determine the reasons for the observed differences between the two organisms. The results of these experiments will be considered in Chapters V and VI.

CHAPTER V.

METABOLIC CHANGES ASSOCIATED WITH AGEING: LETHAL FACTORS FOR PS. AERUGINOSA CELLS.

The viability curves recorded in the last chapter indicated that, although the ageing Ps. aeruginosa cultures went into a decline irrespective of the environmental conditions, continuous incubation under strictly anaerobic conditions produced an unusually rapid mortality. The lower mortality rate in the aerobic cultures may have been due to the continued growth of the survivors, resulting in an apparently higher survival rate by the ageing cells. On the other hand, the anaerobically incubated cultures were unable to continue multiplication in the absence of the nitrate ions, and so the decline was probably a truer estimate of the mortality of the ageing cells of this organism. The extremely rapid decrease in viability observed in these cultures appeared to follow the exhaustion of the nitrate supply, but this may not have been a true cause and effect, as many other factors could equally well have been involved. Experiments to elucidate some of these points were conducted with growing cultures and with resting cell suspensions of Ps. aeruginosa using S. bethesda as a control.

1. Experiments with Growing Cultures.

(a) The effect of nitrate on viability.

(1) Continued addition of nitrate. When grown in 0.5% nitrate broth, Ps. aeruginosa cultures usually destroyed the nitrate within 14 hours. Viable counts carried out on nitrate enriched cultures showed that the population rose to a maximum of 20×10^9 cells per ml. and then slowly declined so that by 192 hours only 10% of the

TABLE 1.

Total cell counts done on anaerobically incubated cultures of *Ps. aeruginosa*.

Age in hours	Number of cells x 10^9 per ml. pH = 6.5	Number of cells x10^9 per ml. pH = 8.5
24	3.6	3.65
48	3.6	-
72	3.0	-
96	2.0	1.15
105	1.8	1.05
120	1.9	1.05
127	2.0	0.90
144	1.65	0.65
192	1.70 (47%)	0.58 (16%)

The figures in parenthesis refer to the percentage of the 24 hour count.

\- = not done

cells were still viable (Fig. 11). The effect of the strongly alkaline pH (9.6 after 100 hours) on the viability was demonstrated in a repeat experiment in which a calculated volume of concentrated HCl was added with each nitrate enrichment. The absence of decline in the neutralised culture (Fig. 11) suggested that the strongly alkaline reaction was at least partially responsible for the observed decline in viability of the non-acid treated cultures. However, addition of acid without further nitrate enrichment still resulted in a rapid decline once the nitrate supply was exhausted. Thus, the continued absence of nitrate from the anaerobic medium appeared to be the main lethal factor.

Autolysis was more extensive in the strongly alkaline control cultures than it was in the acid treated cultures in which the pH was maintained at 6.8 to 7.0 (Table 1). Some autolysis still occurred in the acid treated cultures however. Furthermore, very little autolysis was observed in the plaque-free cultures (Fig. 6) or in the S. bethesda cultures (Fig. 8) despite the strongly alkaline reactions (8.0 to 8.5) observed in these cultures. Thus, as was to be expected, other factors beside the pH shift were involved in the lysis of the plaque strain cells.

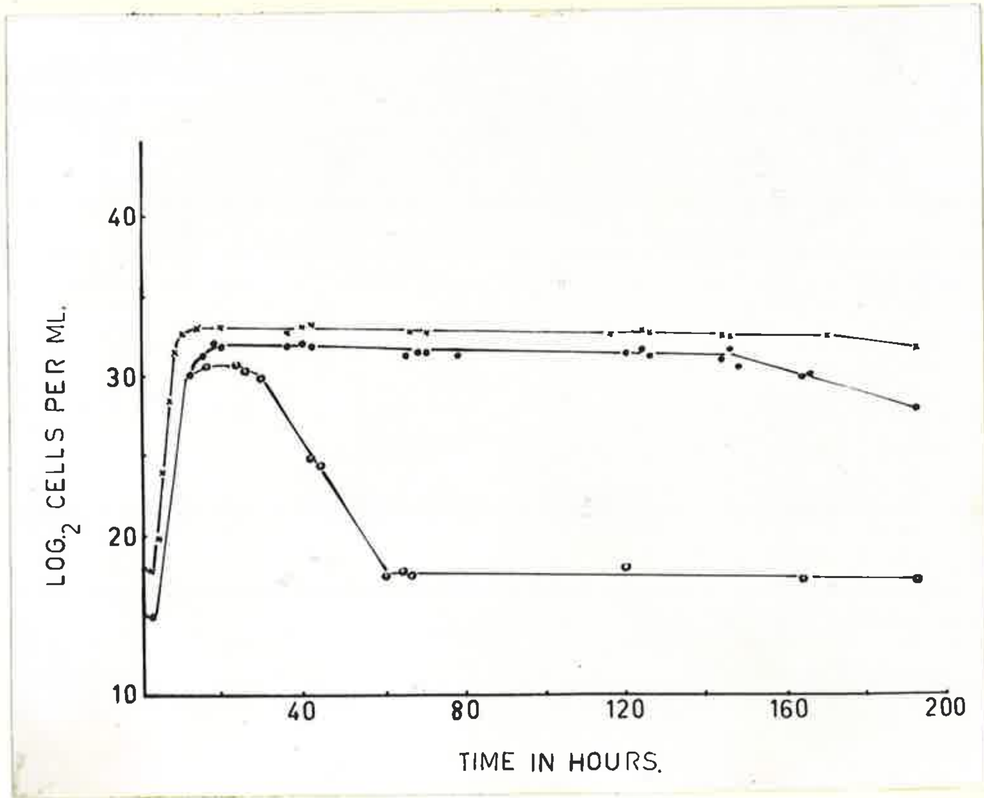
2. Addition of nitrate to decline phase cultures. By the use of a standard inoculum, closely reproducible growth curves could be obtained so that the commencement of the phase of rapid decline could be predicted within a few hours. The declining culture was enriched with various materials before the curve finally flattened out. The percentage increase or decrease in the viable counts was plotted against time (Fig. 12 and 13). The un-enriched control

Fig. 11. The viable population in anaerobic *Ps. aeruginosa* (plaque strain) cultures continuously enriched with sodium nitrate.

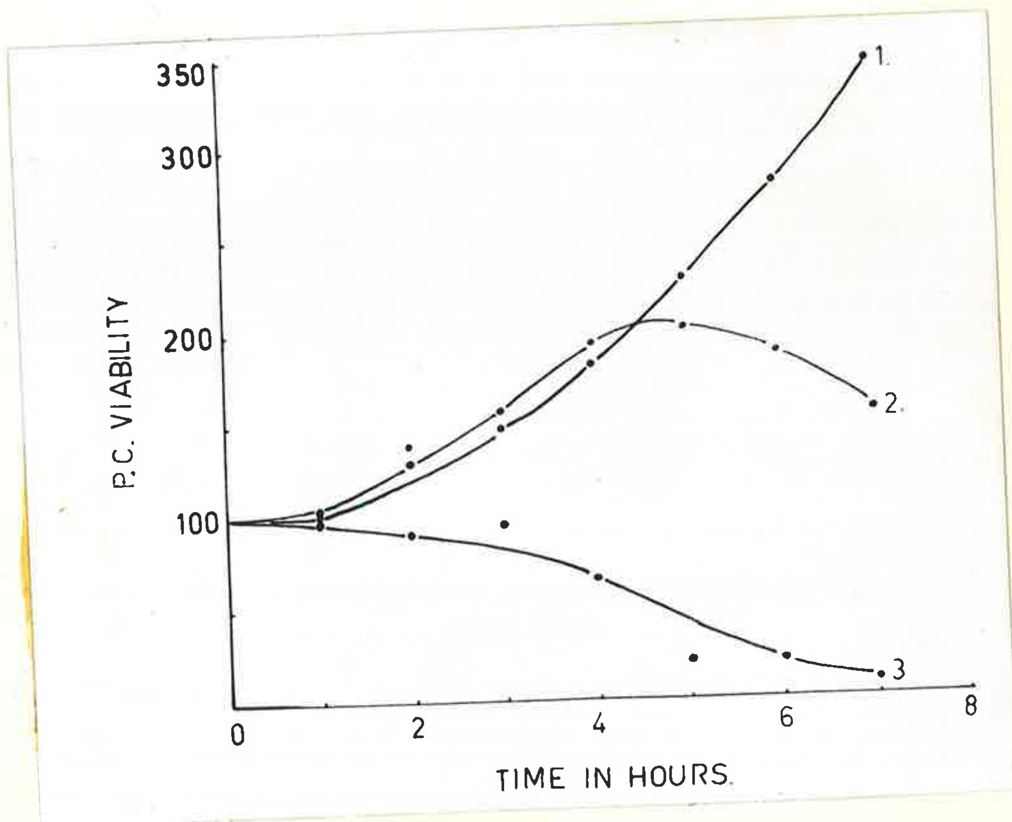
X —— X Nitrate broth enriched with sodium nitrate and HCl.
● —— ● Buffered nitrate broth enriched with sodium nitrate.
○ —— ○ No nitrate additions.

Fig. 12. The variations in the viability of *Ps. aeruginosa* (plaque strain) cells incubated anaerobically in the presence of various additives.

1. Fumaric acid.
2. Sodium nitrate.
3. Control. No additions.



40
30
20
10
LOG₂ CELLS PER ML.
40
80
120
160
200
TIME IN HOURS.



350
300
200
100
P.C. VIABILITY
1.
2.
3
0
2
4
6
8
TIME IN HOURS.

decreased to 10% of the initial population over a seven hour period. However, if nitrate was introduced into the culture, the viability increased to 185% over a five hour period. The viable population then decreased slightly during the final two hours, probably due to the highly alkaline pH (9.5) of the medium. When the pH swing was corrected by a calculated volume of HCl the viability continued to increase to 250% of the original value (Fig. 13). When acid was added alone, the viability declined slightly. Thus a strongly alkaline reaction (pH 8.4) appeared to have a slight bacteriocidal effect.

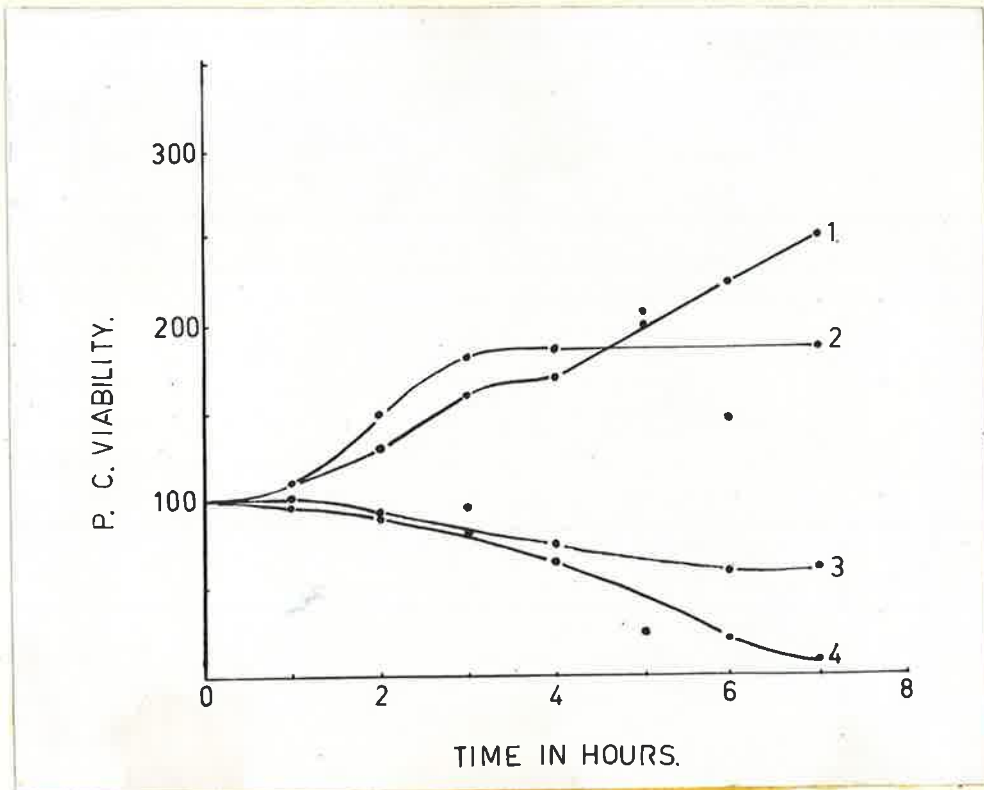
In some experiments, the HCl was replaced by fumaric, succinic, maleic or citric acids. The only acid which stimulated regrowth in the declining cultures was fumaric acid, which resulted in a 350% increase in viability over a seven hour period (Fig. 12). No evidence was obtained for the recovery of apparently dead cells analogous to the report by Heinmets, et.al., (1954). The addition of a dense suspension of heat killed Ps. aeruginosa cells was followed by a 190% increase in viability (Fig. 13). The latter effect was replicated several times, and demonstrated that the surviving decline phase cells were apparently able to utilise the dead or lysed cells for limited regrowth, even under strictly anaerobic conditions.

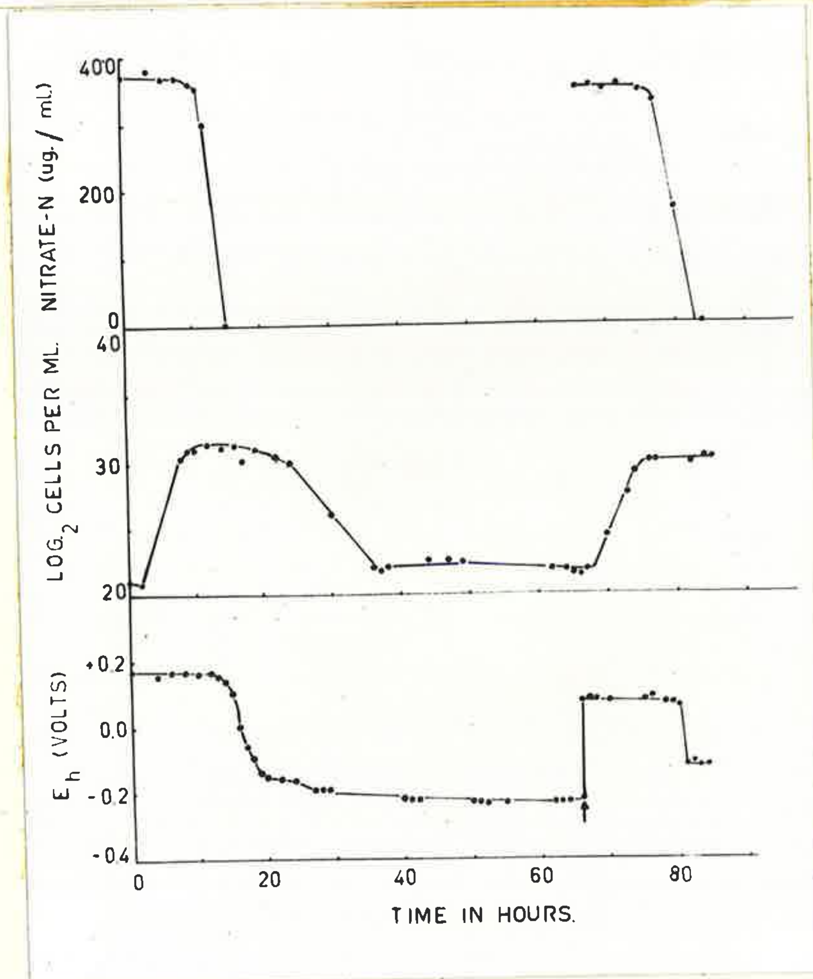
b. The effect of nitrate on the E_h of anaerobic cultures. Following the exhaustion of the nitrate supply, the E_h of the anaerobic Ps. aeruginosa culture rapidly fell from + 0.17 to-0.22 volts (Fig. 14). The addition of further nitrate resulted in a rise in E_h to + 0.05 volts within 30 minutes. The "poising" of the E_h in the presence of nitrate was similar to that observed by Elema, et.al., (1934). Comparison of the E_h and growth curves showed that cell division ceased at the same time

Fig. 13. The variations in viability when *Ps. aeruginosa* cells were incubated anaerobically in the presence of a number of additives.

1. Sodium nitrate with HCl.
2. Heat killed bacterial suspension.
3. Hydrochloric acid.
4. Control. No additions.

Fig. 14. Variations in the E_h of the culture medium of anaerobic *Ps. aeruginosa* (plaque strain) cultures in buffered nitrate broth. The viable population and the nitrate content of the medium were also recorded for comparative purposes. More nitrate was added to the culture after 66 hours incubation (shown by an arrow).


P. C. VIABILITY.
300
200
100
0
2
4
6
8
1
2
3
4
TIME IN HOURS.


NITRATE-N (ug./ ml.)
400
200
0
LOG$_2$ CELLS PER ML.
40
30
20
E_h (VOLTS)
+0.2
0.0
-0.2
-0.4
0
20
40
60
80
TIME IN HOURS.

that the E_h decreased sharply. Thus, the removal of the nitrate was associated with strongly reducing conditions in the medium which seemed to coincide with the inhibition of further cell division.

The inability of Ps. aeruginosa to multiply under strictly anaerobic conditions could have been due to an inability of the strictly aerobic organism to utilise the substrates in the medium to any significant extent in the absence of some external hydrogen acceptor. In an attempt to verify this, carbon balances were prepared for ageing cultures of Ps. aeruginosa. The results of these experiments will be considered in the next section.

c. The effect of nitrate exhaustion on glucose utilisation.
Detailed carbon balances for anaerobic cultures of Ps. aeruginosa harvested during the late logarithmic, stationary and decline phases, together with an aerobic control, were recorded in table 2. Glucose utilisation by the anaerobic cultures continued until the nitrate supply was exhausted. Thereafter, the rate of glucose utilisation was considerably reduced. The decreased glucose uptake occurred before the increasing death rate in the nitrate-free culture could have had any great effect. Thus complete anaerobiosis appeared to inhibit glucose utilisation quite rapidly. On the other hand, the aerated control utilised glucose at a rapid rate throughout the experiment but the fate of a large proportion of the carbon (43%) was not discovered. Only 50% of the utilised glucose was accounted for as CO_2-C, and cellular protoplasm (Table 2). On the other hand, carbon recoveries of 97 to 102% were obtained for all the anaerobic cultures. Estimation of the volatile acid carbon showed that approximately equal amounts of this fraction were produced under both aerobic and anaerobic conditions. When expressed in terms of utilised

TABLE 2.

Carbon recoveries (in mg.) from *Ps. aeruginosa* cultures grown in glucose medium.

	Logarithmic	Anaerobic Stationary A	Anaerobic Stationary B	Decline A	Decline B	Aerobic Logarithmic
Glucose- C. (initial)	1320	1320	1320	1320	1360	2040
Glucose-C. (residual)	1135	910	900	875	885	1770
Glucose utilised	185	410	420	445	475	270
Gaseous CO_2-C.	16.2	41.7	44.0	29.2	41.2	103
CO_2-C. in solution	91.4	196	191	158	185	6.7
Total CO_2-C.	108	238	235	187	226	110
% of utilised C.	58	58	56	42	48	41
Bacterial-C.	52.0	178	206	216	200	21.5
% of utilised C.	28	44	49	48	42	8.0
Volatile acid-C.	18.0	12.7	6.2	15.0	16.4	20.4
Non volatile acid-C.	1.5	3.5	2.5	41.9	34.3	4.1
% of utilised C as acid-C.	1.0	4.0	2.1	12.8	11.0	9.0
Total carbon recovery	179	432	450	460	477	156
Total carbon accounted for	1310	1340	1350	1330	1360	1930
% of total-C.	99	102	102	101	100	95

TABLE 3.

Organic acids (mg.) produced by *Ps. aeruginosa* cells grown in glucose medium.

Growth phase		Volatile acid-C	Fumaric acid-C	Lactic acid-C	α keto glutaric-C	Oxal = acetic-C	Glycollic acid-C	Malic acid-C	Citric acid-C	Total C
Anaerobic										
Logarithmic		15.0	0.1	0.0	0.0	0.0	0.9	0.2	0.3	16.5
Stationary	A	12.7	0.1	2.0	0.0	0.0	1.0	0.0	0.0	16.2
	B	6.2	0.0	0.6	0.0	0.0	1.6	0.0	0.3	8.7
Decline	A	15.0	0.6	34.7	0.4	0.5	4.0	0.6	1.1	56.9
	B	16.4	0.2	16.0	0.0	0.1	14.8	0.4	2.8	50.7
Aerated										
Logarithmic		20.4	0.3	1.2	0.0	0.1	2.1	0.1	0.3	24.5

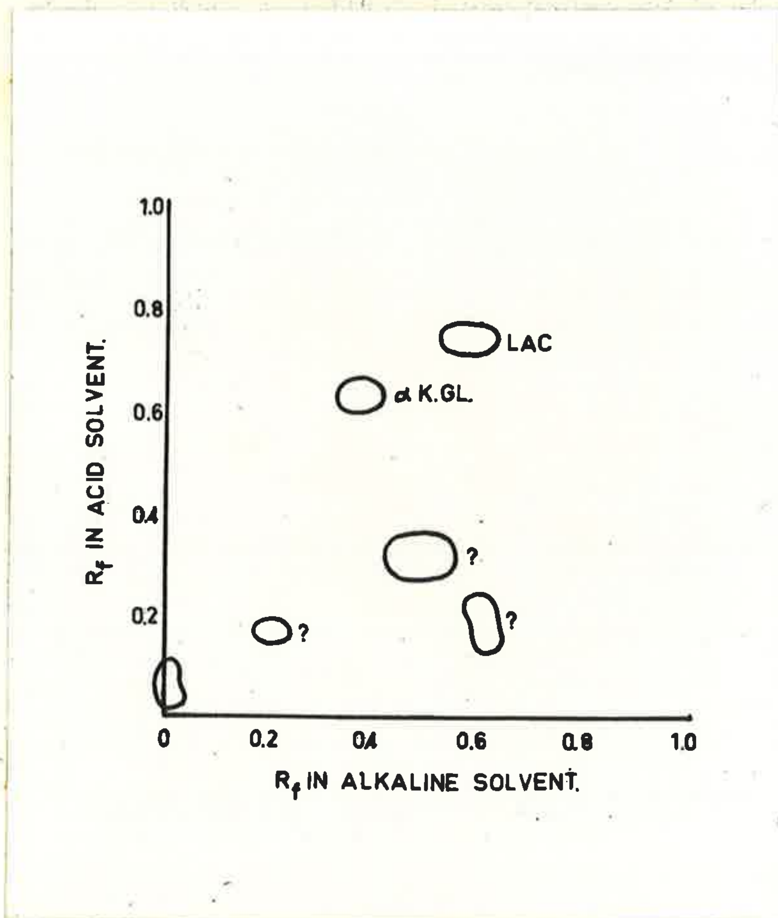
glucose however, the aerobic culture produced almost twice the amount of volatile acid as that found in any of the anaerobic cultures, with the exception of the logarithmic phase culture.

The non-volatile acids in the culture media varied considerably in both type and amount as the cells aged. The most common acids detected were lactic, glycollic, malic and citric acids, with traces of keto glutaric and oxalacetic acids (see Table 3). The carbon recoveries in this fraction increased with the age of the culture, until, by the decline phase, it accounted for 8% of the glucose-carbon. The anaerobic logarithmic and stationary phase cultures closely resembled the aerobic control both in the type and in the amount of the non-volatile acids present in the medium. This can best be seen by a comparison of the results for the column and paper chromatograms recorded in Figs. 15 to 18. The non-volatile acid fraction was disregarded as a significant end product of glucose metabolism in the logarithmic and stationary phase cultures. The decline phase culture contained considerable amounts of lactic, citric and glycollic acids as well as smaller amounts of malic and oxalacetic acids. Two unknown acids were also detected in the paper chromatograms (Fig. 18). These acids may have represented substituted gluconic acid derivatives, but their Rf values were too high for them to have been gluconic acid itself. When the volatile acid carbon in the decline phase culture was included with this fraction, 12% of the utilised glucose was accounted for, and, collectively, these acids therefore constituted significant end products of the glucose metabolism of *Ps. aeruginosa* under anaerobic conditions. The high carbon recoveries for the anaerobic cultures indicated that other undetected end products of glucose metabolism were unlikely to be present.

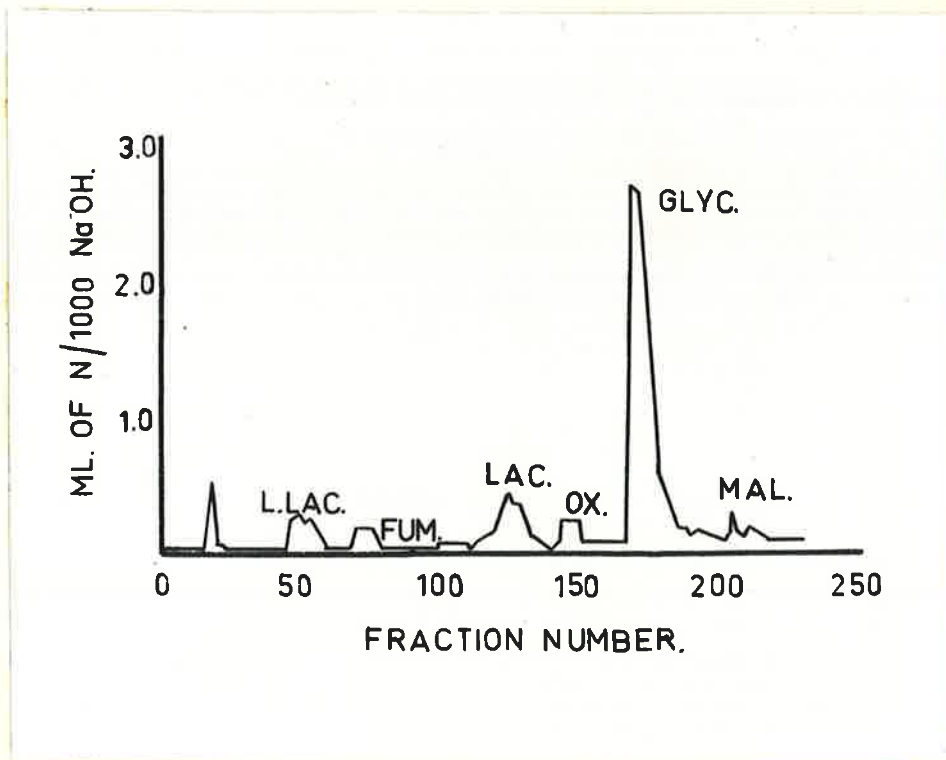
Fig. 15a. Two dimentional paper chromatogram of the ether extract of the aerobic logarithmic phase culture medium.

Fig. 15b. Columnchromatogram of the ether extract of the aerobic logarithmic phase culture medium.

Lac: lactic acid. K.Gl: α keto glutarate.
Mal: malic acid. Fum : fumaric acid.
? : unknown acids. Ox : oxalacetic acid.
L.Lac: Lactyl lactic acid produced from the lactic acid during the extraction process.



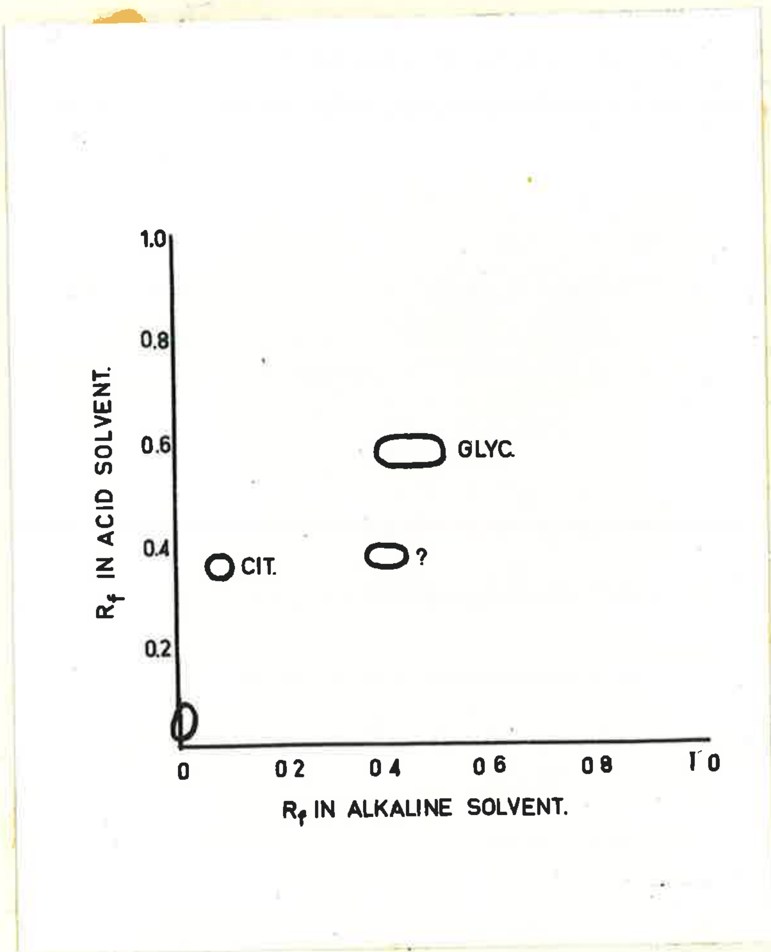
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
R_f IN ACID SOLVENT.
R_f IN ALKALINE SOLVENT.
LAC
α K.GL.
?
?
?



3.0
2.0
1.0
ML. OF N/1000 NaOH.
0
50
100
150
200
250
FRACTION NUMBER.
GLYC.
L.LAC.
FUM.
LAC.
OX.
MAL.

Fig. 16a. Two dimentional paper chromatogram of the ether extract of the anaerobic logarithmic phase culture medium.

Fig. 16b. Column chromatogram of the ether extract of the anaerobic logarithmic phase culture medium.


1.0
0.8
0.6
0.4
0.2
Rf IN ACID SOLVENT.
GLYC.
CIT.
?
0
0 2
0 4
0 6
0 8
1 0
Rf IN ALKALINE SOLVENT.

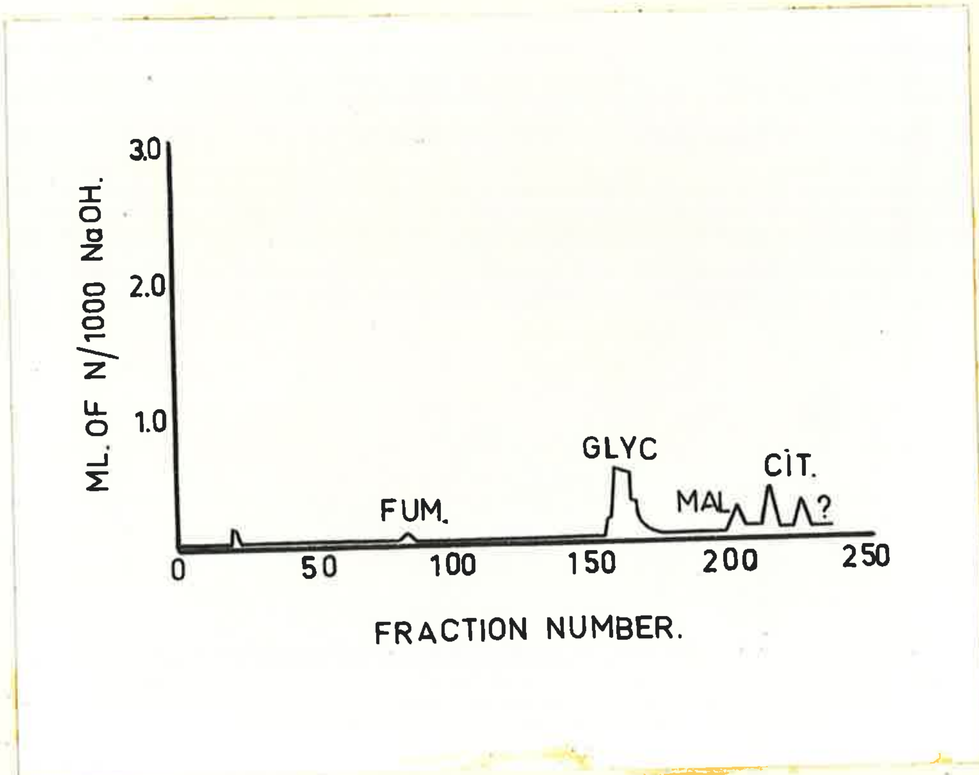
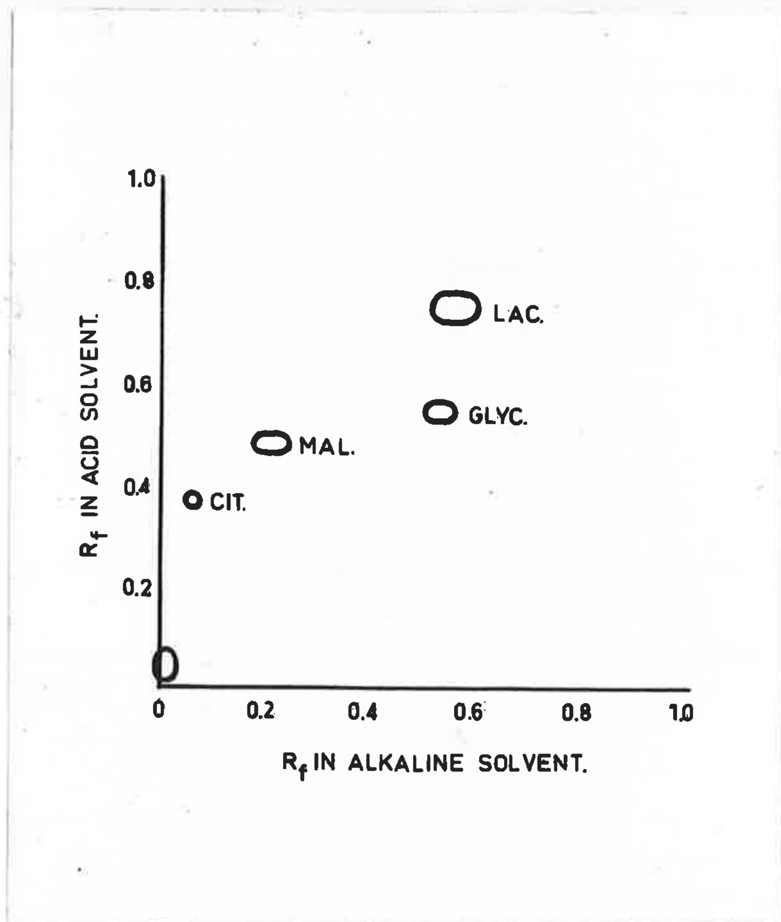

3.0
2.0
1.0
ML. OF N/1000 NaOH.
FUM.
GLYC
MAL
CIT.
?
0
50
100
150
200
250
FRACTION NUMBER.

Fig. 17a. Two dimentional paper chromatogram of the ether extract of the anaerobic stationary phase culture medium.

Fig. 17b. Column chromatogram of the ether extact of the anaerobic stationary phase culture medium.


1.0
0.8
0.6
0.4
0.2
0
Rf IN ACID SOLVENT.
LAC.
GLYC.
MAL.
CIT.
0.2
0.4
0.6
0.8
1.0
Rf IN ALKALINE SOLVENT.

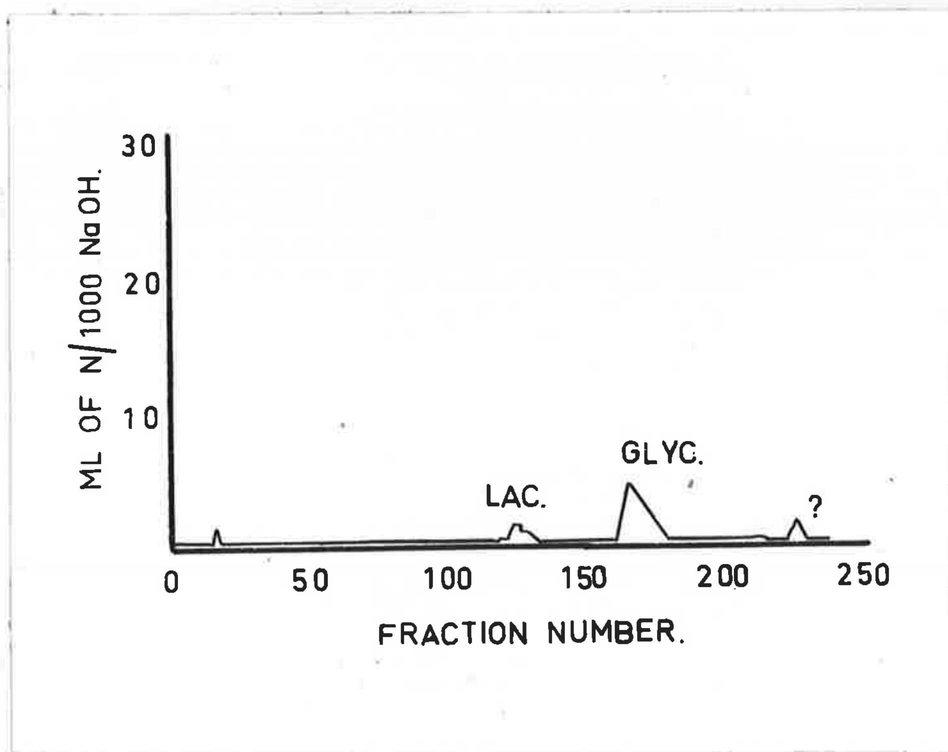
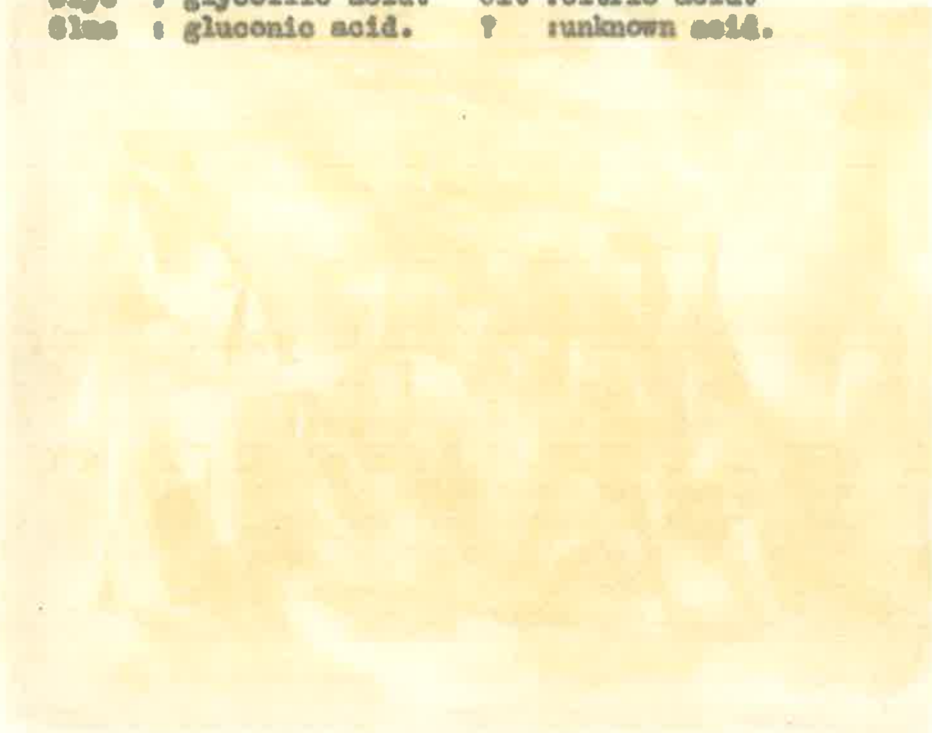
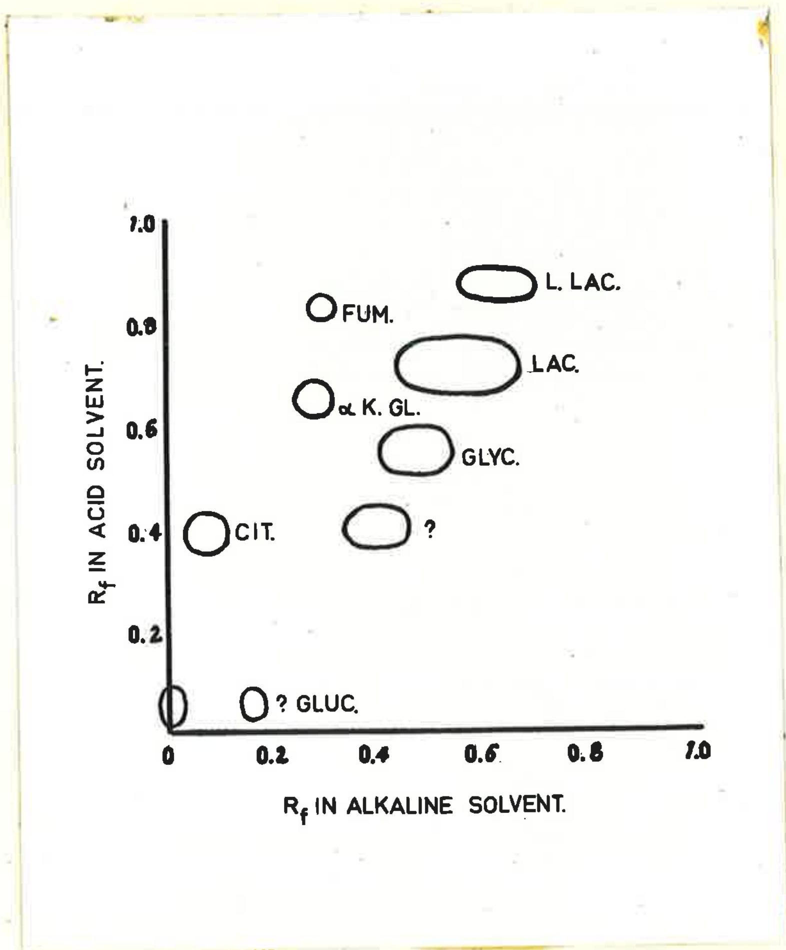

30
20
10
ML OF N/1000 NaOH.
LAC.
GLYC.
?
0
50
100
150
200
250
FRACTION NUMBER.

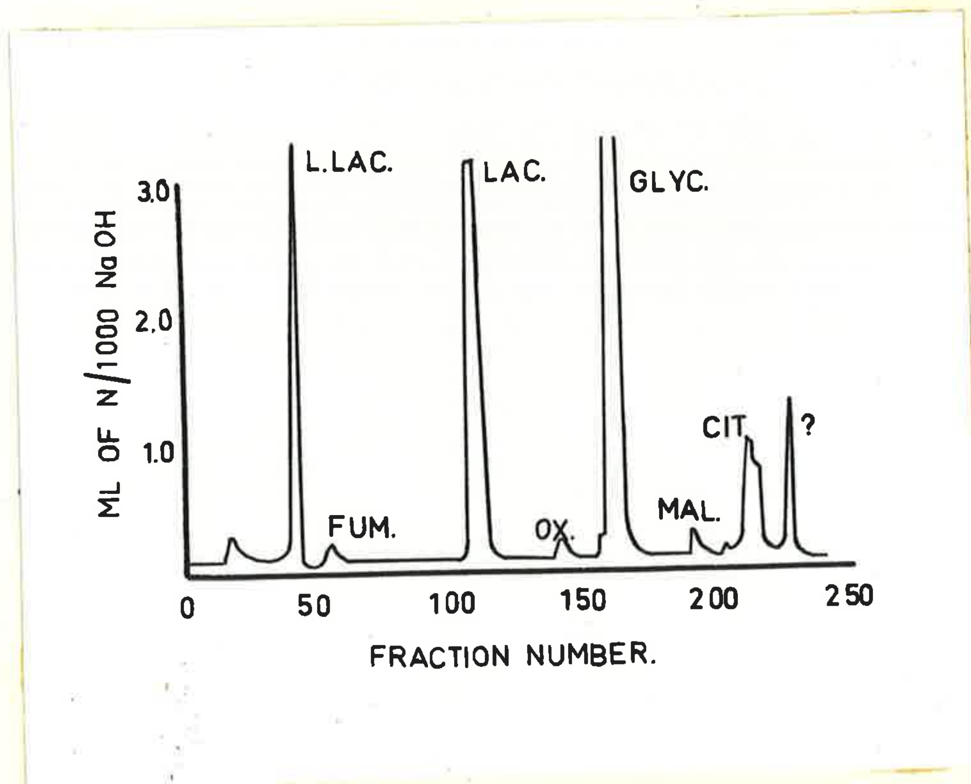
Fig. 18a. Two dimentional paper chromatogram of the ether extract of the anaerobic decline phase culture medium.

Fig. 18b. Column chromatogram of the ether extract of the anaerobic decline phase culture medium.

L. Lac: lactyl acid. Lac: lactic acid.
Fum : fumaric acid. K.Gl: α keto glutaric acid.
Glyc : glycollic acid. Cit :citric acid.
Gluc : gluconic acid. ? :unknown acid.




1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
L. LAC.
FUM.
LAC.
α K. GL.
GLYC.
CIT.
?
? GLUC.
R_f IN ACID SOLVENT.
R_f IN ALKALINE SOLVENT.


3.0
2.0
1.0
0
50
100
150
200
250
L.LAC.
LAC.
GLYC.
FUM.
OX.
MAL.
CIT.
?
ML OF N/1000 Na OH
FRACTION NUMBER.

These results made it apparent that prolonged incubation of the culture under strictly anaerobic conditions affected the normal glucose metabolism of the organism. Incubation under strictly anaerobic conditions induced the death and lysis of the cells in a nutrient medium capable of supporting further growth of the cells when oxygen or nitrate was present. The observed decline of such cultures could have been due to the inability of the organism to multiply in the anaerobic culture. Comparison was therefore made between the behaviour of the ageing broth cultures and non-proliferating or resting cell suspensions made up in various diluents and stored under aerobic and anaerobic conditions. The results of these studies will be considered in the next section.

2. Experiments with Resting Cell Suspensions.

a. Killing curves for Ps. aeruginosa and S. bethesda.

The decrease in viability of distilled water, saline, 0.067 M. phosphate buffer (pH 7.4), glucose basal medium and broth suspensions was recorded in Fig. 19. Distilled water and saline were moderately bacteriocidal for Ps. aeruginosa, reducing the viable population in 120 minutes by 60% and 44% respectively. Phosphate buffer and the basal medium had little or no lethal effect over the same period. Phosphate buffer was selected as a suitable diluent for the later tests.

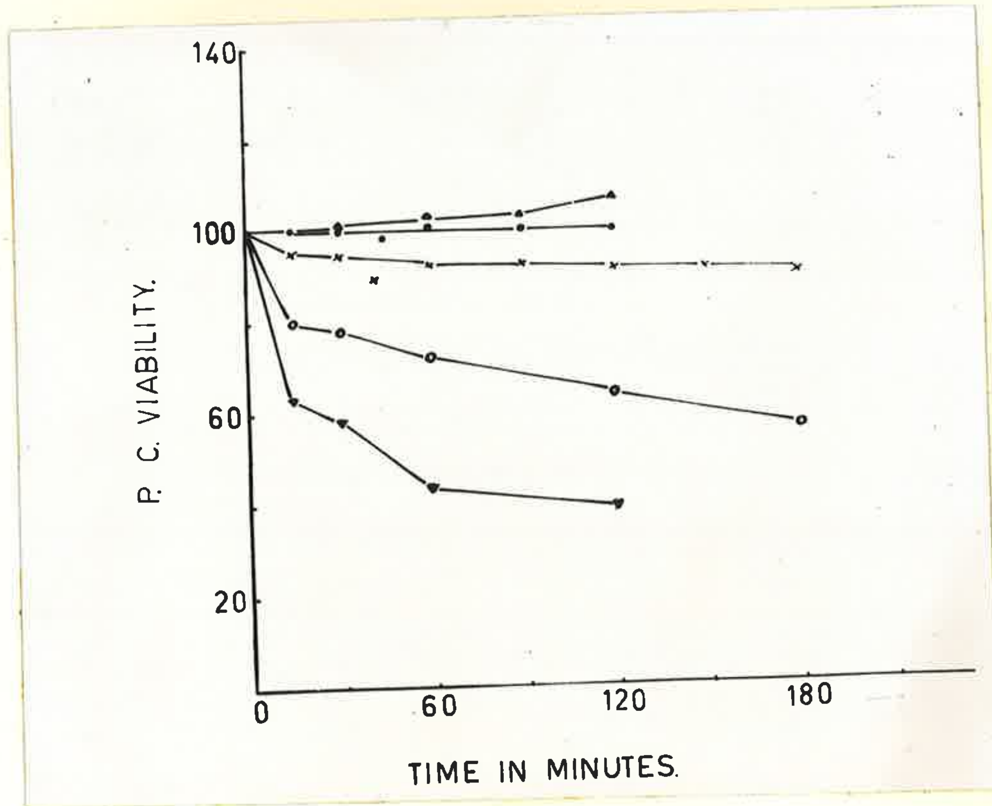
The corresponding S. bethesda suspensions were somewhat more sensitive to dilution in distilled water, saline and buffer (70%, 60% and 40% decrease in viability over a 120 minute period respectively). The viability of S. bethesda in the basal medium was not greatly affected over the two hour period (Fig. 20). Despite its slightly bacteriocidal effect on S. bethesda suspensions, phosphate buffer was used as the diluent for the later experiments.

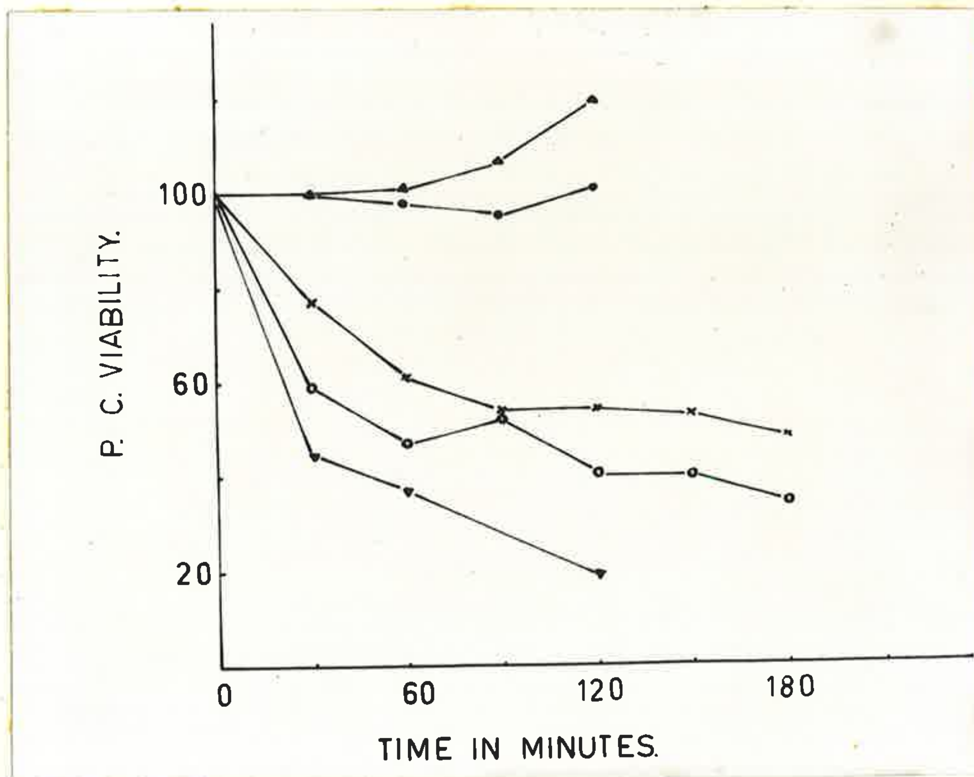
Fig. 19. The changes in the viability of *Ps. aeruginosa* (plaque strain) cells suspended in various diluents and incubated aerobically at 37°C.

▲——▲ Digest broth.
●——● Glucose-nitrate basal medium.
X——X 0.067M. phosphate buffer (pH. 7.4).
⊙——⊙ Saline (0.85%).
▼——▼ Distilled water.

Fig. 20. The changes in viability of *S. bethesda* cells suspended in various diluents and incubated aerobically at 37°C.

▲——▲ Digest broth.
●——● Glucose-nitrate synthetic medium.
X——X 0.067M. phosphate buffer (pH. 7.4).
⊙——⊙ Saline (0.85%).
▼——▼ Distilled water.


140
100
60
20
P. C. VIABILITY.
0
60
120
180
TIME IN MINUTES.


100
60
20
P. C. VIABILITY.
0
60
120
180
TIME IN MINUTES.

b. The effect of oxygen and nitrate on the viability of Ps. aeruginosa and S. bethesda suspensions. Resting suspensions of both organisms were treated as stated in Chapter III, Section 8c. The changes in the viability of the four Ps. aeruginosa suspensions was recorded in Fig. 21 and those for S. bethesda in Fig. 22.

The viability of the aerobic Ps. aeruginosa suspensions remained high whether nitrate was present or not, whereas in the anaerobic, nitrate-free cells it fell to one per cent of the original after 48 hours. The lethal effect of the anaerobic incubation was inhibited by the presence of nitrate in the suspending medium. The four S. bethesda suspensions were not greatly affected by the presence or the absence of either nitrate or oxygen. The observed decrease in cell viability in these cultures was largely ascribed to the action of the phosphate buffer.

c. The effect of the culture medium on the viability of the Ps. aeruginosa suspensions. The possibility that the rapid anaerobic death of the nitrate-free suspensions was partly due to toxic factors in the broth had to be considered. The viability of the cells in fresh and in staled medium determined under aerobic and anaerobic conditions in the presence and absence of nitrate was recorded graphically in Fig. 23. The viability of the anaerobic suspensions rapidly decreased both in the stale and fresh broth. The addition of nitrate to the anaerobically incubated suspensions resulted in the maintenance of a high viability over the three day period, irrespective of the type of broth used as the diluent. Thus, despite the alkaline p^{H} of the "stale" broth, little toxic effect on the Ps. aeruginosa suspensions was noted, provided that air or nitrate was also present. Thus the results for the resting cell suspensions of Ps. aeruginosa agreed with those

Fig. 21. Viability curves for resting suspensions of *Ps. aeruginosa* (plaque strain) incubated under aerobic and anaerobic conditions in the presence and absence of nitrate.

1. Shaken in air or shaken in N_2 .
   Io = cell numbers present at any one time.
   I = initial counts.

2. Shaken under N_2 with nitrate addition.

3. Shaken under N_2.

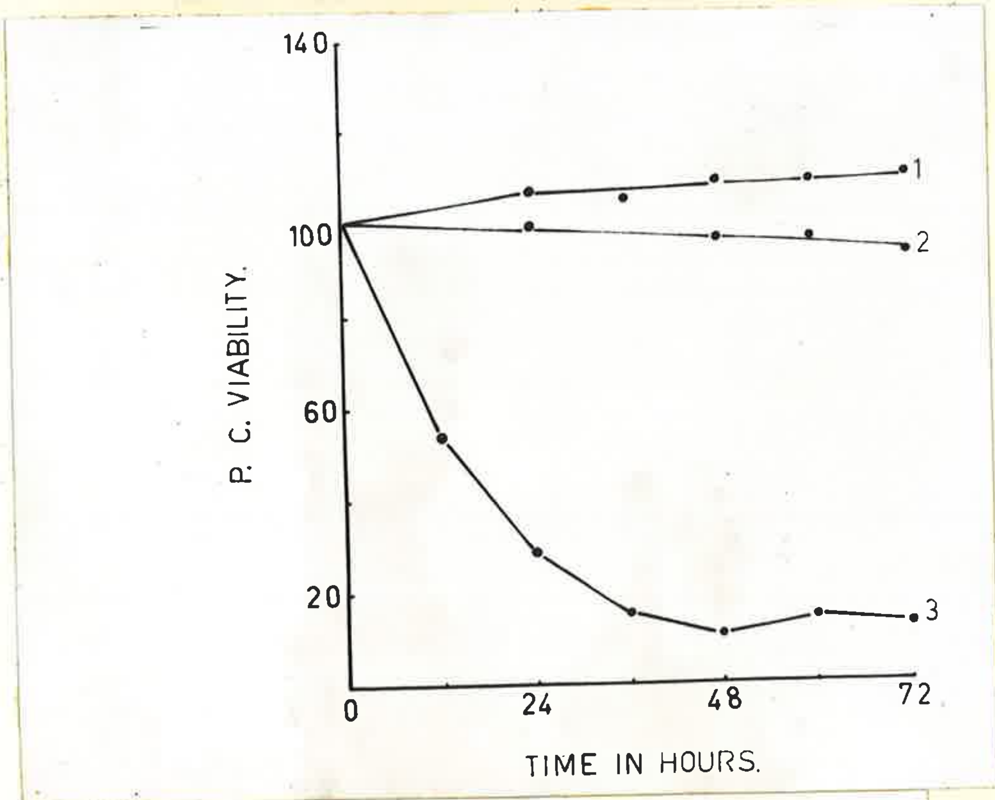
Fig. 22. Viability curves for resting suspensions of *E. bethesda* incubated under aerobic and anaerobic conditions in the presence and absence of nitrate.

●——● Shaken in air.

⊙——⊙ Shaken in air with nitrate addition.

X——X Shaken under N_2.

△——△ Shaken under N_2 with nitrate addition.


140
100
60
20
P. C. VIABILITY.
1
2
3
0
24
48
72
TIME IN HOURS.

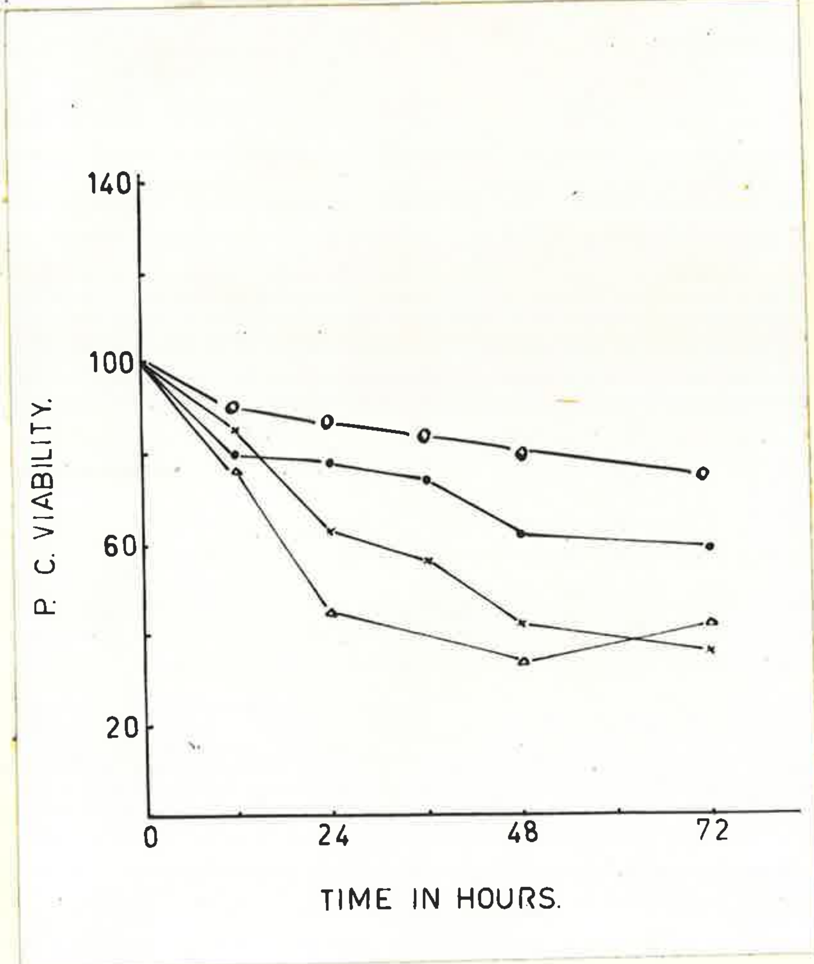
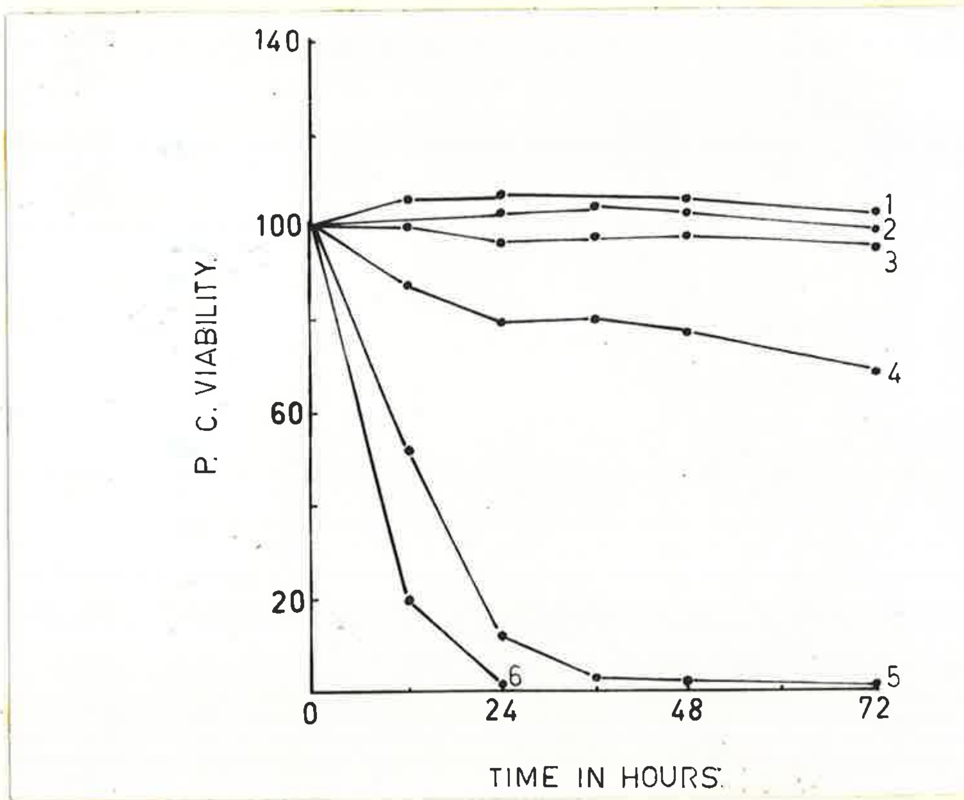

140
100
60
20
P. C. VIABILITY.
0
24
48
72
TIME IN HOURS.

Fig. 23. Effect of "staled" culture medium on the viability of *Ps. aeruginosa* (plaque strain) suspensions incubated under aerobic and anaerobic conditions in the presence and absence of nitrate.

1. "Fresh": shaken in air.
2. "Stale": shaken in air.
3. "Fresh": shaken under N_2 with nitrate.
4. "Stale": shaken under N_2 with nitrate.
5. "Fresh": shaken under N_2.
6. "Stale": shaken under N_2.


140
100
60
20
0
P. C. VIABILITY
24
48
72
TIME IN HOURS
1
2
3
4
5
6

obtained with the growing cultures.

As was expected, the S. bethesda suspensions displayed very little dependence on the presence or absence of an external hydrogen acceptor for continued high viability. The anaerobically incubated suspensions of Ps. aeruginosa therefore contrasted sharply with suspensions of the facultative anaerobe, and these differences were presumably due to fundamental physiological differences between the two organisms. Our present limited knowledge of the internal organisation of the cell makes it pointless to consider this aspect any further. Attention was therefore turned to the second difference observed in the decline phase of the Ps. aeruginosa and S. bethesda cultures. This study concerned the lytic changes observed in the ageing Ps. aeruginosa cultures and experiments carried out on this process will be considered in the next chapter.

CHAPTER VI.

METABOLIC CHANGES ASSOCIATED WITH AGEING: LYTIC FACTORS FOR PS.AERUGINOSA CELLS.

The extensive decrease in total cell numbers which occurred two to four days after the decline in viability was thought to be due to the release of an autolytic enzyme by the cells. Analysis of total counts carried out on the aerobic cultures demonstrated the fact that extensive cell lysis occurred in these cultures also, and so autolysis appeared to be a general characteristic of Ps. aeruginosa and was not dependent on strictly anaerobic conditions for its induction. The behaviour of the ageing cultures of S. bethesda was in contrast to that of the Pseudomonad, and therefore further investigations into the lysis of ageing cultures of both organisms were carried out.

In the past, several lytic systems have been described for Ps. aeruginosa cultures. Recently, phage lysis has been well documented for Ps. aeruginosa cultures isolated from a wide variety of sources. This lytic phenomenon was normally associated with young actively multiplying cells, rather than with senescent cells, and for this reason bacteriophages were thought unlikely to be the direct cause of the currently observed lytic changes. The "irridescent" lytic changes were observed in old cultures of Ps. aeruginosa and a brief investigation of this phenomenon was therefore carried out in the present study although it was uncertain what part the "irridescent" changes could play in the lysis of anaerobic liquid cultures. This chapter therefore begins with an account of these investigations.

1. The Lysis of Ps. aeruginosa Cultures on Solid Media.

Ps. aeruginosa grew well on a basal glucose-nitrate salts agar.

The growth on the aerobic plates after 36 hours incubation was bright green in colour and the colonies exhibited a typical rough "beaten copper" surface (Plate 1). After 36 to 48 hour's incubation small "irridescent" oval to round lytic plaques developed in the dense growth, and these slowly enlarged, often until the lysis was confluent. The floor of the plaque consisted of a thin layer of apparently intact growth which was not completely removed even after prolonged incubation. There was no evidence of regrowth from this apparently resistant core. Transfer of cells from this core to a fresh plate resulted in growth which resembled the parent culture in all respects. More than 30 such transfers had no appreciable effect on the number of "irridescent" patches produced by the culture compared with the original. The "irridescent" material floated off on sterile saline and could be transferred to a nearby plaque-free area on the colony, but no new plaque was produced.

Anaerobic growth on the nitrate-enriched agar was not as luxuriant as on the aerobic plates. The colonies were small, without pigment and free from "irridescent" lytic patches. Comparison of the aerobic and anaerobic plates suggested that the "irridescent" plaques appeared only when the growth reached a certain critical density. However, technical difficulties prevented quantitative verification of this observation.

The localised nature of the plaques strongly suggested a phage actuated mechanism, and despite the lack of success reported by earlier workers, attempts were made to isolate a propagating strain of a phage producing the "irridescent" plaques.

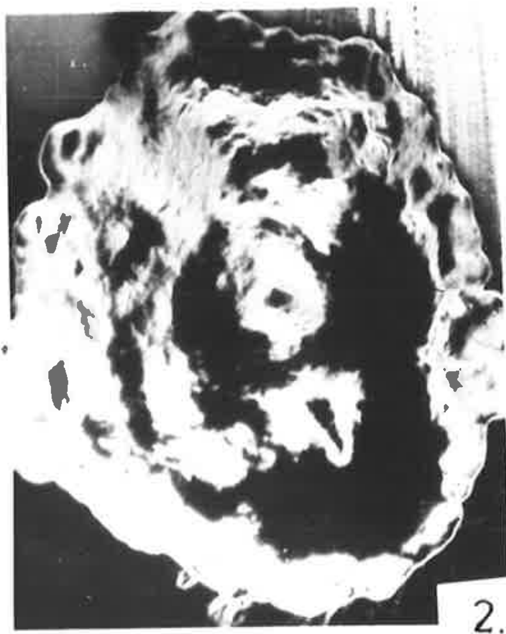
Plate 1. 1. Colonies of Ps. aeruginosa(plaque strain) grown on TSA for four days at 37°C. X 10.

2. A single colony of Ps. aeruginosa (plaque strain) grown on TSA for two days at 37°C. Note the rough irregular "beaten copper" surface. X 40.

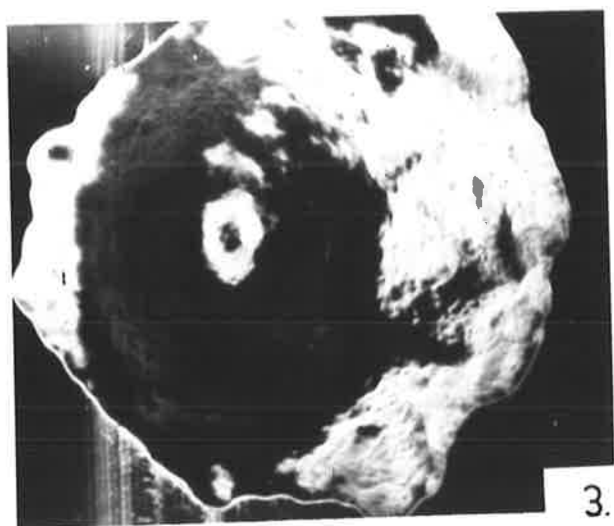
3. A single colony of Ps. aeruginosa (plaque-free strain) grown on TSA for four days at 37°C. Note the absence of plaques and the more regular appearance of the surface. The bright circle in the centre is an illumination artefact. X 40.

4. A single colony of S. bethesda grown on TSA for two days at 37°C. Note the smooth shiny surface and the absence of plaques. The bright ring is an illumination artefact. X 40.

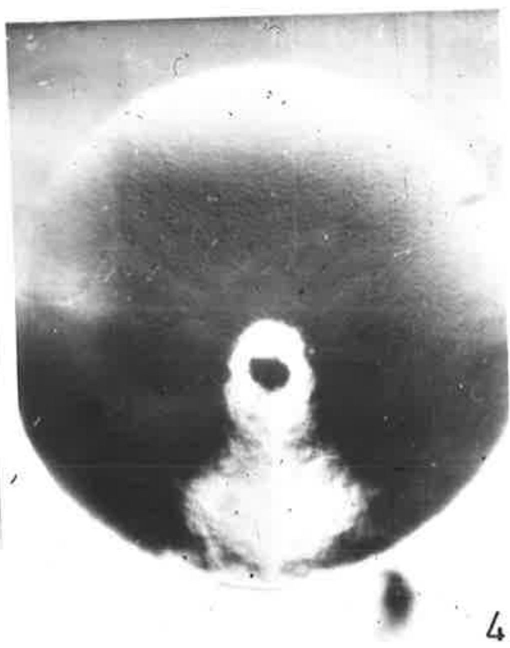
1.



2.



3.



4.

2. Propagation Experiments.

Strains Th5, Foggo, Hayes and S1 did not produce "irridescent" plaques when grown on TSA plates. Growth of these strains with the plaque-forming strains did not result in the transfer of the ability to form plaques to these four organisms. Repeated cross inoculation tests between the plaque and plaque-free strains gave uniformly negative results. In view of the similarly negative results by other workers in the field, further work along these lines did not seem to be justified.

3. Isolation of a Plaque-free-Mutant of Ps. aeruginosa.

The occurrence of the natural plaque-free strains of Ps. aeruginosa led to the isolation of a similar mutant of the N.C.T.C. strain 6750. Direct comparison between the two Ps. aeruginosa strains could then be made. The parent organism was "tagged" with resistance to 50 µg. streptomycin per ml. to assist in the later identification of the mutant. The parent was sensitive to five µg. of streptomycin per ml. The mutant strains were non-pigmented and the increased streptomycin resistance of the plaque-free cultures showed that these strains had been derived from the plaque strain culture and were not chance non-pigmented aerial contaminants.

Growth of Ps. aeruginosa in digest broth for three days before transference to fresh medium resulted in the selection of a plaque-free mutant after three or four sub-cultures. On the other hand, serial daily transfers of the parent in digest broth over a period of three months did not affect plaque production. The mutant produced large pink colonies on TSA and no "irridescent" plaques were detected even when incubation was continued for seven days.

The plaque-free culture did not revert to the parent strain even after ultra-violet irradiation. About one per cent of colonies of chloramphenicol resistant mutants of the plaque-free strain produced pyocyanin and "irridescent" plaques, however. The plaque forming revertants were still resistant to 50 µg. of streptomycin per ml. which made it unlikely that the pigmented colonies were chance aerial contaminants of the wild type organism. Increased resistance to penicillin or terramycin did not result in any detectable reversion to plaque formation by the plaque-free cells. The reversion to plaque formation following chloramphenicol treatment was confirmed by two repeat experiments. There is an obvious need for further study of this puzzling phenomenon but this work was not carried any further as it seemed to be outside the general scope of this thesis.

4. Mixed Cultures of the Plaque and Plaque-free Strains.

The two strains grew readily as mixed cultures on TSA plates. After 48 hours, the pigmented colonies produced "irridescent" plaques which slowly enlarged, often until most of the surface of the colony was destroyed (Plate 1). The plaque-free colonies were pale pink in colour and so were easily identified even when growing alongside the pigmented plaque formers. In mixed colonies, the plaques which appeared in the pigmented growth usually spread only as far as the interface between the two colonies. The presence of numerous plaque-free colonies did not inhibit the formation of plaques by the parental strain. Thus, no evidence was found that the plaque-free cells produced any extracellular inhibitor of plaque formation.

Growth experiments in which Ps. aeruginosa was grown on a basal

TABLE 4.

Lytic activity of *Ps. aeruginosa* cultures on whole cells and cell wall preparations.

Organism added to the agar	Age in hours	Preparation	Plaque strain	Plaque-free strain
Ps. aeruginosa plaque	24 hr.	whole cells	+	-
Ps. aeruginosa plaque-free	24 hr.	whole cells	++	-
S. bethesda	24 hr.	whole cells	+	-
Ps. aeruginosa plaque	24 hr.	cell walls	+	-
Ps. aeruginosa plaque-free	24 hr.	cell walls	+	-

++ = zone of clearing 1 - 2 m.m. in width

\+ = zone of lysis 1 m.m. in width

\- = no zone of lysis

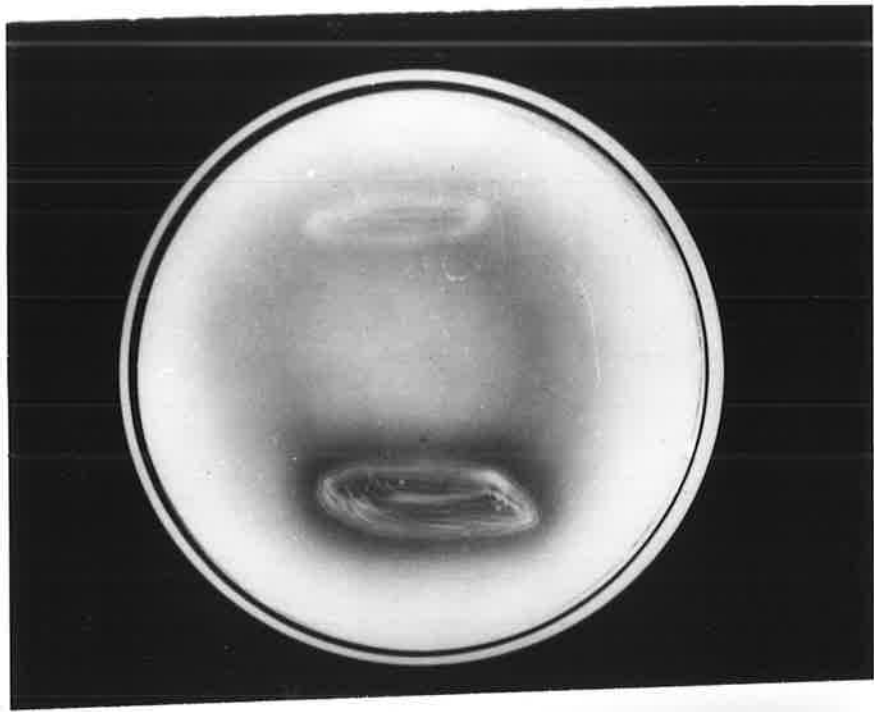
agar containing dead *Ps. aeruginosa* or *S. bethesda* whole cells as energy source suggested that the plaque strain (but not the plaque-free strain) produced a diffusible lytic enzyme which formed a clear halo around the surface colony (Plate 2). No zone of clearing could be discerned around the plaque-free colonies even after incubation for seven days. Similar results were obtained when pure cell walls were used as the energy source (Table 4). The clearing noted when the plaque strain was grown on the plaque-free cell walls was somewhat diffuse as compared with that with the plaque strain walls, but the size of the zone was about the same for both preparations. The plaque-free cells were unable to lyse the cell walls of either strain (Plate 2). Thus the plaque strain culture produced a diffusible lytic factor which spread away from the growing colony. The extracellular lytic enzyme (autolysin) could be detected in the culture media of both aerated and anaerobic cultures. Attempts were made to concentrate the enzyme by ammonium sulphate precipitation (*vide* Chapter III, Section 10), and some of the properties of the crude enzyme preparation were then determined. These properties will be discussed in the next section.

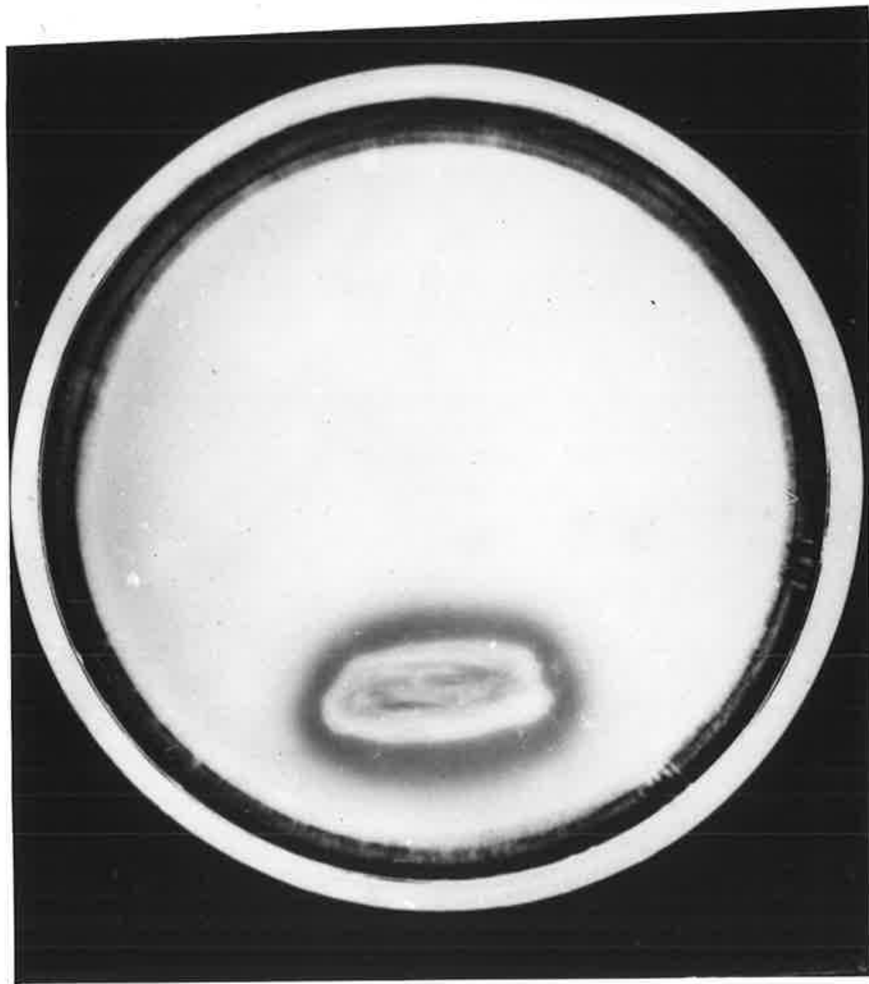
5. Lysis of Resting Cell Suspensions of *Ps. aeruginosa*.

Incubation of living suspensions of *Ps. aeruginosa* with the autolysin preparation at 37°C. was found to be unsatisfactory, as limited cell growth usually occurred making the interpretation of the results very difficult. Incubation was therefore carried out at 45°C., this temperature being sufficiently high to inhibit cell multiplication without resulting in an excessively high death rate in the control suspensions. The decrease in the turbidity and the

Plate 2. Top: Growth of *Ps. aeruginosa* (plaque strain) and *Ps. aeruginosa* (plaque-free strain) on a salts agar medium containing heat killed *Ps. aeruginosa* (plaque strain) whole cells as the sole source of nutriment. The plaque-free strain grew very poorly and there was a complete absence of clearing around the growth. This contrasted directly with the plaque strain (top). The cleared area shows up darkly on the photograph because it was made as a direct contact print of the plate.

Bottom: Growth of *Ps. aeruginosa* (plaque and plaque-free strains) on salts agar medium containing a dense suspension of *Ps. aeruginosa* cell walls as the sole source of nutriment. The plaque strain (top) grew well producing a large zone of clearing in the agar. The plaque-free strain (bottom) produced little growth and no area of clearing appeared even after seven days incubation.





viability of the culture in the presence and the absence of the autolysin (three mg. per ml.) was recorded in Fig. 24.
Both the total and the viable populations decreased rapidly in the presence of the crude enzyme, so that, after 60 minutes, only 20% of the cells were still intact. The turbidity of the control suspension containing phosphate buffer in place of the enzyme preparation decreased by only 10% over the same period. The rate of lysis of the enzyme suspension decreased after the first hour, so that by four hours, ten per cent of the cells were still intact. However, during this period the viability fell to one per cent. The close similarity between the viable and the total curves suggested that death of the cells may have been due to cell lysis rather than to the presence of a distinct bacteriocidal agent in the crude enzyme concentrate. Thus it appeared that the ageing cultures of *Ps. aeruginosa* (plaque strain) produced at least one factor able to bring about the lysis of living *Ps. aeruginosa* cells.

Versene was found to greatly increase the rate of lysis of the resting *Ps. aeruginosa* cells by the enzyme concentrate (Fig. 25). The turbidity decreased by 60% within two minutes, after which the curve flattened out. The controls containing versene alone, or versene and boiled enzyme showed a decrease of about 20% over the same period of time, while the cells to which only Tris buffer had been added showed very little change in turbidity. The crude enzyme concentrate was almost as active for the versene treated plaque-free strain cells as it was for those of the homologous strain. The extract was almost twice as active against young cells (24 to 72 hours) as against the old ones (192 hours), as can be seen from the histogram in Fig. 26.

Fig. 24. The lytic and lethal effects of the concentrated autolysin produced from ageing Ps. aeruginosa (plaque strain) cultures on resting cell suspensions of the homologous organism.

1. Total cell changes in absence of the enzyme preparation.

2. Viable cell changes in absence of the enzyme preparation.

3. Total cell changes in the presence of the enzyme preparation.

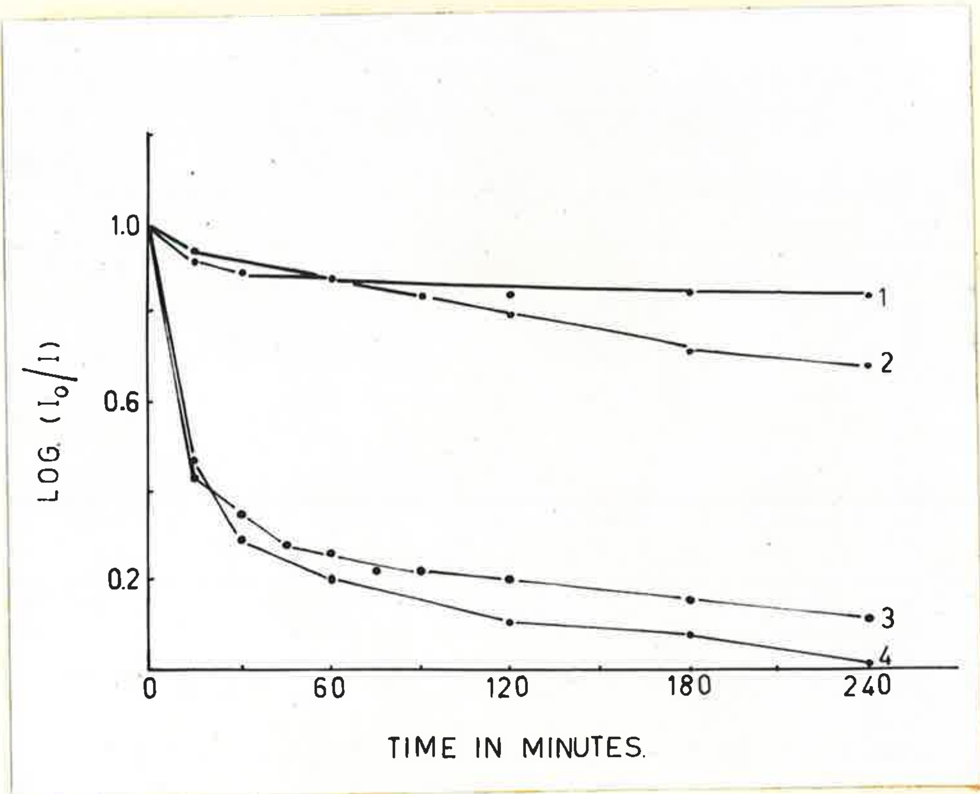
4. Viable cell changes in the presence of the enzyme preparation.

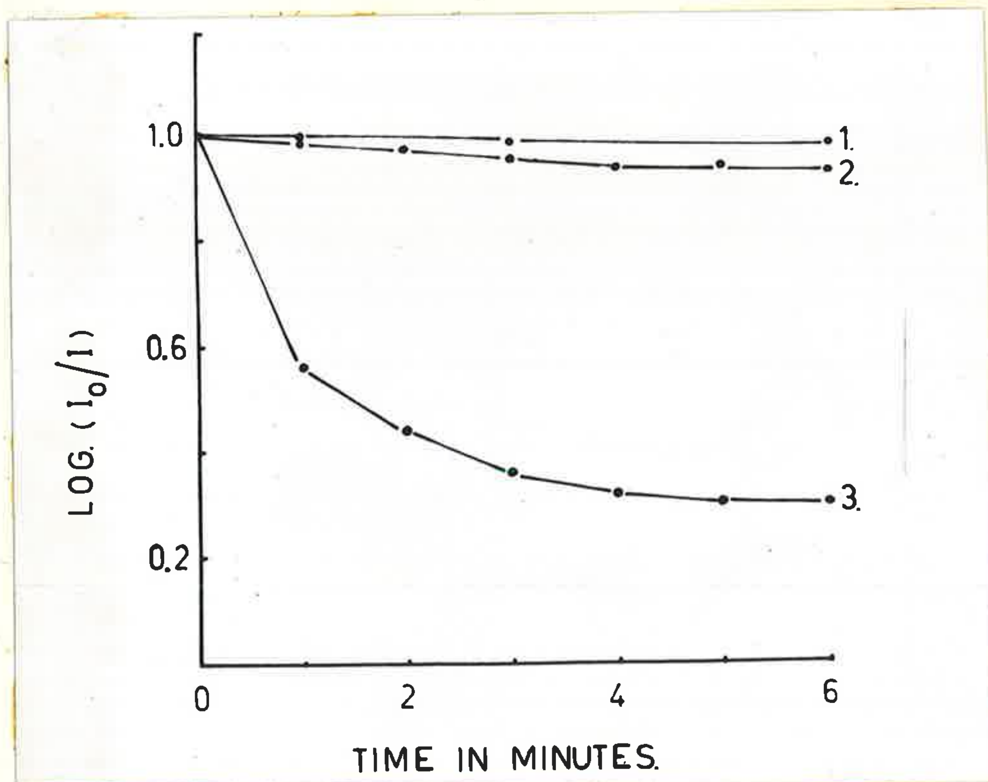
Fig. 25. Lysis curve obtained when a resting suspension of Ps. aeruginosa (plaque strain) cells were incubated with autolysin concentrate (3.0 mg. per ml.) in the presence of 0.01 M. Tris buffer (pH 8.0) and versene (100 µg. per ml.).

1. Control Tris buffer only.

2. Versene and Tris buffer.

3. Autolysin, versene and Tris buffer.


1.0
0.6
0.2
LOG. (I_0/I)
1
2
3
4
0
60
120
180
240
TIME IN MINUTES.


1.0
0.6
0.2
LOG. (I_0/I)
1.
2.
3.
0
2
4
6
TIME IN MINUTES.

The crude enzyme concentrate was also tested against a dense suspension of M. lysodeikticus cells using crystalline egg white lysozyme (100 µg.) as a control. The enzyme concentrate exhibited little or no lytic activity against the Micrococcus, demonstrating that the Ps. aeruginosa enzyme preparation was not likely to contain a bacterial lysozyme. The M. lysodeikticus suspensions was sensitive to the egg white lysozyme, however.

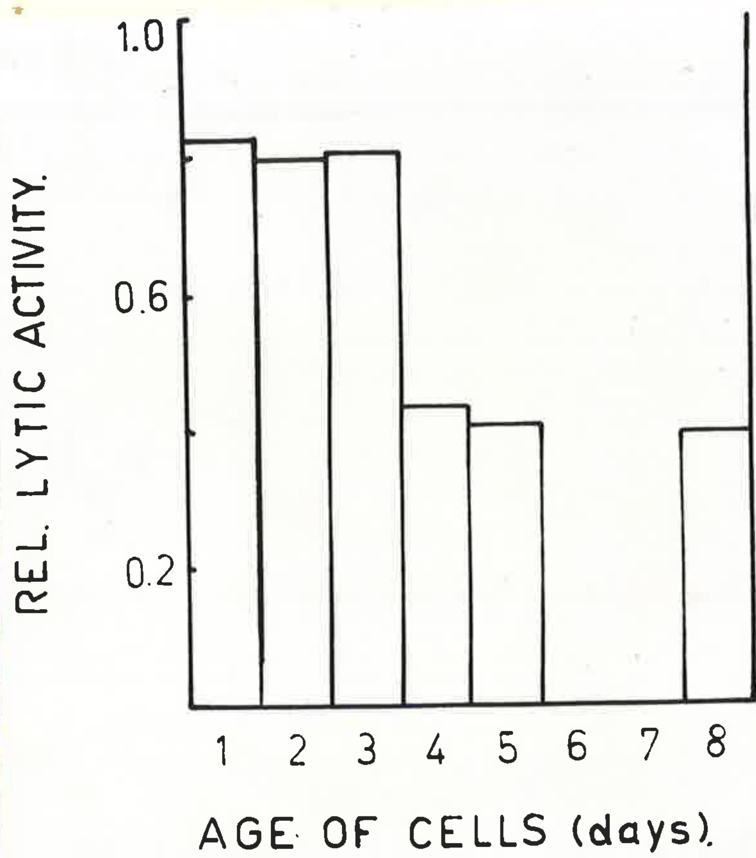
Tests were carried out to determine the relative rate of accumulation of the lytic enzyme in aerobic and anaerobic ageing cultures of Ps. aeruginosa. The relative lytic activities of dialysed extracts prepared from 24, 72 and 120 hour aerobic cultures and also 72, 120 and 192 hour anaerobic cultures were recorded in Fig. 27. The lytic activity of the aerobic culture preparations reached a maximum at 72 hours, whereas the anaerobic culture was inactive until 120 hours, after which it increased slowly. The appearance of the lytic enzymes in the anaerobic culture medium could be correlated with the commencement of cell lysis. The presence of lytic enzymes in the aerobic media explained the appearance of large numbers of cell "ghosts" in electron micrographs of stationary phase broth cultures (Plate 3). The absence of detectable amounts of enzyme in the anaerobic cultures was similarly confirmed by the absence of large numbers of cell ghosts until some 120 hours or more had elapsed (Plate 4). No lytic activity could be demonstrated at any time in the crude extracts of the aerobic or anaerobic cultures of the plaque-free strain of Ps. aeruginosa. This absence of enzyme activity correlated well with the previously observed absence of lysis in the ageing cultures of this organism

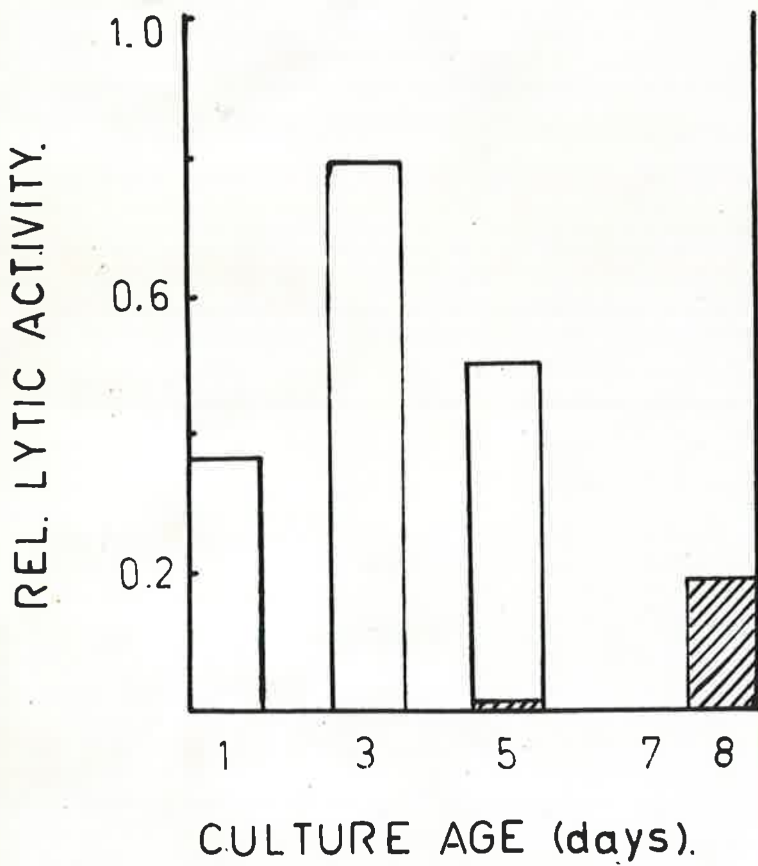
Fig. 26. Histogram of the relative sensitivity of ageing *Ps. aeruginosa* whole cells to the lytic action of the crude autolysin concentrate prepared from a five day broth culture of the plaque forming strain.

Fig. 27. Histogram of the effect of ageing under aerobic and anaerobic conditions on the relative rate of release of autolytic enzymes into the medium.

Aerobic cultures.

Anaerobic cultures.


REL. LYTIC ACTIVITY.
1.0
0.6
0.2
1
2
3
4
5
6
7
8
AGE OF CELLS (days).


REL. LYTIC ACTIVITY.
1.0
0.6
0.2
1
3
5
7
8
CULTURE AGE (days).

(Figs. 6 and 7).

The autolysin concentrate reduced the opacity of a plaque strain cell wall suspension by 15% over a 60 minute period. Over the same time period lysozyme (100 µg.) was almost twice as effective. "Protoplasts" of Ps. aeruginosa were slowly lysed by the autolysin irrespective of whether lysozyme or glycine had been used in their preparation. Over a 120 minute period, the opacity of the "protoplast" suspensions were reduced by about 90%. The control suspensions containing boiled enzyme were not affected. Addition of the crude enzyme to a sucrose stabilised suspension of whole cells resulted in a 40% reduction in the turbidity of the preparation within 90 minutes. Examination of the suspension under phase contrast illumination did not show the presence of "protoplasts" in the suspension. Attempts to demonstrate "protoplasts" in ageing aerobic or anaerobic nitrate broth cultures of Ps. aeruginosa (stabilised with 5%, 10%, 15%, or 20% sucrose) were unsuccessful. The turbidity of the sucrose stabilised cultures decreased by 50% to 70% over the eight day period of the experiment, but examination of the cultures by phase contrast microscopy failed to demonstrate any structures resembling "protoplasts". The crude enzyme concentrate apparently hydrolysed both the cell wall and the cytoplasmic membrane, thus resulting in the complete lysis of the cell, even in osmotically stabilised media.

Finally, attempts were made to determine whether more than one enzyme was present in the autolysin concentrate. A number of possible substrates were tested against the crude enzyme preparation, and the results of these tests will now be considered.

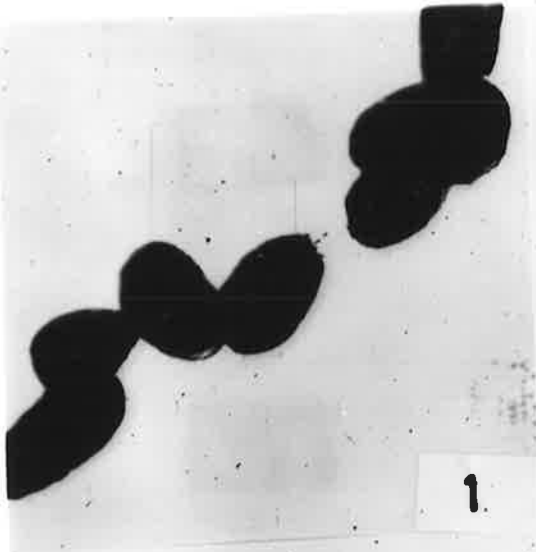
Plate 3. Electron micrographs of *Ps. aeruginosa* and *S. bethesda* whole cells shadowed with palladium.

1. *Ps. aeruginosa* (plaque strain) cells after 24 hours anaerobic incubation at 37°C. X 20,000.

2. *Ps. aeruginosa* (plaque strain) cells after 24 hours aerobic incubation at 37°C. X 20,000. Note the presence of many empty "ghosts" together with a number of electron dense whole cells. Older cultures showed essentially the same picture.

3. *Ps. aeruginosa* (plaque-free) cells after 48 hours anaerobic incubation at 37°C. X 20,000.

4. *S. bethesda* cells after 48 hours anaerobic incubation at 37°C. X 30,000.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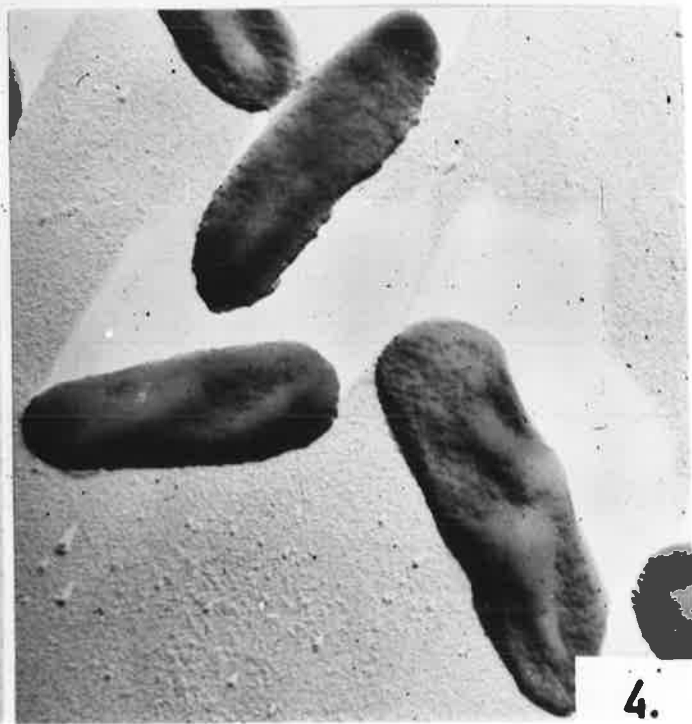
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6. Activity of the Enzyme Concentrates against Known Substrates.

a. Mucinase activity. The crude enzyme preparation was found to contain an active mucinase which rapidly hydrolysed a standard preparation of ovomucin. The results of the test were recorded in Table 5. The enzyme preparation obtained from the 72 hour aerobic culture (80 µg. of protein per ml.) completely hydrolysed the standard ovomucin solution in 60 minutes, whereas the corresponding preparation obtained from a plaque-free culture was completely inactive, even when 600 µg. of protein per ml. were present. The anaerobic plaque strain culture was not as active as the aerobic culture, but definite activity was detected, thus supporting the earlier findings concerning the lytic ability of these preparations against whole cells.

b. Proteinase activity. Only slight proteinase activity was detected in the plaque strain enzyme concentrates, and the plaque-free preparations were completely inactive.

c. Nuclease activity. No RNase or DNase activity could be demonstrated in the enzyme concentrates of either strain of *Ps. aeruginosa*.

d. Esterase activity.

(1) Acetyl cholinase. The enzyme concentrates showed no detectable activity against the substrate.

(2) Lethicinase. *Ps. aeruginosa* (plaque strain) produced a large diffuse zone of white precipitate around the colonies on agar containing 20% human serum. The plaque-free strain did not produce any corresponding change in the medium.

When the plaque strain enzyme concentrate was incubated with the

Plate 4. Electron micrographs of *Ps. aeruginosa* (plaque strain) whole cells shadowed with palladium.

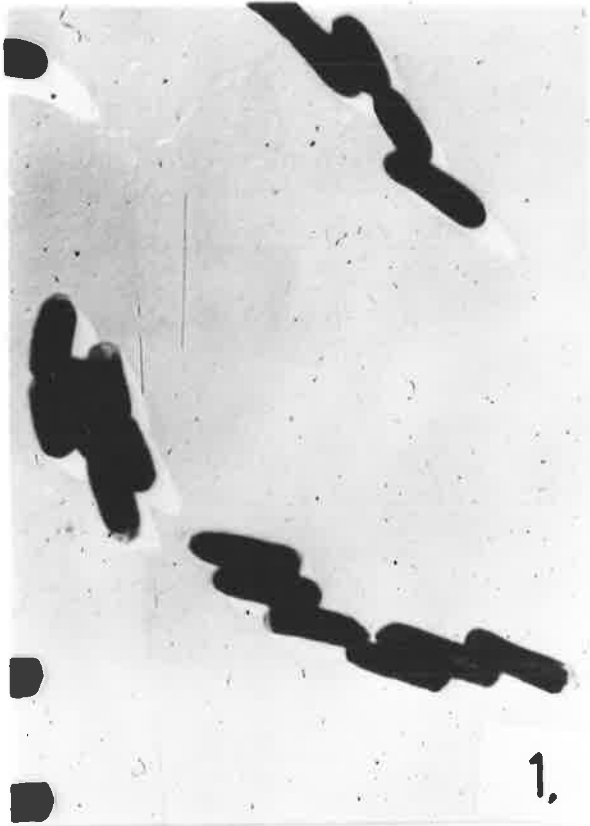
1. 24 hours anaerobic incubation at 37°C. X 10,000.

2. 72 hours anaerobic incubation at 37°C. X 10,000.

3a. 96 hours anaerobic incubation at 37°C. Note the extrusion of cellular contents and the continued presence of electron dense granules within the cell X 18,000.

3b. 120 hours anaerobic incubation at 37°C.

4. 192 hours anaerobic incubation at 37°C. X 35,000.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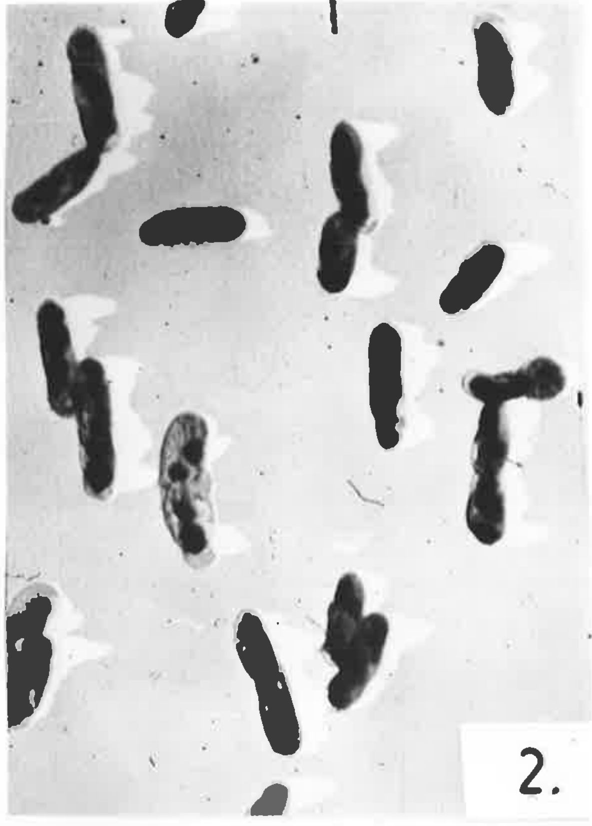
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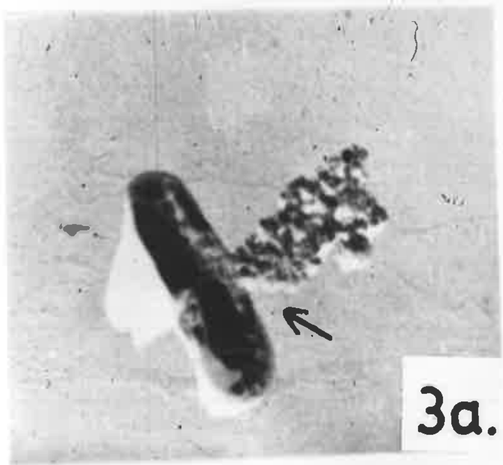
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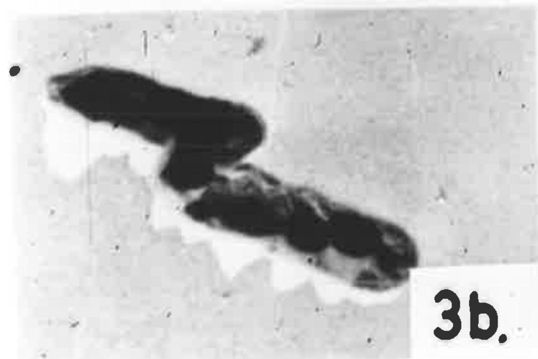
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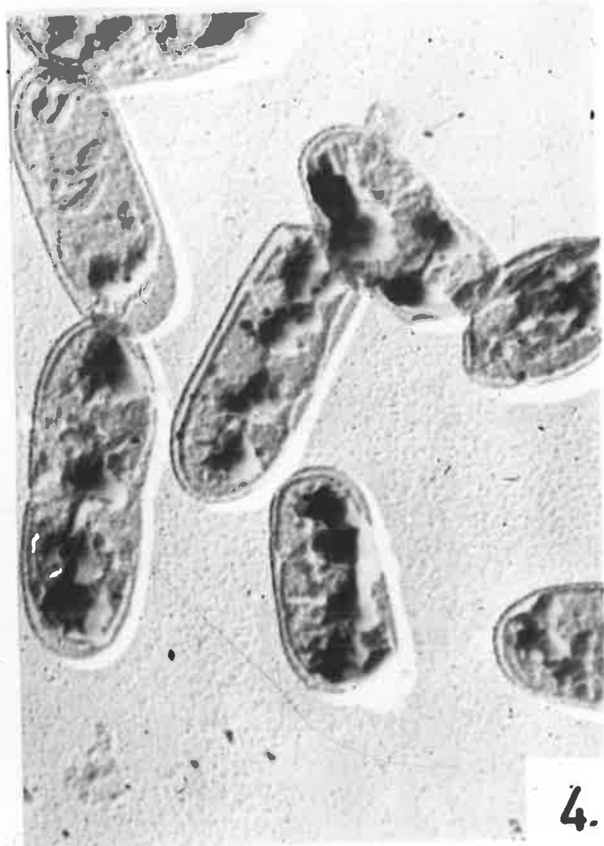
4.

heat inactivated serum the lethicin content was reduced from 230 µg. to 100 µg. while the lethicin content of the heated controls did not change appreciably. At the completion of the test, the remaining lethicin was precipitated prior to its quantitative estimation. The preparation incubated with the enzyme concentrate contained 20 µg. of free choline. The supernatant fluid from the boiled enzyme controls and the plaque-free enzyme concentrate did not contain detectable quantities of free choline. Thus the lytic activity of the concentrates from the ageing culture media could be correlated with the presence or absence of mucinase and esterase activity in the preparations. The presence of these enzymes in the medium does not necessarily mean that the mucinase and the esterase are specifically concerned with the induction of cellular lysis in the plaque strain cultures of _Ps. aeruginosa_. However, examination of the changes in the chemical composition of the cell walls of _Ps. aeruginosa_ following treatment with a preparation of _Ps. aeruginosa_ autolysin (_vide_ Chapter IX) strengthened the suggestion that these enzymes were at least partially involved in the initiation of cellular lysis of _Ps. aeruginosa_.

Conclusions to Chapters IV, V, VI.

The experiments detailed so far in this thesis have demonstrated that extensive variations may occur in the characteristic growth, death and subsequent lysis of different bacterial species. Thus, the process of ageing in the _Ps. aeruginosa_ cultures differed extensively from that observed with cultures of _S. bethesda_ cultivated under similar conditions. By appropriate manipulations it was possible to isolate a mutant strain of _Ps. aeruginosa_ which was still subject to the lethal effects of prolonged anaerobic incubation but not to the later autolytic changes.

Mucinase activity of crude enzyme obtained from aged liquid cultures of *Ps. aeruginosa*.

Preparation	1/5	1/10	1/20	1/40	1/80	1/160	Enzyme control	Ovomucin control
Ps. plaque 72 hr. aerobic	+	+	+	+	±	-	+	-
Ps. plaque 96 hr. aerobic	+	+	+	+	±	-	+	-
Ps. plaque 120 hr. anaerobic	+	±	-	-	-	-	+	-
Ps. plaque 192 hr. anaerobic	+	+	±	±	-	-	+	-
Ps. plaque-free 72 hr. aerobic	-	-	-	-	-	-	+	-
Ps. plaque-free 192 hr. anaerobic	-	-	-	-	-	-	+	-
S. bethesda 192 hr. anaerobic	-	-	-	-	-	-	+	-
V.cholerae mucinase x	+	+	+	+	+	+	+	-

+ = completely clear. No strands.

± = opalescence.

- = Dense opalescence and strands.

x By the courtesy of Dr. E.L. French, Melbourne.

Examination of the culture filtrates of the two strains demonstrated that the plaque strain was subject to lytic changes brought about by enzymes released into the culture medium. The mutation which resulted in the loss of the ability to produce the "irridescent" plaques was also associated with an inability of these cells to produce diffusible lytic enzymes. The mutant cells were lysed by the plaque strain autolysin so that the change from plaque to non-plaque producer was not due to a chemical change which rendered the cells completely resistant to autolytic digestion. Apparently the change was a more subtle one involving some alteration to the cellular metabolism which prevented the release of active autolytic enzymes into the medium. Thus, the present study indicated that a number of metabolic changes can occur during the process of ageing in liquid cultures. At present it is impossible to define these differences in precise chemical terms. However, the investigations which will be considered in Chapters VII, VIII and IX of this thesis have been made as a contribution to the chemistry of the Ps. aeruginosa cell in the hope that it may ultimately be of help in the understanding of some of the cellular changes associated with the ageing and lysis of this organism.

CHAPTER VII.

CHEMICAL CHANGES ASSOCIATED WITH AGEING: THE CRUDE CHEMICAL COMPOSITION OF PS. AERUGINOSA AND S. BETHESDA CELLS IN LIQUID CULTURES.

The growth experiments recorded in Chapter IV showed that extensive variations occurred in the rate of the decline and death of different bacterial cultures incubated in a nutrient medium for an extended period of time. Chemical assays were carried out on the ageing whole cells in an attempt to correlate any changes in composition with the induction of cellular death and lysis. The resistance of the _S. bethesda_ cells to the early death and extensive lytic changes in anaerobic cultures made this organism a suitable control for these investigations.

Because of the lack of detailed information regarding the chemical composition of _Ps. aeruginosa_ cells, it was necessary to begin this investigation with whole cells before any attempt could be made to study the composition of the cell walls of the ageing cells. The chemical assays described in this section have been divided into four parts dealing respectively with the protein, carbohydrate, lipid and nucleic acid composition of the two _Ps. aeruginosa_ strains and _S. bethesda_. The total nitrogen, total phosphorous and hexosamine contents of the cells were also determined, and the total recovery of cell substance estimated.

1. Total Nitrogen and Protein.

The amount of total nitrogen present in ageing cells of both of the _Ps. aeruginosa_ strains and of _S. bethesda_ varied from 11 to 13 mg.%. However, examination of the detailed figures in Table 6 did not reveal any obvious trends corresponding with the ageing of the cells. The results of the protein estimations varied considerably,

TABLE 6.

Total- N and protein composition of *Ps. aeruginosa* and *S. bethesda* whole cells in complex and defined media.

(mg. per 100 mg. dry weight of cells).

Age of cells	Medium	*Ps. aeruginosa* Plaque strain			*Ps. aeruginosa* Plaque-free strain			*S. bethesda*		
		Total- N	Folin- C[x]	Biuret	Total- N	Folin- C	Biuret	Total- N	Folin- C.	Biuret
				Aerobic cultures						
24	C.N.T.	11.4	72	68	11.2	67	65	-	-	-
24	Broth	12.7	65	56	11.4	71	63	11.3	65	54
96	Broth	11.8	68	65	13.0	79	68	12.2	71	60
24 (rough)	Broth	-	-	-	-	-	-	11.0	47	43
				Anaerobic cultures						
24	Broth	9.6	66	-	-	-	-	-	-	-
72	Broth	10.0	74	47	-	-	-	-	-	-
96	Broth	11.6	80	60	-	-	-	-	-	-
192	Broth	12.0	78	60	-	-	-	-	-	-
24	C.N.T.	11.4	60	52	11.8	67	60	-	-	-
48	C.N.T.	11.6	68	60	11.0	82	74	12.4.	73	64
64	C.N.T.	11.6	71	52	-	-	-	-	-	-

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TABLE 6 (cont'd)

Age of cells	Medium	*Ps. aeruginosa* Plaque strain			*Ps. aeruginosa* Plaque-free strain			*S. bethesda*		
		Total- N	Folin- C[x]	Biuret	Total- N	Folin- C	Biuret	Total- N	Folin- C	Biuret
96	C.N.T.	11.6	69	62	9.0	83	70	11.6	73	66
120	C.N.T.	-	-	-	11.0	89	73	-	-	-
192	C.N.T.	13.0	80	75	11.5	94	73	10.0	72	61

x Folin- C = Folin Ciocalteau reagent.

- = not done.

depending on the reagent used, but, irrespective of the medium,the young *Ps. aeruginosa* cells contained between 50% and 60% of their dry weight in the form of protein. The Biuret estimations were consistently 10 to 15 mg.% lower than the corresponding Folin-Ciocalteau figures and an average of the two was taken as the best protein estimation available. These figures were recorded in Tables 9 and 10. The young *S. bethesda* cells contained somewhat more protein (65%) than the two *Ps. aeruginosa* strains.

As the cells aged, so the protein content of the *Ps. aeruginosa* plaque strain cells slowly increased, until after 192 hours it accounted for almost 80% of the dry weight of the cell. The plaque-free cells also increased slowly in their protein content. On the other hand, the protein content of the *S. bethesda* cells did not alter to any appreciable extent as the cells aged. However, when the Salmonella became antigenically rough there was a sharp increase in the protein content of the cells, although no corresponding increase in total nitrogen could be detected (Table 6).

Ps. aeruginosa cellular proteins contained between 15.1% and 15.5% nitrogen, while the corresponding figure for *S. bethesda* was 15.5%. Thus between 80% and 90% of the total nitrogen of the cell was accounted for as protein-N. As the cells aged, the non-protein nitrogen content of the cells slowly declined, until after 192 hours, almost 100% of the total nitrogen was accounted for as protein. Less than one per cent of the total nitrogen of the young cells was present as free ammonia, while the remainder of the non-protein nitrogen was accounted for as nucleic acids and amino sugars.

2. Total Carbohydrate.

The figures in Table 7 demonstrated that considerable variations in the estimates of the carbohydrate content could be obtained when different assay methods were used. For this reason, routine determinations were made by the Anthrone as well as by the total reducing sugar methods, and the two extreme values for the cellular carbohydrate composition were recorded in Table 8. The total hexosamine content of the cells after culturing for 24 hours and 192 hours was also determined. The total carbohydrate content of the ageing cells decreased slightly over the eight day period of the experiment, irrespective of the assay method used.

The aerobic cells usually contained slightly less carbohydrate than the corresponding anaerobic cultures, but it was doubtful if this difference had any real significance for the present problem. The reducing sugar content of the Ps. aeruginosa strains varied from 5% to 8% of the dry weight, but the corresponding figures for the S. bethesda cells were somewhat higher (12% to 15%). The difference in the cellular carbohydrate content of the two organisms could be partly explained in terms of the difference in the antigenic smoothness of the Salmonella cells as compared with that of the Ps. aeruginosa strains. The loss of the smooth "O" antigens in the S. bethesda cell wall should result in a decreased carbohydrate content in the cell. The carbohydrate content of a rough variant of S. bethesda was only two mg.% when estimated by the Anthrone reagent, while the total reducing sugars accounted for 6.3% of the dry weight of the cells (Table 8). Thus the carbohydrate content of the rough S. bethesda cells closely resembled that found in the partially rough Ps. aeruginosa strains. Attempts to smooth the

TABLE 7.

Carbohydrate composition of *Ps. aeruginosa* (plaque strain) cells and cell walls estimated by four methods using a glucose standard.

	Ps. aeruginosa (plaque strain) 24 hours.	
	Whole cells.	Cell walls.
Anthrone reagent	3.2 mg.% x	4.0 mg.%
Dische reagent	3.3 mg.%	4.2mg.%
Orcinol reagent	5.7 mg.%	5.5 mg.%
Total reducing sugar	7.5 mg.%	7.0 mg.%

x mean of two or three replicates.

TABLE [illegible].

Carbohydrate composition of *Ps. aeruginosa* and *S. bethesda* whole cells in complex and defined media.

(mg. per 100 mg. dry weight of cells).

Age of cells	Medium	*Ps. aeruginosa* plaque strain			*Ps. aeruginosa* plaque-free strain			*S. bethesda*		
		Anthrone	Reducing*	Hexosamine	Anthrone	Reducing	Hexosamine	Anthrone	Reducing	Hexos.
					Aerated cultures					
24	Broth	2.2	5.2	-	1.8	5.4	0.7	8.0	12.1	0.8
96	Broth	2.3	2.9	1.3	1.5	5.5	0.6	5.8	8.4	0.5
24 (rough)	Broth	-	-	-	-	-	-	1.6	6.3	0.6
24	C.N.T.	3.4	6.0	-	2.0	5.2	-	-	-	-
					Anaerobic cultures					
24	Broth	3.6	9.0	1.0	-	-	-	-	-	-
72	Broth	4.0	-	-	-	-	-	-	-	-
96	Broth	3.8	-	-	-	-	-	-	-	-
192	Broth	3.0	8.1	0.8	-	-	-	-	-	-
24	C.N.T.	3.0	7.8	0.9	1.6	6.1	0.6	-	-	-
48	C.N.T.	3.2	-	-	2.5	-	-	4.8	14.5	1.3
64	C.N.T.	2.4	-	-	-	-	-	-	-	-

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TABLE 8 (cont'd)

Age of cells	Medium	*Ps. aeruginosa* plaque strain			*Ps. aeruginosa* plaque-free strain			*S. bethesda*		
		Anthrone	Reducing x	Hexosamine	Anthrone	Reducing	Hexosamine	Anthrone	Reducing	Hexos.
96	C.N.T.	2.2	-	-	2.4	-	-	4.4	-	1.2
120	C.N.T.	-	-	-	2.2	-	-	-	-	-
192	C.N.T.	2.1	7.0	0.5	2.6	6.3	0.6	4.5	12.0	-

x Reducing = total reducing sugar.

- = not done.

two _Ps. aeruginosa_ strains by serial passage through white mice were only partially successful, and the total carbohydrate content of the cells was not greatly increased by 10 such passages.

The hexosamine content of the three strains was approximately one mg.% (Table 8), and, except for the 45% decrease recorded by the anaerobic culture of _Ps. aeruginosa_ (plaque strain), did not vary greatly as the cells aged. The overall carbohydrate content of the strains was thus not greatly affected by the ageing.

3. _Total Lipid._

The three present strains contained considerable quantities of lipid. About half of the total lipid (four to five mg. per cent) could be extracted with cold ether (Tables 9 and 10). Hydrolysis of the cells with 6N. HCl at 100°C. then released a further three to six per cent lipid. The total lipid content of the anaerobic cultures of _Ps. aeruginosa_ decreased slowly as the cells aged. This was in contrast to a slight increase found in the ageing _S. bethesda_ cultures (Table 10). The free lipid content of all three strains remained constant throughout the experiment, irrespective of the presence of aerobic or anaerobic conditions. The smooth to rough variation in _S. bethesda_ cells was not accompanied by any detectable change in the free or total lipid content of the cells.

4. _Total Phosphorous and Nucleic Acids._

a. _The total phosphorous_ content of aerobically grown cells varied between one and two mg.% (Table 9) while the corresponding figures for the anaerobic cells varied between two and three mg.% (Table 10). The phosphorous content of cells grown in digest broth (high phosphate content) did not differ significantly from those

TABLE 9.

Composition of whole cells of Ps. aeruginosa and S. bethesda grown in complex and defined media under aerated conditions. (mg. per 100 mg. dry weight of cells).

Age of cells	Strain	Medium	Protein (aver.)	Total- P	Nucleic acid RNA	Nucleic acid DNA	Lipid Free	Lipid Total	Carbohydrate[x]	Total %
24	Ps.plaque	Broth	60	1.8	15.0	4.5	-	8.3	5.2	95
96	Ps.plaque	Broth	66	-	3.9	2.3	-	5.8	2.9	83
24	Ps.plaque	CNT	70	2.6	4.6	3.0	5.4	6.4	6.0	93
24	Ps.pl-free	Broth	67	1.6	10.5	4.1	-	6.2	5.4	95
96	Ps.pl-free	Broth	73	1.6	3.0	1.7	-	6.8	5.5	92
24	Ps.pl-free	CNT	66	1.8	3.0	1.8	-	-	-	-
24	S.bethesda	Broth	59	2.0	10.8	2.8	3.7	5.4	12.1	92
96	S.bethesda	Broth	65	1.1	3.5	2.9	-	4.0	6.4	83
24R	S.bethesda	Broth	45	2.4	14.8	3.3	4.0	5.6	6.3	77

x Estimated as total reducing sugar.

- = not done.

Ps. plaque = Ps. aeruginosa (plaque strain).

Ps. plaue-free = Ps. aeruginosa (plaque-free strain).

Composition of whole cells of *Ps. aeruginosa* and *S. bethesda* grown in complex and defined media under anaerobic conditions (mg. per 100 mg. dry weight of cells).

Age of cells	Strain	Medium	Protein (aver.)	Total-P.	Nucleic acid RNA	DNA	Lipid Free	Total	Carbohydrate[x]	Total %
24	Ps.plaque	Broth	-	2.8	14.9	4.1	3.6	10.0	9.0	-
72	Ps.plaque	Broth	60	2.4	1.1	1.9	3.2	9.8	-	87
96	Ps.plaque	Broth	70	2.6	1.3	2.0	-	8.4	-	92
192	Ps.plaque	Broth	69	2.4	1.8	2.2	4.0	8.0	8.1	91
24	Ps.plaque	CNT	56	2.2	10.0	4.0	4.6	7.0	7.8	87
48	Ps.plaque	CNT	64	3.0	3.1	1.9	5.2	7.4	-	87
64	Ps.plaque	CNT	61	2.4	1.5	1.5	-	6.8	-	80
96	Ps.plaque	CNT	65	2.6	1.1	1.4	-	5.0	-	82
192	Ps.plaque	CNT	77	2.3	1.6	1.4	4.2	4.8	7.0	94
24	Ps.pl-free	CNT	63	2.1	8.6	3.3	5.0	8.1	6.1	91
48	Ps.pl-free	CNT	78	2.5	2.1	2.4	-	-	-	-
96	Ps.pl-free	CNT	76	1.8	0.6	0.6	-	8.0	-	93
120	Ps.pl-free	CNT	81	1.8	0.3	1.5	-	-	-	-
192	Ps.pl-free	CNT	83	2.2	0.5	1.5	4.7	6.8	6.3	100

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TABLE 10 (cont'd)

Age of cells	Strain	Medium	Protein (aver.)	Total-P	Nucleic acid RNA	Nucleic acid DNA	Lipid Free	Lipid Total	Carbohydrate*	Total %
48	*S.bethesda*	CNT	69	2.2	4.9	2.9	3.9	5.9	14.5	99
96	*S.bethesda*	CNT	69	2.0	4.3	2.7	-	6.3	-	-
192	*S.bethesda*	CNT	67	1.9	2.8	2.8	4.5	8.8	12.0	95

* Estimated as total reducing sugar.

- = not done.

Ps. plaque = *Ps. aeruginosa* (plaque strain).

Ps. pl.-free = *Ps. aeruginosa* (plaque-free strain).

grown in CNT medium (no inorganic phosphate). The phosphate content of the three strains was not affected by the process of ageing.

b. The total nucleic acid content of the three strains varied considerably during the growth cycle. During the logarithmic growth phase Ps. aeruginosa cells contained up to 20 mg.% nucleic acid, of which about one third was DNA. The mutation to the plaque-free strains was not accompanied by a significant alteration in the cellular nucleic acid content. However, the change in Ps. aeruginosa from smooth to rough was accompanied by an increase in the RNA content of the cells (Table 9). The nucleic acid phosphorous accounted for about 80% of the total phosphorous in the young cells. However, the rapid decrease in RNA-P associated with the ageing of the cells was not accompanied by any corresponding decrease in the total phosphorous content. Apparently the ageing cells conserved the phosphorous which was therefore not lost by diffusion into the medium.

The RNA and DNA content of replicate 24 hour old cell samples varied considerably in different experiments (Tables 9 and 10). These variations were at times considerable and could not be explained merely as experimental error. This led to a more detailed investigation of the nucleic acid content of ageing Ps. aeruginosa and S. bethesda cells. The RNA and DNA content of the aerobically and anaerobically incubated Ps. aeruginosa cells increased linearly to a maximum between 7 and 10 hours. This peak coincided with the cessation of logarithmic growth in the cultures. Over this period, the total nucleic acid content of the cells accounted for about 20% of the dry weight of the cells (See Table 11). The amount of residual RNA and DNA in the aerated cells after 192 hour's incubation (47% and 65% respectively) agreed

TABLE 11.

The nucleic acid content of *Ps. aeruginosa* (plaque strain) cells grown under aerobic and anaerobic conditions.

Age of cells	Aerobic culture				Anaerobic culture			
	Viable ¤ $\times 10^6$	Total ¤ $\times 10^6$	RNA-P. mg.P.per 100 mg.cells	DNA-P. mg.P.per 100 mg.cells	Viable $\times 10^6$	Total $\times 10^6$	RNA-P. mg.P.per 100 mg.cells	DNA-P mg.P.per 100 mg.cells
0	300	-	-	-	300	-	-	-
2	324	2,000	0.88	0.22	342	690	1.06	0.23
4	1,430	2,230	0.96	0.23	546	1,100	1.26	0.26
6	11,100	12,230	1.00	0.42	2,130	4,660	0.07	0.40
7½	14,800	13,430	1.20	0.48	4,160	7,130	1.64	0.50
10	15,700	14,090	0.95	0.42	7,060	4,800	0.41	0.56
24	14,000	14,090	0.62	0.43	5,480	7,350	0.21	0.29
48	3,430	13,340	0.62	0.39	229	4,880	0.12	0.22
96	-	-	-	-	0.5	1,950	0.13	0.38
120	600	10,600	0.58	0.48	1.4	1,450	-	0.17
192	170	8,600	0.56	0.32	1.7	521	0.13	0.12

¤ Number of cells $\times 10^6$ per ml. of culture.

- = not done.

fairly closely with the figure of 61% obtained for the total number of surviving whole cells at this time. The intact ageing cells apparently still contained most of their original DNA but their RNA content declined slowly. The aerobic cultures continued to multiply slowly throughout the decline phase so that a proportion of the cells contained a considerable amount of RNA as well as DNA. Electron micrographs of aerobically grown Ps. aeruginosa cultures showed the presence of many electron dense, actively dividing cells, together with a number of empty "ghosts" (Plate 3). This picture contrasted sharply with the anaerobic cultures in which few "ghosts" were visible until much later in the growth cycle (Plate 4). Ageing of the aerobic cultures did not result in any obvious changes in the proportion of intact cells and "ghosts" compared with the 24 hour old cells. Thus, even after 192 hour's incubation, numerous electron dense cells of normal appearance were still observed to be present and no obvious morphological differences could be detected between the young and old aerobic cells in the preparations.

The total nucleic acid content of the anaerobic cells decreased so that by 192 hours only 12% of the 10 hour peak remained (Table 11). The RNA content of the decline phase cells decreased sharply so that by 192 hours only 8% remained. The DNA content decreased by 80% over the same period. The total cell count decreased to seven per cent of the 10 hour maximum and this suggested that some of the lysed cells retained portion of their DNA within the cell envelope. Electron micrographs made from ageing anaerobic Ps. aeruginosa cells showed that very few electron dense cells were still present after 192 hour's incubation. Many of the cells contained one or more electron

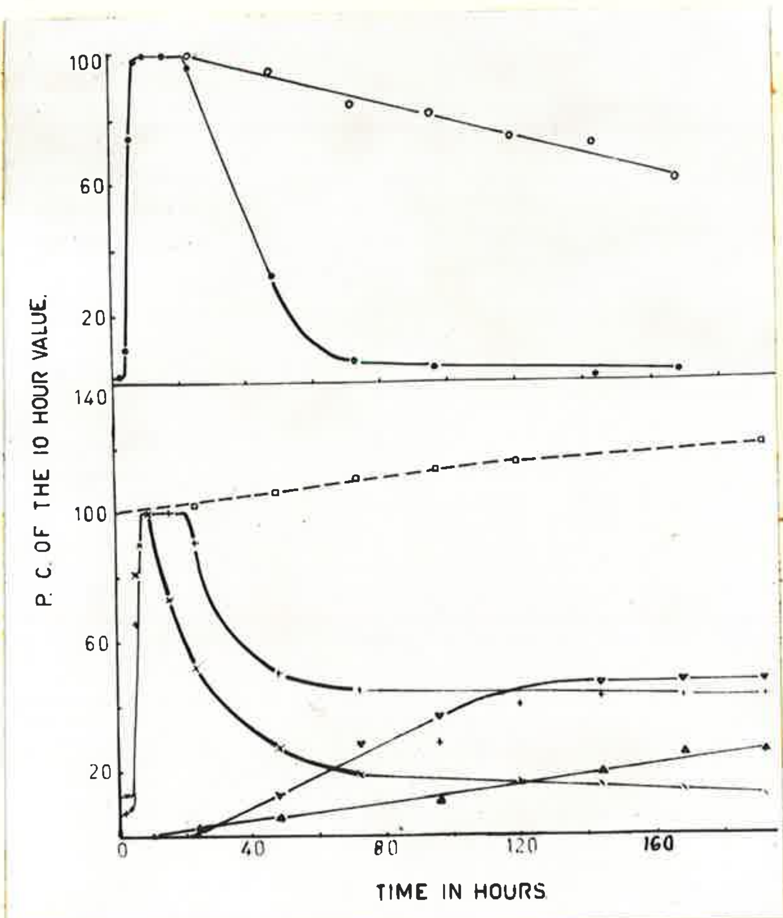
dense granules, often at the poles (Plate 4). The position and size of the granules was similar to that of the bacterial nuclei following staining of the cells by the conventional nuclear staining techniques (Knaysi, 1951). Thus, the ageing and lysing bacterial cells appeared to retain some granular material within the envelope and this may explain the retention of 20% of the intact DNA by the 192 hour old bacterial cells. When the RNA and DNA content of the cells was expressed in terms of a 100 ml. aliquot of the original culture (rather than per 100 mg. dry weight of cells) the DNA curves were found to closely approximate to the total counts (Figs. 28 and 29). The RNA curve fell rapidly in both aerobic and anaerobic cultures although the continued growth of part of the aerated population probably reduced the rate of fall somewhat compared with that observed in the anaerobic medium. The DNA content of the _Ps. aeruginosa_ cells also decreased with age and the rate of decline was almost parallel to the decrease in total cell numbers. Thus, the intact _Ps. aeruginosa_ cells retained their DNA, but not their RNA throughout the growth cycle.

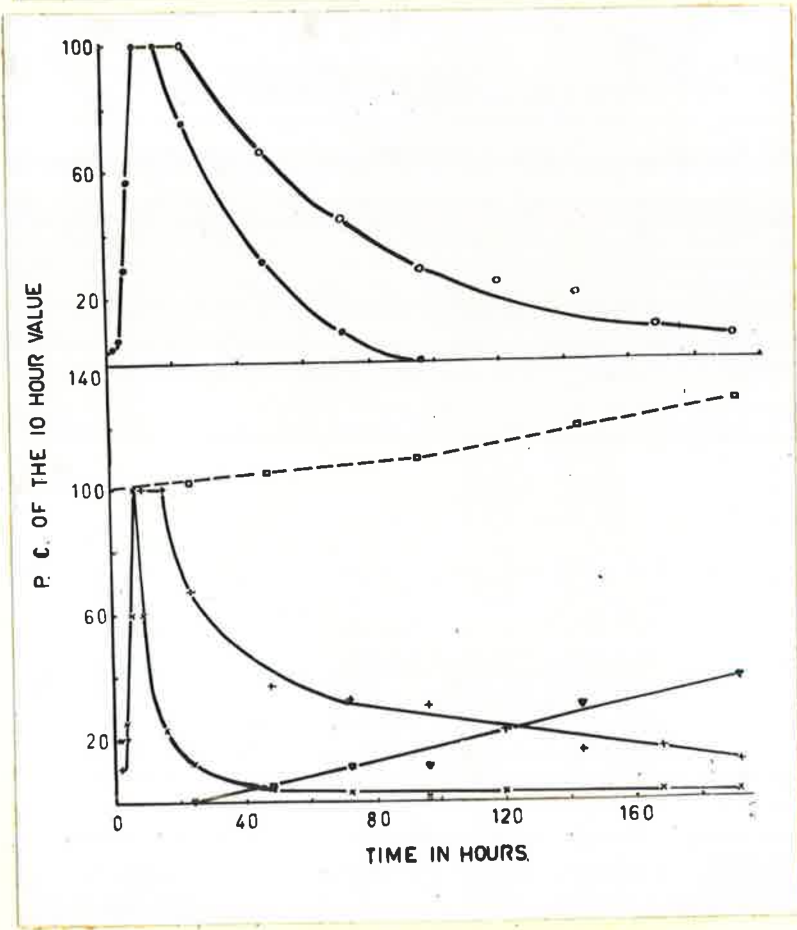
The absorbance at 260 mμ. by the cold three per cent TCA extract of young _Ps. aeruginosa_ and _S. bethesda_ cells demonstrated the presence of free nucleotides in these cells (Fig. 30). After 48 hour's incubation, little or no free nucleotide could be demonstrated in the TCA extracts of aerobically or anaerobically grown cells.

c. _Nucleic acid accumulation in the medium of ageing cultures_. The viscosity of the cell-free broth from both aerobic and anaerobic cultures of _Ps. aeruginosa_ increased as the cultures aged, so that by 192 hours, it was 20% to 25% higher than the sterile broth controls (Figs. 28 and 29). The changes in viscosity after treatment of the culture

Fig. 28. The nucleic acid content of the culture medium and of ageing cells of <u>Ps. aeruginosa</u> incubated in nitrate broth under aerobic conditions.

Fig. 29. The nucleic acid content of the culture medium and of ageing cells of <u>Ps. aeruginosa</u> incubated in nitrate broth under anaerobic conditions.


100
60
20
140
100
60
20
0
40
80
120
160
P. C. OF THE 10 HOUR VALUE.
TIME IN HOURS


100
60
20
140
100
60
20
0
40
80
120
160
P. C. OF THE 10 HOUR VALUE
TIME IN HOURS.

filtrates with six enzyme preparations were recorded in Fig. 31. The V. cholerae mucinase and the Streptomyces albus enzyme were without effect. The pancreatic enzyme and the hyaluronidase reduced the viscosity by 20% to 30%, but both preparations were shown later to contain traces of DNase. The RNase showed little activity, but DNase brought about an 80% decrease in viscosity in 60 minutes. Thus the increased viscosity of the old culture media was mainly due to the presence of extracellular DNA. This was confirmed when it was found that the broth strongly absorbed ultra-violet in the 260 to 270 mμ. range. The results of quantitative RNA and DNA assays carried out on TCA insoluble material obtained from culture filtrates were expressed as mg. per 100 ml. of original culture and were recorded in Figs. 28 and 29. The aerated culture medium contained the equivalent of 25% and 47% respectively of the initial cellular RNA and DNA. The nucleic acids could be detected in the medium only after cellular lysis became appreciable, however. The small amount of RNA in the aerated medium was probably due to the small amount of residual RNA still present in the cells by the time extensive lysis began. On the other hand, the DNA content of the cells was still amost 100% of the logarithmic phase maximum at the time lysis released the cell contents into the medium.

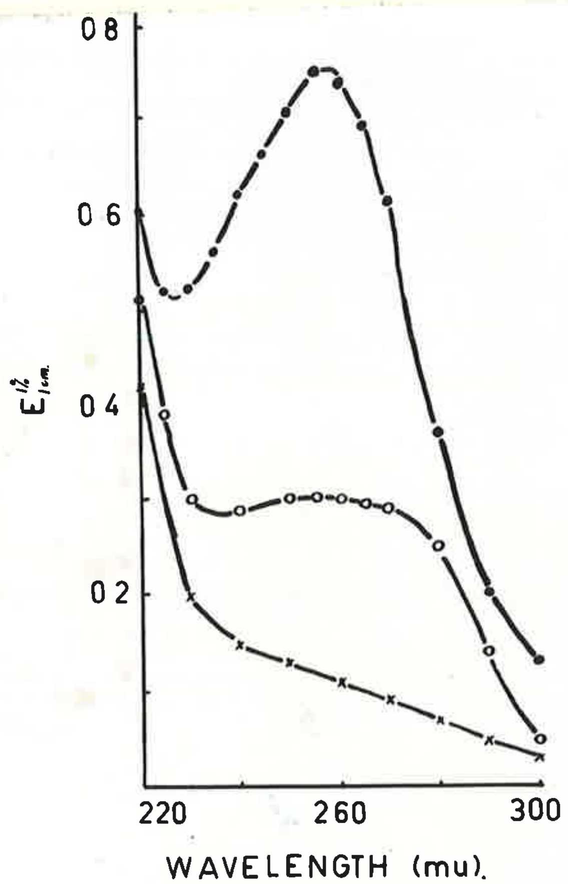
The ageing anaerobic culture medium contained DNA only, and all attempts to detect significant amounts of RNA were unsuccessful. The reason for the absence of RNA from the medium became clearer when it was shown that more than 90% of the intracellular RNA had been destroyed before cellular lysis could be detected. However, considerable amounts of DNA were still present when the cell contents were released

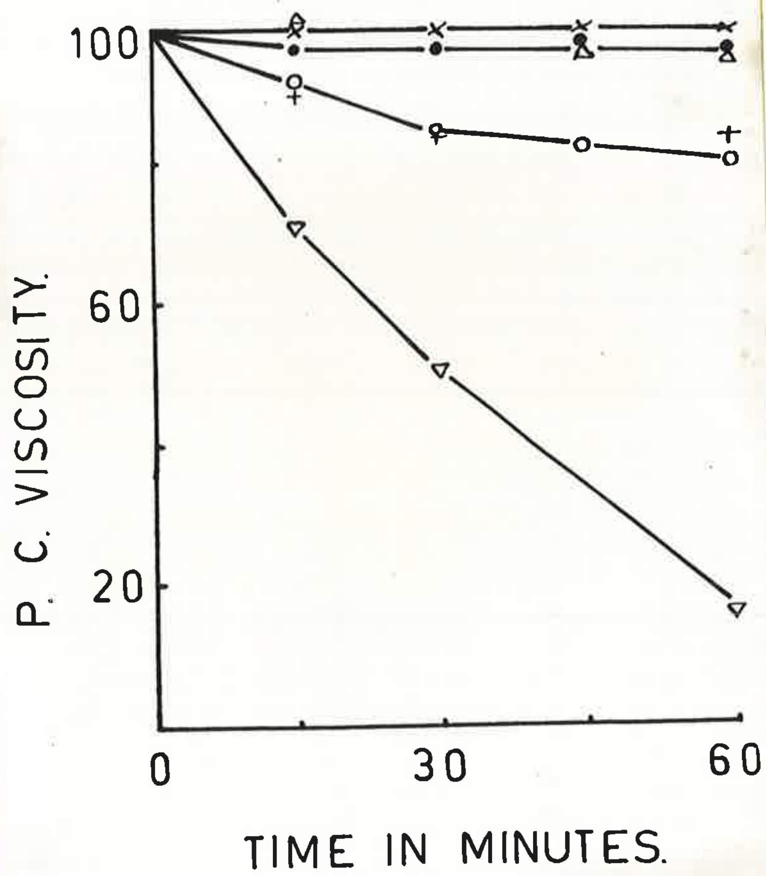
Fig. 30. Ultra-violet absorbance curves of cold 5% TCA extracts prepared from ageing <u>Ps. aeruginosa</u> cells. The TCA was extracted with cold ether.

●——● 24 hour old cells.
⊙——⊙ 48 hour old cells.
x——x 72 hour old cells.

Fig. 31. The percentage changes in viscosity when a cell-free culture filtrate from a five day old <u>Ps. aeruginosa</u> (plaque strain) culture was incubated with various enzyme preparations.

●——● Mucinase.
+——+ Trypsin.
⊙——⊙ Hyaluronidase.
x——x <u>Strept. albus</u> enzyme.
RNase. △——△
DNase. ▽——▽
Control. x——x


0 8
0 6
0 4
0 2
$E^{1\%}_{1 cm}$
220
260
300
WAVELENGTH (mu).


100
60
20
P. C. VISCOSITY.
0
30
60
TIME IN MINUTES.

into the medium (Fig. 29).

Several unsuccessful attempts were made to demonstrate extra-cellular RNA or DNA in old cultures of the plaque-free strain of *Ps. aeruginosa* and in the *S. bethesda* cultures. The absence of nucleic acids from these cultures could be correlated with the absence of extensive lysis by these ageing cultures.

The nucleic acid estimations for both cells and medium were checked by the Schmidt-Thannhauser method to ensure that pentoses released by the disintegrating cell walls were not giving rise to false nucleic acid estimates. The RNA and DNA content of both cells and medium (estimated from their absorbance at 260 mμ. and 268 mμ. respectively) confirmed the colourimetric assays in all respects.

The close correlation between the rate of nucleic acid release into the medium and the decrease in the total number of cells present (Figs. 28 and 29) was presumptive evidence that the nucleic acids were released into the medium only after the rupture of the cell envelope. A further check of this hypothesis was made by a comparison of the base composition of the intra- and extra-cellular nucleic acids. Excellent separation of the bases was obtained using one dimentional ascending paper chromatograms and the bases were readily identified from their Rf values which were almost identical with the corresponding values quoted by Wyatt (1955). The absence of a spot corresponding to thymine in the RNA hydrolysate or to uracil in the DNA preparations indicated that satisfactory separation of the two nucleic acids had been effected by the Schmidt-Thannhauser technique.

d. <u>The base composition of nucleic acids isolated from ageing cells and from the culture medium</u>. The percentage molar proportions

TABLE 12.

Base molar proportions [x] for RNA extracted from *Ps. aeruginosa* (plaque strain) and *S. bethesda* cells grown under aerobic and anaerobic conditions.

Age in hours	Strain	Atmosphere	Guanine	Adenine	Cytosine	Uracil	$\frac{A+U}{G+C}$	$\frac{U}{A}$
24	*Ps.aeruginosa*	Air	27.6	20.9	20.7	29.9	0.95	1.58
72	*Ps.aeruginosa*	Air	26.6	20.9	22.4	30.1	1.00	1.45
120	*Ps.aeruginosa*	Air	28.1	19.8	19.5	32.6	0.82	1.04
24	*Ps.aeruginosa*	N_2	27.5	23.6	21.2	27.7	1.05	1.13
24	*S. bethesda*	N_2	30.3	33.7	15.9	20.0	1.16	0.59
72	*S. bethesda*	N_2	29.8	33.9	16.1	20.2	1.07	0.54
192	*S. bethesda*	N_2	30.6	33.6	14.7	21.0	1.20	0.63

x SD for six replicates $\pm 8\%$

TABLE 13.

Base molar proportions for DNA extracted from *Ps. aeruginosa* (plaque strain) and *S. bethesda* cells grown under aerobic and anaerobic conditions.

Age in hours	Strain	Atmosphere	Guanine	Adenine	Cytosine	Thymine	$\frac{A+T}{G+C}$
24	*Ps.aeruginosa*	Air	34.2	20.4	26.0	19.4	0.69
72	*Ps.aeruginosa*	Air	31.6	19.3	28.6	20.4	0.61
120	*Ps. aeruginosa*	Air	31.0	19.5	27.6	21.7	0.55
192	*Ps.aeruginosa*	Air	32.1	20.1	25.0	23.8	0.76
24	*Ps.aeruginosa*	N_2	30.7	19.9	31.7	20.6	0.56
96	*Ps.aeruginosa*	N_2	32.9	18.4	31.1	17.5	0.54
144	*Ps.aeruginosa*	N_2	31.8	18.1	30.0	20.0	0.69
192	*Ps.aeruginosa*	N_2	31.2	17.3	31.7	19.7	0.76
24	*S. bethesda*	N_2	25.8	26.3	25.5	22.3	0.95
72	*S. bethesda*	N_2	27.3	26.6	24.2	21.7	0.86
120	*S. bethesda*	N_2	27.1	25.2	26.7	20.8	0.84
192	*S. bethesda*	N_2	26.4	25.2	25.4	22.7	0.92

of the four bases of the cellular RNA isolated from aerobic _Ps. aeruginosa_ and _S. bethesda_ cells were recorded in Table 12, while the corresponding values for the DNA bases will be found in Table 13. The base proportions of the cellular RNA did not vary significantly over a five day ageing period. The bases guanine and uracil were present in equimolar proportions in the _Ps. aeruginosa_ RNA. Similarly, the bases adenine and cytosine were present in equimolar amounts. The ratio of uracil to adenine was approximately 1.5 in the young aerated cells, but the ratio decreased slightly as the cells aged. The ratio of $\frac{A+U}{G+C}$ was approximately 1.0 for both the aerobic and the anaerobic cells. The small amount of RNA in the older anaerobic cells made it impracticable to determine the base composition of this material under these conditions. The DNA base proportions were such that approximately equimolar amounts of guanine and cytosine were present with smaller but equivalent quantities of adenine and thymine (Table 13). The ratio of $\frac{A+T}{G+C}$ varied between 0.5 and 0.6. Few differences in base composition due to ageing could be detected, but a slight decrease in the adenine content of the DNA from the anaerobic ageing cells was noted.

The base proportions of the extracellular RNA which accumulated in the aerobic cultures were recorded in Table 14. These figures closely resembled the corresponding ones for the cellular RNA. The extracellular RNA was, therefore, not partially degraded before its release into the medium. The anaerobic cultures did not contain sufficient RNA to enable the base proportions to be determined. The DNA in the _Ps. aeruginosa_ culture medium closely resembled the intracellular DNA in-so-far as the base proportions were concerned (Table 15),

TABLE 14.

Base molar proportions [x] for RNA isolated from the cell free medium after aerobic growth of *Ps. aeruginosa* (plaque strain).

Age in hours	Guanine	Adenine	Cytosine	Uracil	$\frac{A+U}{G+C}$	$\frac{U}{A}$
48	27.0	17.5	23.4	32.0	0.87	1.52
72	26.8	17.2	25.2	29.7	0.98	1.82
192	28.3	18.1	26.3	27.3	0.85	1.38

x SD for six replicates was ± 8%.

TABLE 15.

Base molar proportions for DNA isolated from the cell-free medium after aerobic and anaerobic growth of *Ps. aeruginosa* (plaque strain).

Age in hours	Atmosphere	Guanine	Adenine	Cytosine	Thymine	$\frac{A+T}{G+C}$
48	Air	29.5	17.4	30.2	22.8	0.68
96	Air	29.4	16.7	30.8	23.1	0.66
192	Air	30.1	17.3	30.6	21.8	0.59
96	N_2	30.7	18.9	30.2	20.1	0.76
120	N_2	33.8	19.8	29.5	17.8	0.58
192	N_2	33.5	19.0	29.2	18.2	0.52

suggesting that the deoxyribonucleoprotein was released intact into the medium following the lysis of the cell.

The RNA and DNA content of aerobic and anaerobic S. bethesda cells, together with the corresponding viable and total counts, were recorded in Table 16. The viable population in the aerobic culture decreased by 80% over the eight days of the experiment, but the total counts remained at 100% throughout the experiment. The RNA content of the cells decreased rapidly during the stationary phase, so that less than 15% of the maximum was still present after eight days aerobic incubation. The RNA content of the anaerobically incubated cultures decreased more slowly, so that 30% was still present at 192 hours. The DNA content of both the aerobic and anaerobic cells remained at 100% throughout the experiment. No extracellular RNA or DNA could be detected in the aerobic or in the anaerobic cultures of this organism.

The base composition of the RNA and DNA of the S. bethesda cells showed very little variation due to ageing (Tables 12 and 13). The U/A ratio of the RNA was approximately 0.6 throughout the experiment. The four bases in the DNA were present in approximately equimolar amounts and the ratio $\frac{A+T}{G+C}$ was 1.0, irrespective of the age of the cells.

This concluded the study of the chemical constitution of the whole cells of ageing cultures of Ps. aeruginosa and S. bethesda. A summary of the results of these estimations will be found in Tables 9 and 10. On the whole, the composition of the three strains remained remarkably constant throughout the ageing process. The increase in cellular protein largely compensated for the decrease

TABLE 16.

The nucleic acid content of S. bethesda cells grown under aerobic and anaerobic conditions.

Age of cells	Aerobic culture: Viable ≍ x 10^6	Total ≍ x 10^6	RNA-P. mg.P.per 100 mg.cells	DNA-P. mg.P.per 100 mg.cells	Anaerobic culture: Viable x 10^6	Total x 10^6	RNA-P mg.P.er 100 mg.cells	DNA-P. mg.P.er 100 mg.cells
0	250	-	-	-	320	-	-	-
3	380	1,000	0.25	0.03	760	-	0.11	0.10
5	5,550	6,000	1.02	0.20	760	1,522	0.16	0.17
7	7,200	12,000	0.09	0.30	840	2,558	0.41	0.26
10	11,000	14,200	0.63	0.25	960	2,029	0.90	0.19
16	8,600	9,980	0.74	0.28	-	-	-	-
24	8,210	14,400	0.42	0.07	800	3,670	0.99	0.16
72	8,420	14,000	-	0.20	458	3,730	0.69	0.14
120	3,000	15,300	0.18	0.28	183	3,400	0.59	0.17
192	2,170	13,880	0.15	0.31	163	3,040	0.31	0.25

≍ number of cells x 10^6 per ml. of culture.

- = not done.

in RNA, so that total recoveries of 80% to 95% of the cell substance were recorded throughout. The inability to detect extensive changes in the gross chemical composition of the ageing cells led to further investigations of the problem using the more sensitive techniques of immuno-chemistry. The results of some of these experiments will be considered in the next chapter.

CHAPTER VIII.

CHEMICAL CHANGES ASSOCIATED WITH AGEING: IMMUNO-CHEMICAL STUDIES.

The chemical changes which accompanied the ageing of Ps. aeruginosa cells were not sufficiently extensive to be detected by conventional chemical assay methods. However, autolysis of the cell would be expected to result in a number of changes in the cell wall composition. Since the cell wall contained a number of antigenic materials, attempts were made to detect some of these cell wall changes by the more sensitive techniques of immuno-chemistry.

1. Agglutination Experiments.

The three strains were grown anaerobically in CNT medium for 192 hours. The S. bethesda suspensions were stable, irrespective of the age of the cells. However, normal saline suspensions of both strains of Ps. aeruginosa auto-agglutinated within four hours. When the suspensions were made up in 0.3% saline they remained stable for 18 hours, and the agglutinating titre of the ageing suspensions of the three strains was therefore determined and recorded in Table 17. Suspensions of aerobically grown cells gave similar results to those recorded for the anaerobic ones, so that the results for the aerobic suspensions were not recorded.

The titres for the plaque strain suspensions were slightly lower when tested against the plaque-free strain specific antiserum, than with the homologous serum. On the other hand, the plaque-free suspensions were agglutinated equally well by both sera. Irrespective of the strain of organism used, the titres for both sera decreased two to four fold as the cells aged. This was interpreted as a progressive change to a less smooth state on ageing. Similar results

TABLE 17.

Agglutinating titres of suspensions of ageing cells tested against antisera produced in rabbits against the three strains.

Suspension	Reciprocal of the end titre.		
	Plaque antiserum	Plaque-free antiserum	*S. bethesda* antiserum
Ps. aeruginosa plaque vaccine	320	40	-
Ps. aeruginosa plaque 24 hours	320	80	-
Ps. aeruginosa plaque 96 hours	320	40	-
Ps. aeruginosa plaque 144 hours	160	40	-
Ps. aeruginosa plaque 192 hours	160	20	-
Ps. aeruginosa plaque-free vaccine	320	160	-
Ps. aeruginosa plaque-free 24 hours	320	320	-
Ps. aeruginosa plaque-free 96 hours	40	40	-
Ps. aeruginosa plaque-free 144 hours	80	40	-
Ps. aeruginosa plaque-free 192 hours	80	40	-
S. bethesda vaccine	-	-	2560
S. bethesda 24 hours	-	-	1280
S. bethesda 96 hours	-	-	640
S. bethesda 144 hours	-	-	640
S. bethesda 192 hours	-	-	320

- = not tested.

were obtained with ageing S. bethesda suspensions (Table 17), where a four fold decrease in titre occurred over the eight day period of the experiment. These experiments indicated that alterations to the chemical structure of the cell wall accompanied the process of ageing. These alterations were not obvious when the whole cell was examined chemically, but the falling titres suggested that further chemical studies of the cell walls themselves might disclose some change in composition.

One cell wall component which was of considerable immunological interest was the lipopolysaccharide fraction. Experiments were carried out to determine whether any quantitative changes could be detected in the lipopolysaccharide content of the ageing cell walls. The three strains were grown anaerobically in CNT medium for 24 and 192 hours. The cell walls were isolated after sonic disruption of the cells. Electron micrographs indicated that the walls of both the young and the old cells were almost completely free of electron dense intracellular granules (Plate 5). The cell walls accounted for 10 to 15% of the original dry weight of the cells. The cell walls were extracted with hot 90% phenol and protein, total nitrogen and carbohydrate estimations were carried out on the various fractions, and a balance sheet was drawn up. The details of these analyses will be considered in the next section.

2. The Composition of Ageing Cell Walls Determined by Phenol/Water Partition.

The dry weights of the three fractions obtained when approximarely 0.5 to 1.0 g. of cell wall was extracted with phenol, were estimated as percentages of the original cell wall and recorded

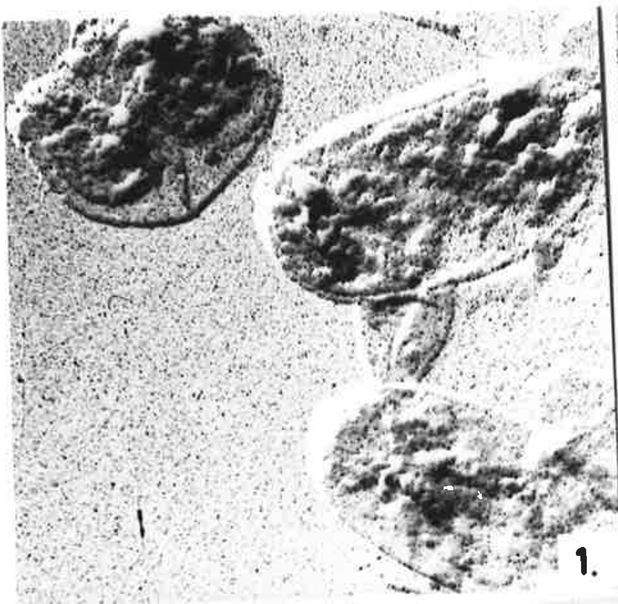
Plate 5. Electron micrographs of cell wall preparations shadowed with palladium.

1. Ps. aeruginosa (plaque strain) cell walls from a 24 hour anaerobic culture. X 40,000.

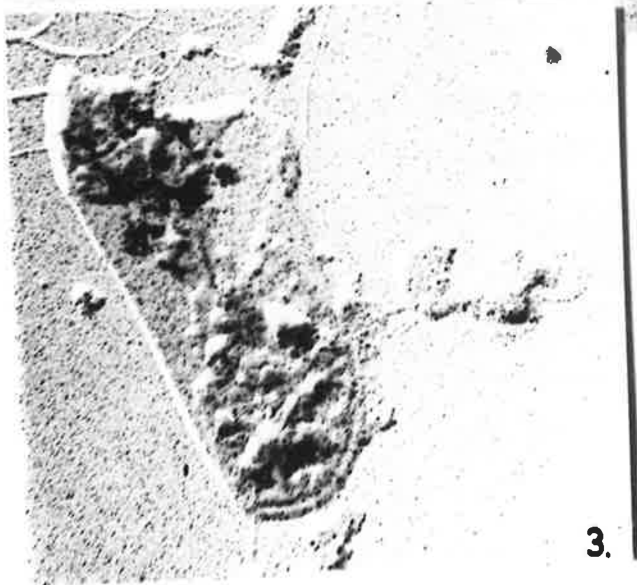
2. Ps. aeruginosa (plaque strain) cell walls from a 192 hour anaerobic culture. X 40,000.

3. Ps. aeruginosa (plaque-free strain) cell walls from a 24 hour anaerobic culture. X 40,000.

4. S. bethesda cell walls from a 24 hour anaerobic culture. X 20,000.


1.

2.

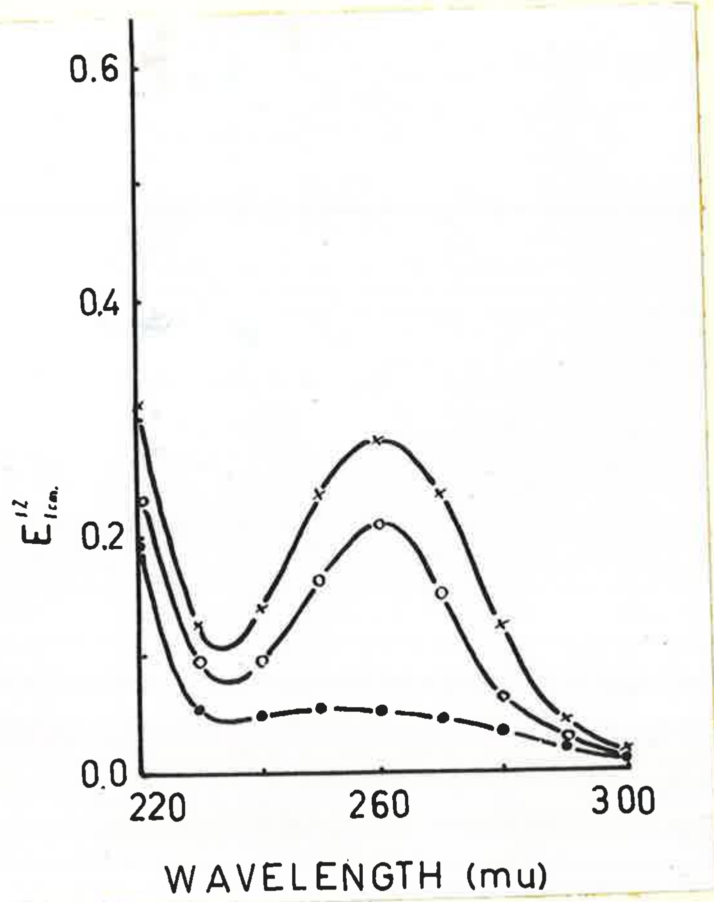

3.

4.

in Table 18. The amount of cell wall substance which passed into the three fractions did not vary significantly with increasing cell age, or within the different strains tested. The phenol residue accounted for about 40% of the cell wall substance, while the remaining fractions accounted for approximately 50%. The aqueous phase contained some protein, large amounts of carbohydrate material, the lipopolysaccharide and a trace of RNA (Fig. 32). The supernatent fluid, after centrifugation at 30,000 r.p.m. for 120 minutes, contained considerable amounts of carbohydrate as well as the RNA. The lipopolysaccharide pellet dissolved readily in distilled water to give an opalescent solution which was free of nucleic acid. About 10% of the material in the aqueous phase was lipopolysaccharide (Table 18). The results of the protein and the total nitrogen estimations on the three fractions were recorded in Table 19. Several of the Folin-Ciocalteau estimations recorded that apparently more than 100% of the solids present consisted of protein. Such a result was obviously impossible and was due to the use of standard curve prepared against serum albumin to estimate the amount of bacterial protein present. The total N. figures for this fraction confirmed that the phenol extracted almost pure protein from the cell walls. No variations in the protein content of this fraction were noted as the cells aged. The phenol residue contained between 40% and 60% protein together with considerable amounts of non-protein nitrogen. The S. bethesda residue had a slightly higher protein content than the corresponding Ps. aeruginosa fractions, but ageing did not appear to greatly alter the results with either organism. The aqueous phase always contained some protein. The S. bethesda aqueous contained

Fig. 32. Ultra-violet absorbance curves for the aqueous phase and lipopolysaccharide preparation obtained after phenol/water partition of *Ps. aeruginosa* (plaque strain) cell walls.

X —— X Yeast RNA.
⊙ —— ⊙ Aqueous extract.
● —— ● Lipopolysaccharide.



0.6
0.4
0.2
0.0
220
260
300
WAVELENGTH (mu)

TABLE 18.

Composition of *Ps. aeruginosa* and *S. bethesda* cell walls determined by phenol/water partition expressed as mg.% of dry wt. of cell wall.

Preparation	Phenol layer	Phenol residue	Aqueous layer	Spinco supernatent	Lipopoly-saccharide
Ps. plaque 24 hours	30	45	20	8.8	1.6
Ps. plaque 192 hours	32	40	20	11.4	1.6
Ps. plaque-free 24 hours	30	35	16	19	1.18
Ps. plaque-free 192 hrs.	30	43	20	11	0.9
S. bethesda 24 hours	25	37	32	12	1.2
S. bethesda 192 hours	31	44	21	9.5	1.1

TABLE 19.

Protein ‡ composition of Ps. aeruginosa and S. bethesda cell walls determined by phenol/water partition.

Preparation	Cell wall	Phenol layer	Phenol residue	Aqueous layer
Ps. plaque 24 hours	57%	100% (100%)[x]	40% (61%)[x]	45%
Ps. plaque-free 24 hrs.	69%	105% (102%)	52% (77%)	69%
Ps. plaque-free 192 hrs.	70%	94% (96%)	38% (64%)	60%
S. bethesda 24 hours	54%	120% (95%)	61% (65%)	22%
S. bethesda 192 hrs.	63%	97% (100%)	- (80%)	33%

x estimated from Total - N.

‡ estimated by the Folin-Ciocalteau reagent.

\- = not done.

somwhat less protein than the corresponding plaque strain preparation, which was only about half that found in the plaque-free fraction. The protein remained in solution when the lipopolysaccharide was precipitated with alcohol, and both the lipopolysaccharide and the Spinco supernatents were protein free.

The carbohydrate estimations carried out on the various fractions were made by the Anthrone method as a matter of routine, although the lipopolysaccharides were also tested with the Orcinol reagent. The total carbohydrate content of the various fractions were estimated as glucose, and the results were recorded in Table 20. The partition of the cell walls resulted in very little carbohydrate remaining in the phenol layer or in the cell residue, and later assays showed that about 70% passed into the aqueous phase. The considerable species differences in carbohydrate content already noted between the Ps. aeruginosa and S. bethesda cell walls were responsible for the large increase in the carbohydrate content of the aqueous phase of the S. bethesda preparations. Most of this carbohydrate material remained in solution when the lipopolysaccharide was precipitated with alcohol. Examination of the final lipopolysaccharide preparations in the Spectrophotometer failed to show any absorption peak in the 260 to 270 mµ. range (Fig. 32).

The carbohydrate content of the lipopolysaccharides varied from 15% to 26% when determined by the Anthrone reagent, and from 24% to 33% with the Orcinol test. The total reducing sugar content of the lipopolysaccharides could not be determined because of the limiting amounts of material available. For this reason, the individual sugar components, and particularly the colitose content was not

TABLE 20.

Carbohydrate composition of phenol/water partition fractions from *Ps. aeruginosa* and *S. bethesda* cell walls. Expressed as mg.% of cell wall dry weight.

Preparation	Cell wall	Phenol layer	Phenol residue	Aqueous layer	Spinco supern.	Lipopolysaccharide Anthrone	Orcinol
Ps.plaque 24 hrs.	3.5	0.02	0.72	2.40	0.4	0.32	0.42
Ps.plaque 192 hrs.	3.5	-	-	-	1.9	0.24	0.355
Ps.pl.-free 24 hrs.	3.2	0.10	0.82	2.30	1.37	0.22	0.31
Ps.pl.-free 192 hrs.	3.3	0.06	0.90	2.20	1.07	0.17	-
S. bethesda 24 hrs.	12.0	0.09	0.72	9.01	4.3	0.31	0.40
S. bethesda 192 hrs.	11.0	0.05	-	6.3	3.4	0.24	-

Expressed as percent of the dry weight of each fraction.

Preparation	Phenol layer	Phenol residue	Aqueous layer	Spinco supern.	Lipopolysaccharide Anthrone	Orcinol
Ps.plaque 24 hrs.	0.1%	1.6%	12%	4.4%	20%	26%
Ps.plaque 192 hrs.	-	-	-	13.9%	15%	24%
Ps.pl.-free 24 hrs.	0.3%	2.5%	11.5%	7.7%	19%	25%
Ps.pl.-free 192 hrs.	0.2%	2.1%	10.8%	9.6%	18%	-
S. bethesda 24 hrs.	0.3%	2.0%	28%	33%	26%	33%
S. bethesda 192 hrs.	0.1%	-	30%	37%	23%	-

- = not done.

determined. The lipopolysaccharide content in the walls varied from 1.0% to 1.6% and the amount present did not vary during ageing (Table 18). Thus the progressive loss of antigenic smoothness observed in the ageing cell suspensions was not due to changes in the amount of lipopolysaccharide present in the cell wall, but was more likely due to changes in the carbohydrate composition of the existing lipopolysaccharide. In the past, the phenol/water partition has been used mainly as a preparative technique, but the present attempts to use the technique as a semi-quantitative assay of the cell wall lipopolysaccharide content appeared to be successful and the constancy of the recoveries from the ageing cell walls of the three species was very gratifying.

Quantitative serological techniques can also be used to demonstrate small differences in the lipopolysaccharides of young and old cells. However, preliminary precipitin tests made with the Ps. aeruginosa lipopolysaccharide preparation (0.5 mg. dry weight per ml.) against a one in two saline dilution of the specific rabbit antiserum gave only a faint precipitate after 24 hours. As expected, the Ouchterlony (1949) gel diffusion plates were also negative.

The plaque strain lipopolysaccharide inhibited the haemagglutinatic of washed sheep erythrocytes when tested by the method of Crumpton, et.al., (1958). Such inhibition experiments proved that immunologicall active material was present in the lipopolysaccharide preparations, but did not demonstrate whether ageing produced chemical changes in the lipopolysaccharide molecule. Proof that chemical changes did occur with ageing would require considerably larger amounts of material

than were available at the time. Such investigations would involve quantitative analyses of the individual sugar components of the polysaccharide moiety, together with a study of the lipid A fraction of the molecule. Finally, the preparation of antisera with high precipitin titres would enable a study of the lipopolysaccharides from young and old cells by means of quantitative precipitin curves (Kabat and Meyer, 1948). Studies along these lines would perhaps offer the most precise immuno-chemical data on the nature of the possible variations in the lipopolysaccharide composition of ageing cells. While the results from such a study would be of undoubted interest, particularly from the point of view of the progressive antigenic changes from smooth to rough, they did not seem to be sufficiently relevant to the present study to justify the preparation of new antisera and further lipopolysaccharide material. Attention was therefore turned to the overall chemical composition of the cell walls of young and old cells and the results of these chemical analyses will be considered in detail in the following chapter.

CHAPTER IX.

CHEMICAL CHANGES ASSOCIATED WITH AGEING: VARIATIONS IN THE CHEMICAL COMPOSITION OF THE CELL WALLS OF PS. AERUGINOSA AND S. BETHESDA.

The balance sheets obtained for whole cells of Ps. aeruginosa and S. bethesda failed to disclose any changes of great significance. However, since cell lysis probably followed extensive alterations to the cell wall architecture, chemical assays were carried out with purified cell wall preparations in an effort to determine the nature and possible significance of these changes.

1. Ageing Cultures of Ps. aeruginosa and S. bethesda.

The cell wall preparations were free of electron-dense granules when checked for purity with the electron microscope (Plate 5). The composition of the cell walls was determined as total nitrogen and protein, total carbohydrate, total lipid and total phosphorous. These assays will now be considered in detail.

a. The total nitrogen content of the cell walls of Ps. aeruginosa and S. bethesda varied from 8% to 11% of the dry weight (Table 21). The cell walls prepared from aerobic cells contained somewhat more nitrogenous material than the anaerobic preparations. On the other hand, the plaque strain walls recorded consistently lower figures for this fraction than were found with the other two organisms, but these differences were never very great. Little or no change in the total nitrogen content of the cell walls accompanied the ageing of the cells, either in complex or in defined media.

(1) Protein determinations made by the Folin-Ciocalteau and by the Biuret techniques were recorded in Table 21. The protein content of the plaque-strain cell walls varied from 50 to 60 mg.% depending on

TABLE 21.

Total- N and protein composition of Ps. aeruginosa and S. bethesda cell walls prepared from cells grown aerobically and anaerobically in complex and defined media. (mg. per 100 mg. dry weight of cell wall).

Age of cells	Medium	Ps. aeruginosa plaque strain			Ps. aeruginosa plaque-free strain			S. bethesda		
		Total- N	Folin C.	Biuret	Total- N	Folin C.	Biuret	Total- N	Folin C.	Biuret
					Aerated cultures					
24	C.N.T.	11.0	60	55	8.5	74	58	8.8	64	50
96	C.N.T.	10.2	60	53	12.2	76	60	9.6	60	50
					Anaerobic cultures					
24	Broth	6.2	60	40	-	-	-	-	-	-
72	Broth	8.8	60	57	-	-	-	-	-	-
96	Broth	8.8	65	58	-	-	-	-	-	-
192	Broth	8.9	71	59	-	-	-	-	-	-
24	C.N.T.	8.4	57	50	10.1	73	65	-	-	-
48	C.N.T.	8.4	52	47	-	-	-	8.0	59	50
64	C.N.T.	8.2	61	-	-	-	-	-	-	-
96	C.N.T.	8.8	65	56	10.5	75	70	9.0	67	59
120	C.N.T.	-	-	-	10.0	75	62	-	-	-
192	C.N.T.	8.7	68	60	10.9	75	65	9.0	68	58

- = not done

the assay method used. The nutritional complexity of the medium did not affect the protein content of the young cell walls. The relative protein content of the anaerobically grown cell walls increased as they aged. Thus, the protein content of the 192 hour plaque strain cell walls varied from 65 to 70 mg.%. In contrast, the plaque-free cell walls contained 65% to 70% protein throughout the growth cycle, irrespective of the age of the cells (Table 21). Thus the mutation by <u>Ps. aeruginosa</u> to the plaque-free strain resulted in an increase of 10 to 15 mg.% in the protein content of the cell wall. The <u>S. bethesda</u> cell walls consisted of about 50% protein, and this amount increased only slightly as the cells aged. The increased protein content of the plaque-free cell walls largely accounted for the higher total nitrogen figures recorded for these preparations (Table 21). In general, the protein -N accounted for about 90% of the total nitrogen in the cell wall preparations, indicating that only small amounts of other nitrogenous materials in the cell walls were present.

The protein content of the ageing cell walls increased rather than decreased over the period when the most extensive cell lysis was observed. Autolysis of <u>Ps. aeruginosa</u> cells did not, therefore, involve the removal of extensive amounts of protein from the cell wall. Experience with Gram positive bacteria (Salton, 1958) suggested that the removal of certain key cell wall amino acids and amino sugars could be involved in the process of cell lysis in the present study. Attempts were therefore made to determine whether such changes did, in fact occur during the autolysis of <u>Ps. aeruginosa</u> cultures.

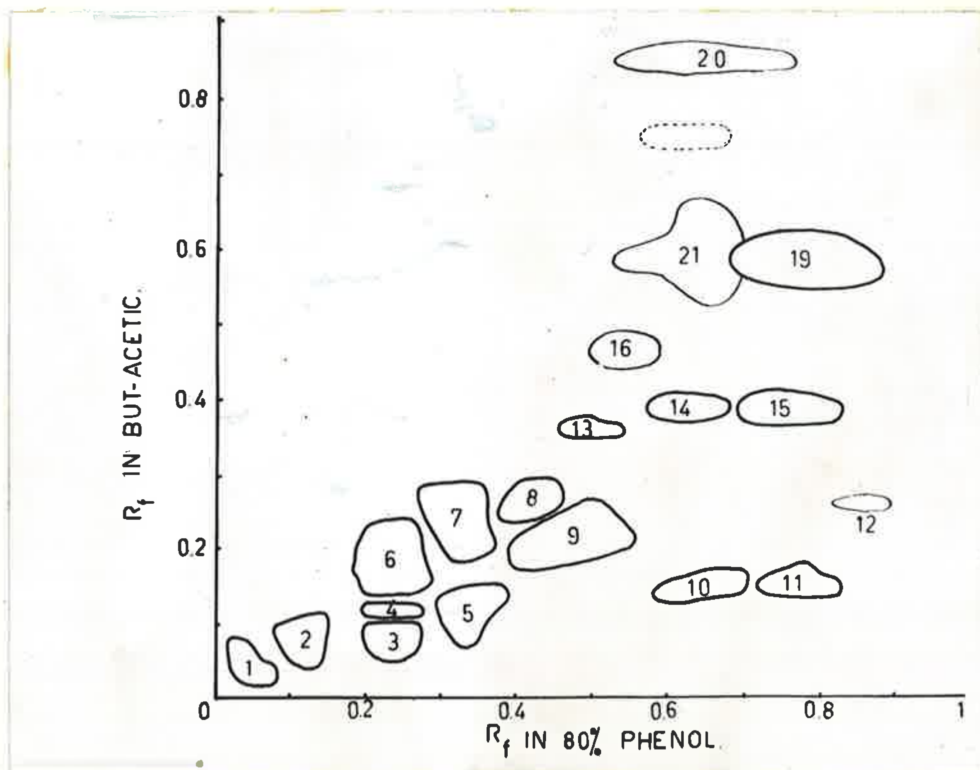
(2) <u>Amino acid determinations</u> were carried out on the ageing cell walls of the three strains grown in aerobic and anaerobic

cultures. A preliminary experiment was carried out to determine the completeness of hydrolysis of the bacterial proteins over increasing periods of time at 100°C. Hydrolysis was 75% complete after one hour, but several large spots (Fig. 33), present on the chromatogram were identified as incompletely hydrolysed peptides. Prolonged hydrolysis resulted in an increase in the amount of serine, aspartic acid, valine and methionine in the hydrolysates (Table 22). The increase in methionine and serine corresponded with the disappearance of the peptide 1 (Fig. 33) while the rise in aspartic acid and valine in the 20 hour hydrolysate corresponded with the disappearance of peptide 2 (Fig. 33). Thus hydrolysis in 6 N. HCl at 100°C. for 20 hours resulted in the complete breakdown of the cell wall proteins to free amino acids.

Trytophane was destroyed after four hours hydrolysis at 100°C. although a faint spot corresponding to this amino acid could be detected after one hour. Some difficulty was experienced in completely separating the lysine from the glycine (not estimated quantitatively for this reason), and also the leucine from the isoleucine. However, the addition of two per cent lutidine to the ninhydrin solution enabled these amino acid pairs to be clearly distinguished qualitatively. The most abundant amino acids in the Ps. aeruginosa cell walls were aspartic and glutamic acids, alanine, phenyl alanine, lysine, serine and DAP. Smaller amounts of valine, methionine, tyrosine and leucine were also present. In all, twenty amino acids were detected in the hydrolysates. The amounts of the various amino acids agreed surprisingly well with corresponding figures (Table 22) quoted for E. coli cell walls by Salton (1960). The total amino acid recovery after 20 hours hydrolysis was approximately 50 mg.% which agreed very favourably with the known protein content of

33. Two dimentional ascending paper chromatogram of 24 hour old *Ps. aeruginosa* (plaque strain) cell wall material following hydrolysis with 6N. HCl at 100°C. for one hour. The papers were developed with ninhydrin at room temperature for 24 hours. The numbers refer to the following amino acids.

1. Cysteic acid.
2. Diamino pimelic acid.
3. Lysine.
4. Glycine.
5. Serine.
6. Aspartic acid.
7. Glutamic acid.
8. Alanine.
9. Tryosine.
10. Histidine.
11. Arginine.
12. Proline.
13. α amino butyric acid.
14. Valine.
15. Methionine.
16. Tryptophane.
17. Leucine.
18. Iso-leucine.
19. Phenyl alanine.
20. Peptide 1.
21. " 2.



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0.6
0.4
0.2
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0.2
0.4
0.6
0.8
1
R_f IN BUT-ACETIC
R_f IN 80% PHENOL

TABLE 22.

Amino acid composition of *Ps. aeruginosa* (plaque strain) cell walls.
(mg. of amino acid per 100 mg. dry weight of cell wall.) [x]

Amino acid	Time of hydrolysis			*E. coli* Salton (1960)
	1 hour	4 hours	20 hours	16 hours
DAP	2.6	3.1	2.0	-
Lysine	5.5	5.4	4.4	4.0
Glycine	+	+	+	3.1
Serine	2.9	4.2	3.7	3.7
Aspartic acid	5.5	6.5	10.7	7.1
Glutamic acid	6.5	7.2	9.3	6.9
Alanine	4.0	4.5	3.9	5.6
Tyrosine	1.3	2.1	1.8	3.3
Proline	+	+	+	1.5
Arginine	0.4	0.8	0.7	3.8
Histidine	0.5	1.0	0.8	0.9
α amino butyric acid	0.7	1.3	0.9	-
Valine	0.3	0.9	1.1	3.4
Methionine	0.3	0.8	1.0	0.7
Leucine	1.8	2.4	1.9	5.3
Phenyl alanine	4.0	4.6	4.0	3.0
Iso leucine	+	+	+	3.7
Peptide 1	+	+	±	-
Peptide 2	+	-	-	-
Total amino acid	36.3	44.8	46.2	56.0

x error for estimations ± 10%

\+ = spot identified but not estimated quantitatively.
± = trace only.

\- = not detected.

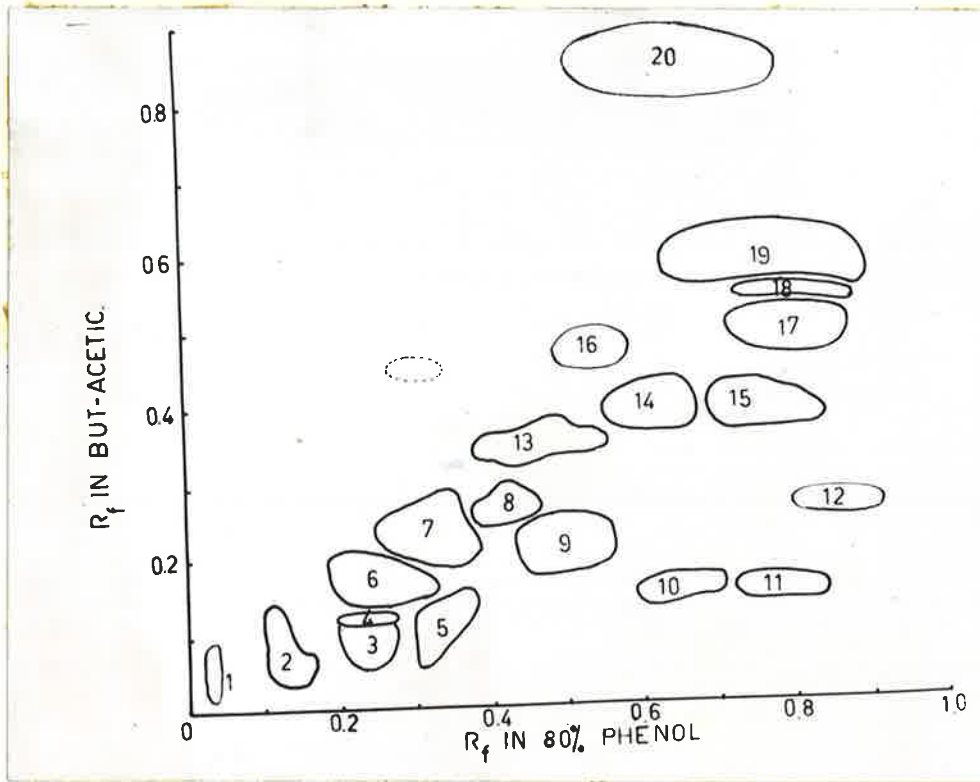
Ps. aeruginosa cell walls.

Attention was turned to the amino acid composition of ageing cell walls of the two *Ps. aeruginosa* strains and of *S. bethesda*. The *Ps. aeruginosa* (plaque strain) cell wall amino acid composition was recorded in Table 23. The amino acid recovery for the ageing cell walls was about 50 mg.%. The decrease in the amino acid recovery recorded for the four day old aerobic cell wall preparation was thought to be due to an error in the dry weight determination rather than to a real decrease in amino acid content. The total number of amino acids in the ageing cell walls remained the same (c.f. Figs. 34 and 35), but a number of changes occurred in the concentration of individual amino acids present. The content of aspartic acid, phenyl alanine, leucine and histidine increased as the anaerobic cells aged (Table 23) whilst a simultaneous decrease was recorded in the amounts of DAP, lysine, alanine and serine. The decreases occurred mainly between 88 and 192 hours, coinciding with the period of cell lysis. Similar decreases in DAP, lysine and serine were also observed in the aerated cell walls.

The amino acid composition of the corresponding plaque-free cell walls was recorded in Table 24. The amino acid content of the ageing anaerobic cell walls increased by 15%. There was no sudden decrease in DAP, serine or lysine corresponding to that found in the plaque strain preparations. No spot corresponding to glycine could be detected in the chromatogram (c.f. Figs. 34 and 36). The amounts of the other individual amino acids present were very similar to those of the plaque strain except that the arginine and methionine content of the plaque-free cell walls was almost double that found in the plaque strain walls (Table 24). The amino acid recovery was equivalent to 80% to 90% of the

Fig. 34. Two dimentional ascending paper chromatogram of 24 hour old Ps. aeruginosa (plaque strain) cell wall material following hydrolysis with 6N.HCl at 100°C. for 20 hours. The numbers refer to the amino acids listed in Fig. 33.

Fig. 35. Two dimentional ascending paper chromatogram of 192 hour old cell walls of Ps. aeruginosa (plaque strain) following hydrolysis with 6N. HCl at 100°C. for 16 hours. The numbers refer to the amino acids listed in Fig.33.



20
0.8
0.6
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17
16
R_f IN BUT-ACETIC
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0.2
6
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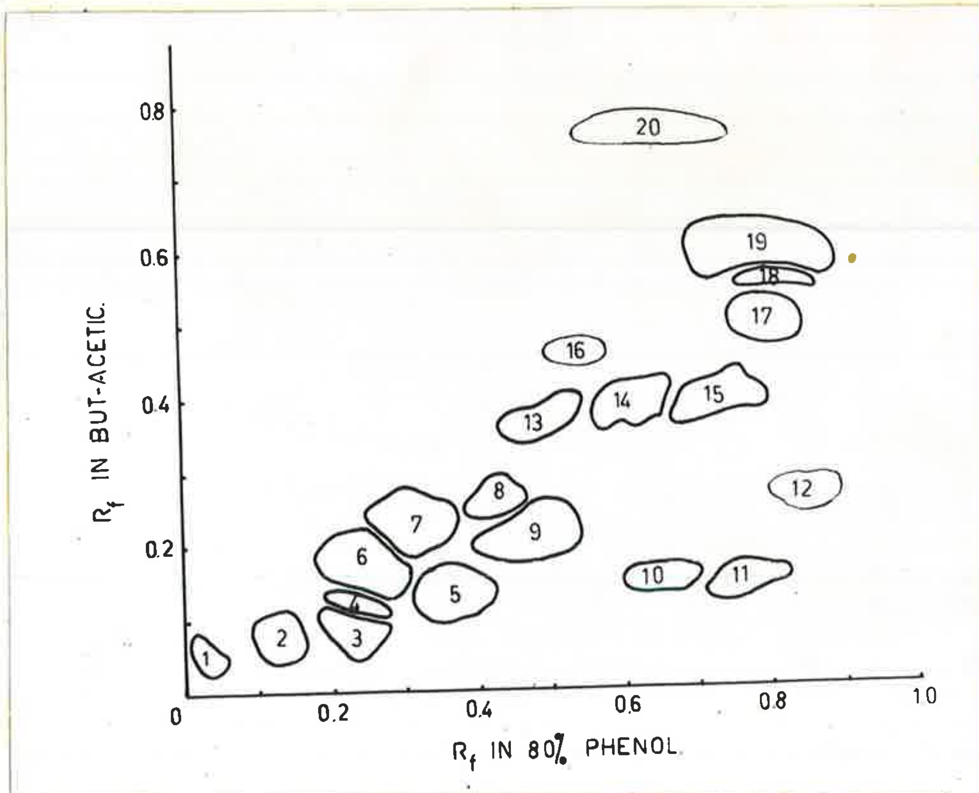
1
0
0.2
0.4
0.6
0.8
1.0
R_f IN 80% PHENOL



0.8
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0.6
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R_f IN BUT-ACETIC
0.4
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12
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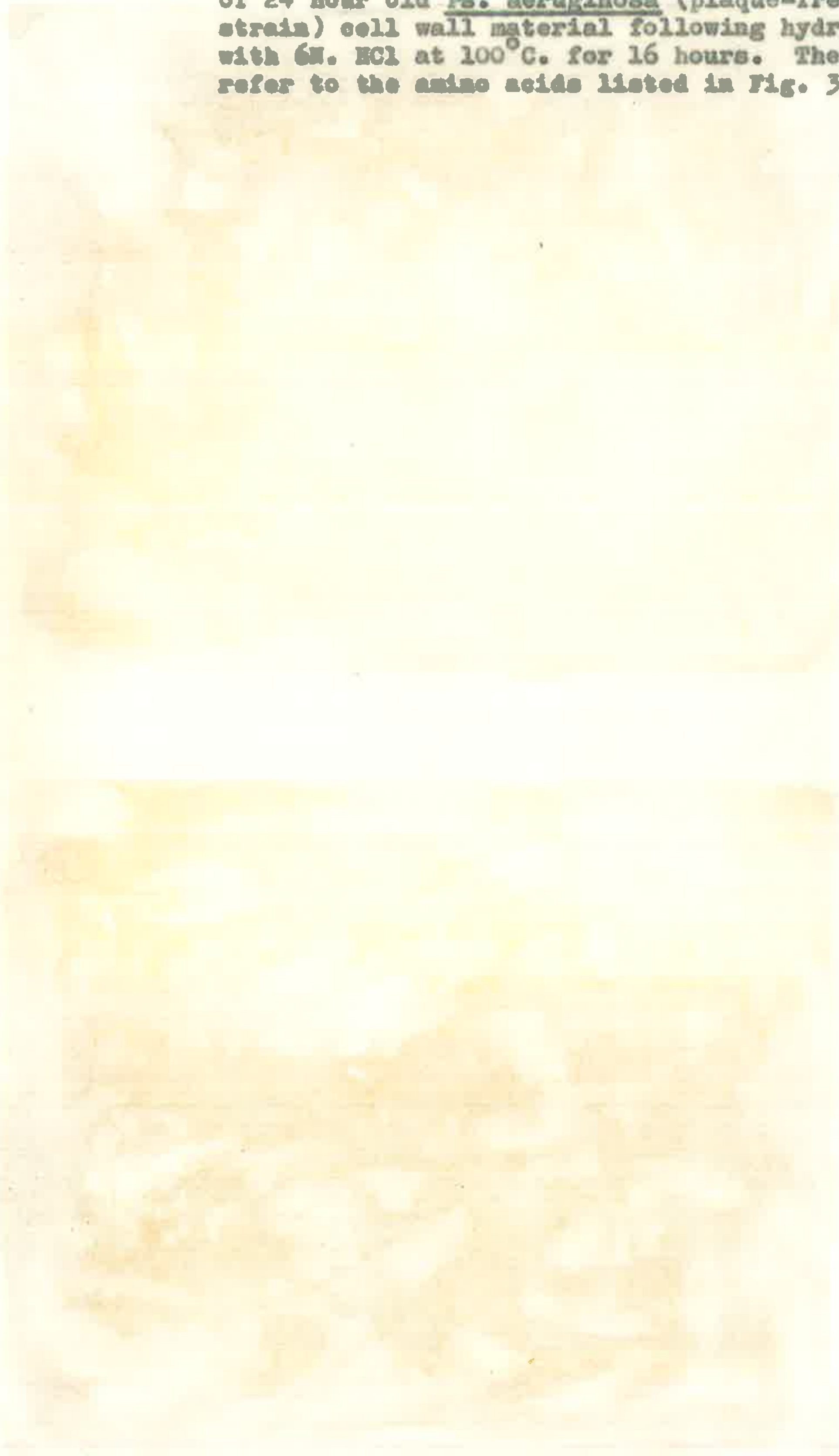
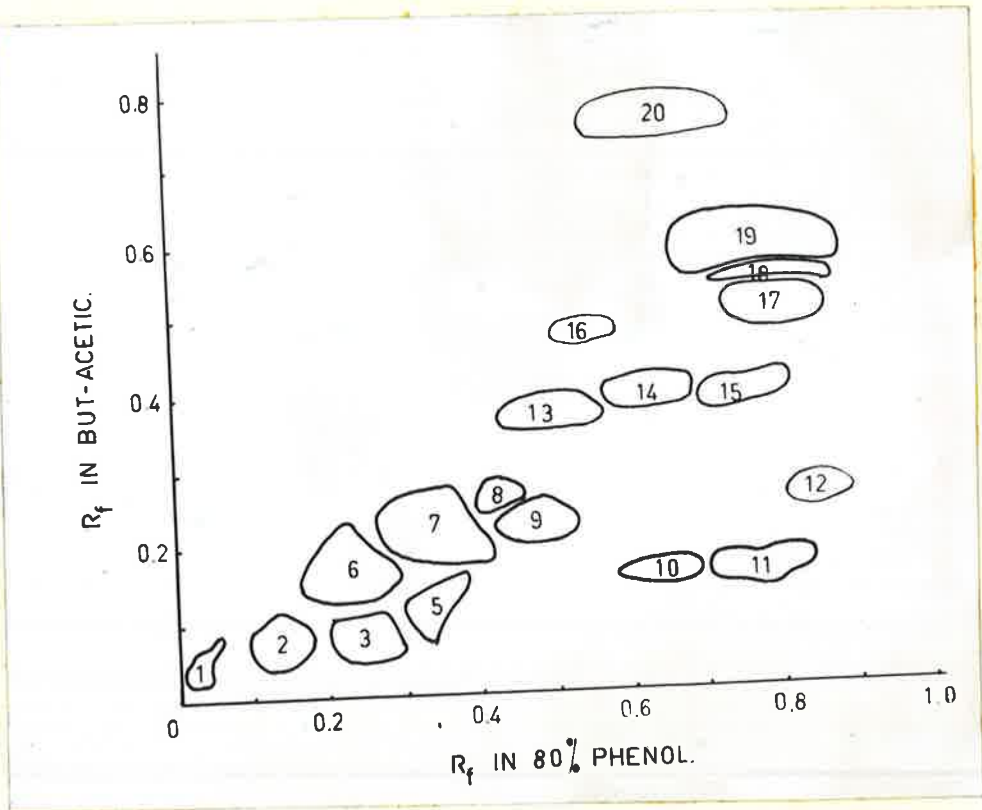
9
0.2
6
10
11
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
R_f IN 80% PHENOL

Fig. 36. Two dimentional ascending paper chromatogram of 24 hour old *Ps. aeruginosa* (plaque-free strain) cell wall material following hydrolysis with 6N. HCl at 100°C. for 16 hours. The numbers refer to the amino acids listed in Fig. 33.





0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
R_f IN BUT-ACETIC.
R_f IN 80% PHENOL.
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TABLE 23.

Amino acid composition of ageing cell walls of *Ps. aeruginosa* (plaque strain) expressed as mg. per 100 mg. of cell wall.

	Anaerobic					Aerated	
	24*	40	64	88	192	24	96
Cysteic acid	+	+	+	+	-	+	+
DAP	3.0	2.6	2.6	1.9	1.4	5.4	3.6
Lysine	5.4	4.7	4.7	4.7	3.1	6.7	4.8
Glycine	+	+	+	+	++	+	+
Serine	5.2	7.3	3.8	4.4	3.8	7.8	4.8
Aspartic acid	10.7	10.4	10.3	11.1	12.0	9.6	6.1
Glutamic acid	9.3	9.3	9.2	8.1	8.6	6.4	3.2
Alanine	3.5	4.9	3.5	3.2	3.0	4.1	2.4
Tyrosine	2.2	2.1	1.6	1.8	+	2.3	1.6
Proline	+	+	+	+	+	+	+
Histidine	0.8	0.7	0.8	1.0	1.3	1.5	1.6
Arginine	0.6	0.6	0.6	0.8	1.0	1.0	1.0
α amino butyric acid	0.9	0.9	0.9	1.0	1.2	2.0	1.4
Valine	1.1	1.1	1.0	1.5	1.1	1.7	1.9
Methionine	0.9	1.0	1.0	1.4	1.1	1.7	1.8
Leucine	1.9	2.9	3.3	4.3	4.4	2.5	3.0
Phenyl alanine	5.4	5.5	6.0	8.1	8.2	4.6	4.9
Iso leucine	1.0	1.4	1.7	2.0	2.1	1.1	1.1
Tryptophane	-	±	+	-	-	+	+
Peptide 1	±	-	±	±	±	+	+
Total amino acid	52	55	51	55	53	58	41

* age of cell walls in hours.

+ = spot identified but not estimated quantitatively.
± = trace only.
- = not detected.

TABLE 24.

Amino acid composition of ageing cell walls of *Ps. aeruginosa* (plaque-free strain) expressed as mg.per 100 mg.of cell wall.

Amino acid.	Anaerobic.				Aerated.	
	24*	96	120	192	24	96
Cysteic acid	+	±	-	±	+	-
DAP.	3.1	4.5	5.0	5.0	3.7	4.5
Lysine	5.0	6.9	7.4	7.0	6.4	5.4
Glycine	-	-	-	-	-	-
Serine	5.4	6.2	6.0	6.8	5.3	6.0
Aspartic acid	9.3	7.0	8.4	6.8	9.7	7.9
Glutamic acid	8.1	7.5	8.9	9.8	11.7	6.6
Alanine	3.7	3.2	3.7	5.3	6.2	3.7
Tyrosine	1.7	1.5	1.4	2.5	1.6	1.2
Proline	+	+	+	+	+	+
Arginine	1.3	1.8	1.4	1.7	1.1	1.6
Histidine	2.0	2.8	2.2	2.6	1.5	2.5
α amino butyric acid	1.5	1.8	1.7	2.0	1.7	1.5
Valine	1.9	2.7	2.7	2.4	1.7	2.5
Methionine	1.8	2.6	2.7	2.3	1.6	2.4
Leucine	3.2	3.5	4.4	4.7	3.6	3.9
Phen. alanine	5.8	6.5	8.1	8.6	6.7	7.8
Iso leucine	1.6	1.6	2.1	2.2	1.7	1.9
Tryptophane	+	±		-	+	-
Unknown 1	+	+	±	+	+	+
" 2	-	-	-	-	-	-
Total amino acid	55	60	63	69	64	59

* Age of cell walls in hours.
+ = spot identified but not estimated quantitatively
± = trace. - = not detected.

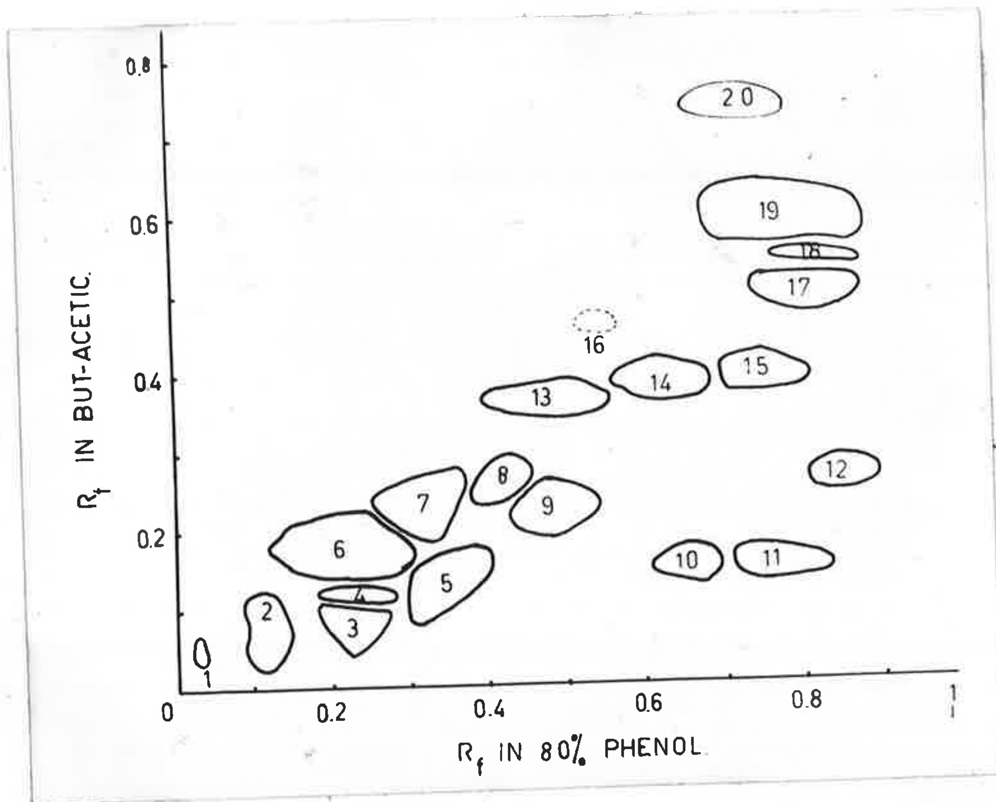
total protein in the cell walls.

A similar pattern of amino acid composition was observed in the *S. bethesda* cell walls, but the exact amounts of the individual components varied somewhat (Table 25). The aspartic acid content was higher and iso-leucine was reduced (Fig. 37). The trytophane spot was larger than for either of the *Ps. aeruginosa* strains, but it was still too faint for quantitative estimation. The serine content of the *S. bethesda* cell walls was somewhat lower than it was for *Ps. aeruginosa* cell walls. The total amino acid recoveries in the ageing anaerobic cell walls remained constant at approximately 45 mg.%. The DAP, alanine, lysine and serine content of the cell walls did not decrease during these experiments.

The coincidence of the decrease in the DAP, alanine, lysine and serine content of the *Ps. aeruginosa* plaque strain cell walls with the initiation of extensive cell lysis was interesting especially in view of the retention of these amino acids in the cell walls of the lysis resistant plaque-free strain and in *S. bethesda*. The observed decrease in the amount of DAP, lysine, serine and alanine present in the plaque strain cell walls led to an examination of the carbohydrate and amino sugar content of the ageing walls. The details of these investigations will be considered in the next section.

b. Total carbohydrate. The plaque strain cell walls contained three to five mg.% of carbohydrate (estimated as glucose by the Anthrone reagent). The corresponding reducing sugar content was 6.0 to 9.6 mg.% (Table 26). Very little change occurred during the ageing of the cells and no significant differences were detected in the carbohydrate content of the plaque strain cell walls prepared from cells grown under aerobic

Fig. 37. Two dimentional ascending paper chromatograms of 24 hour old *S. bethesda* wall material after hydrolysis with 6N. HCl. at 100° C. for 16 hours. The numbers refer to the amino acids listed in Fig. 33.



0.8
0.6
0.4
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0.2
0.4
0.6
0.8
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R_f IN BUT-ACETIC
R_f IN 80% PHENOL
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TABLE 25.

Amino acid composition of ageing cell walls of S. bethesda expressed as mg. per 100 mg. of cell wall.

Amino acid	Anaerobic.			Aerated.	
	48*	88	192	24	96
Cysteic acid	+	±	+	+	+
DAP	2.3	1.2	1.9	2.5	3.3
Lysine	4.9	3.0	3.4	3.4	7.8
Glycine	+	+	+	+	+
Serine	3.0	2.0	3.0	2.1	3.2
Aspartic acid	12.3	12.0	16.0	8.1	11.6
Glutamic acid	6.9	6.2	8.3	6.2	7.6
Alanine	2.1	2.5	1.5	1.8	2.0
Tyrosine	3.0	1.9	1.6	2.5	2.5
Proline	+	+	+	+	+
Arginine	0.5	0.4	0.4	2.8	1.0
Histidine	0.6	0.6	0.6	0.8	1.6
α amino butyric acid	1.0	1.0	0.8	1.8	1.4
Valine	1.1	1.0	0.7	0.6	1.2
Methionine	1.1	1.0	0.6	0.9	1.1
Leucine	1.7	2.5	3.1	2.9	3.6
Phenyl alanine	2.8	3.6	3.9	2.0	4.6
Iso leucine	1.2	2.4	3.1	1.5	3.8
Tryptophane	+	+	+	+	+
Peptide 1	+	±	-	±	-
Total amino acid	45	41	49	40	56

* age of cell walls in hours.

+ = spot identified but not estimated quantitatively
± = trace.
− = not detected.

or anaerobic conditions, either in complex or in defined media.

The plaque-free cell walls contained similar amounts of carbohydrate to the parent strain. Once again the figures remained fairly constant throughout the ageing period. However, the total reducing sugar content of the plaque-free cell walls almost doubled compared with the amount found in the plaque strain preparations. The S. bethesda cell walls contained reducing sugars equivalent to almost 20% of the total dry weight (Table 26). The corresponding figures for the Anthrone tests were somewhat lower, but were still three to four times as high as that found in the Ps. aeruginosa preparations. Cultural conditions and the age of the S. bethesda cell walls had little effect on the total carbohydrate content. Thus, although considerable species differences could be detected, the extent of the changes which could be ascribed to ageing were disappointing.

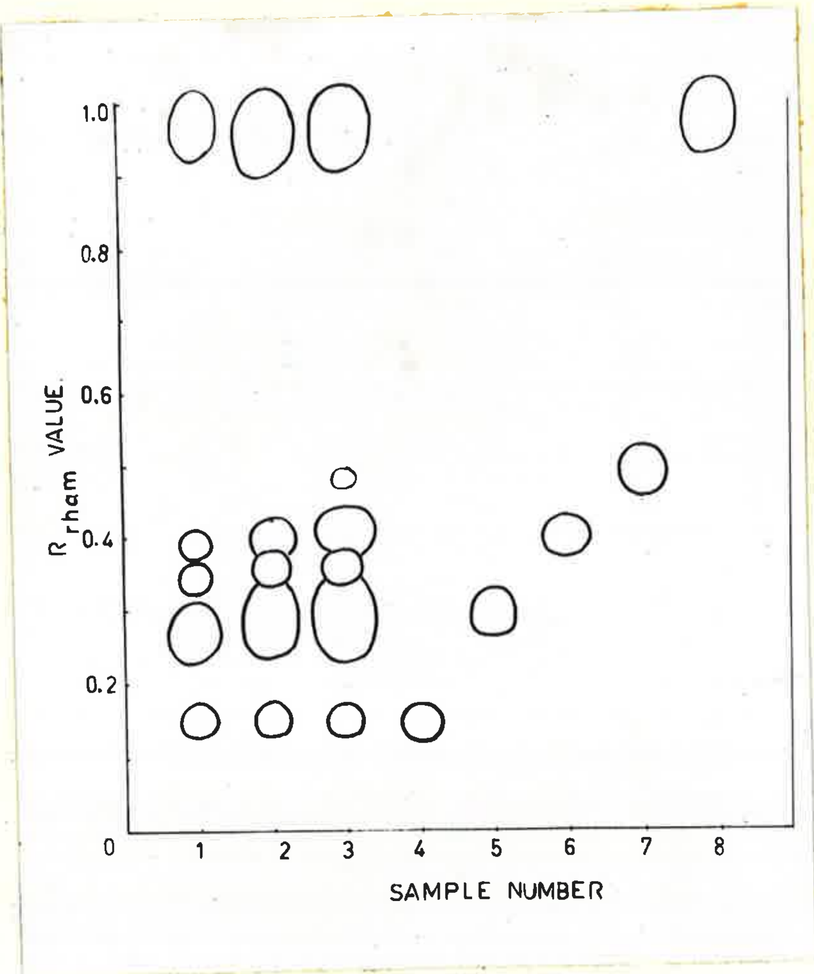
(1) Qualitative chromatographic sugar determinations. The Ps. aeruginosa cell walls contained glucose, rhamnose, glucosamine, muramic acid and a little mannose (Fig. 38). No spots corresponding to galactose, arabinose, xylose or ribose were detected. In addition to the above spots, the S. bethesda cell wall chromatograms contained a small spot corresponding to arabinose.

(2) Quantitative sugar and amino sugar determinations. The muramic acid and glucosamine content of the ageing cell walls of the three strains were recorded in Table 27. The amino sugar content varied between one and two mg.% with approximately equal amounts of glucosamine and muramic acid present in the young cell walls. As the plaque strain walls aged so the glucosamine content decreased sharply but the muramic acid was not greatly affected. The plaque-free strain

Fig. 38. Single dimentional descending paper chromatogram of cell wall material after hydrolysis in 2N. sulphuric acid at 100°C. for two hours.

1. *Ps. aeruginosa* (plaque strain) cell walls.
2. *Ps. aeruginosa* (plaque-free strain) cell walls.
3. *S. bethesda* cell walls.
4. Glucosamine.
5. Glucose.
6. Mannose.
7. Arabinose.
8. Rhamnose.

The spot R_{Rham} = 0.35 was provisionally identified as muramic acid.



1.0
0.8
0.6
0.4
0.2
0
R_{rham} VALUE
1
2
3
4
5
6
7
8
SAMPLE NUMBER

TABLE 26.

Carbohydrate composition of Ps. aeruginosa and S. bethesda cell walls from cells grown in aerated and anaerobic media (mg. per 100 mg. dry weight of cell walls).

Age of cells	Medium	Atmos.	Ps. aeruginosa plaque strain			Ps. aeruginosa plaque-free strain			S. bethesda		
			Anth.	Reducing[x]	Hexos.	Anth.	Reducing	Hexos.	Anth.	Reducing	Hexosamine
						Aerobic cultures					
24	Broth	Air	5.0	8.3	-	5.3	13.0	-	12	18.6	-
96	Broth	Air	5.7	6.9	1.4	5.5	14.2	1.5	10	13.0	1.4
						Anaerobic cultures					
24	Broth	N_2	4.4	9.6	-	-	-	-	-	-	-
72	Broth	N_2	4.0	-	-	-	-	-	-	-	-
96	Broth	N_2	4.6	-	-	-	-	-	-	-	-
192	Broth	N_2	4.4	8.0	-	-	-	-	-	-	-
24	C.N.T.	N_2	3.7	8.0	1.4	3.1	12.1	1.4	-	-	-
48	C.N.T.	N_2	3.4	-	-	-	-	-	12.0	18.3	2.1
64	C.N.T.	N_2	4.0	-	-	-	-	-	-	-	-
96	C.N.T.	N_2	3.6	-	-	3.8	-	-	11.0	-	-
120	C.N.T.	N_2	-	-	-	3.0	-	-	-	-	-
192	C.N.T.	N_2	3.0	6.1	0.8	4.0	13.5	1.1	11.0	15.4	1.6

x Reducing = total reducing sugar

- = not done.

TABLE 27.

Sugar and amino sugar composition of ageing cell walls of *Ps. aeruginosa* and *S. bethesda* grown anaerobically in defined medium.

Age in hours	Organism	Glucos-amine	Muramic acid	Glucose	Mannose	Rhamnose	Arabinose	Total
24	*Ps.aeruginosa*	0.7[x]	0.8	2.9	0.3	2.2	-	6.8
64	plaque	-	-	2.7	0.3	2.0	-	-
88	strain	-	-	1.8	0.3	2.0	-	-
192		0.2	0.6	1.1	0.3	1.8	-	4.0
24	*Ps. aeruginosa*	0.7	0.8	5.0	0.6	3.0	-	10.0
72	plaque-free	-	-	4.4	0.7	3.0	-	-
192	strain	0.5	0.9	3.4	0.5	2.8	-	7.7
48	*S. bethesda*	1.1	1.0	3.9	3.7	9.6	+	19.3
88		-	-	4.8	3.3	8.2	+	-
192		1.6		4.1	3.0	8.1	+	16.7

x mg. per 100 mg. of cell walls.

\+ = detected chromatographically but not estimated quantitatively.

\- = not estimated.

and the _S. bethesda_ cell walls did not show any analogous changes with ageing.

The plaque strain cell walls contained approximately equimolar amounts of glucose and rhamnose with a much smaller amount of mannose (Table 27). As the cell aged the glucose content decreased by 60% without any extensive changes in the rhamnose or mannose contents.

The _Ps. aeruginosa_ (plaque-free) cell walls contained almost twice the amount of glucose and mannose to that found in the parent strain cell walls. On the other hand, the rhamnose content was unaffected by the mutation. The decrease in the cell wall glucose content as the plaque-free cell walls aged was considerably less than for the plaque strain over the same period of time. The chromatographic estimation of the carbohydrate content of the ageing cell walls were consistent with the earlier chemical assays (Tables 26 and 27). They also helped to explain the observed increase in the total reducing sugar content of the plaque-free cell walls in the absence of any corresponding change in the Anthrone figures. The _S. bethesda_ cell walls contained three to four times as much rhamnose as was found in the _Ps. aeruginosa_ preparations. A similar increase was observed in the mannose content (Table 27).

The finding by Brumfitt, _et.al._, (1958) that lysozyme resistance in _M. lysodeikticus_ was associated with changes in the O-acetyl content of the cell walls led to a similar investigation of the plaque and plaque-free cell walls. The O-acetyl content of the cell walls did not change greatly during the process of ageing (Table 28). However, the plaque-free cell walls contained less O-acetyl than the parent strain, but although this difference was interesting, it did not seem to have

TABLE 28.

O-acetyl content of the cell walls of *Ps. aeruginosa* and *S. bethesda*.

Age in hours	Organism	Aerated	Anaerobic
24	*Ps. aeruginosa* plaque strain	0.45 [x]	0.31
96		0.42	-
192		-	0.27
24	*Ps. aeruginosa* plaque-free strain	0.26	0.23
96		0.28	-
192		-	0.19
48	*S. bethesda*	0.40	0.35
96		0.32	-
192		-	0.34

x mg. per 100 mg. of cell wall.

- = not done.

any obvious relevance to the increased resistance to the lytic changes under discussion. Attention was therefore turned to the lipid content of the cell walls of the three strains.

c. Total lipid. The aerobic Ps. aeruginosa plaque strain cell walls recorded a lipid content of 18 mg.% (Table 29), which later decreased slightly as the cells aged. However, the corresponding figures for the plaque-free strain cell walls remained constant at 16 mg.% over the four day period. The young aerobic S. bethesda cell walls contained somewhat less lipid (12 mg.%) and this figure increased slightly as the cells aged. The free lipid content of the anaerobically grown cells of all three strains remained at six to eight mg.% throughout the experiments (Table 30). The complex lipid content of the Ps. aeruginosa (plaque strain) cell walls decreased from seven mg.% at 24 hours to less than two mg.% after eight days. Over the same period of time, the Ps. aeruginosa (plaque-free strain) cell walls showed little change from the eight to ten mg.% normally found in the young cells. The S. bethesda cell walls contained a high proportion of free lipid, but the combined lipid content remained steady at about four mg.% throughout the experiment (Table 30). Thus, in addition to the demonstrated generic differences between the two organisms, a definite ageing effect could be demonstrated for the plaque forming Ps. aeruginosa strain.

d. Total phosphorous. When grown in broth the total phosphorous content of the Ps. aeruginosa (plaque strain) cell walls varied between two and three mg.%. No detectable variations occurred as the cells aged. The cell walls of the organisms taken from the CNT medium contained 1.0 to 1.8 mg.% of phosphorous, and, once again,

TABLE 29.

Composition of cell walls of Ps. aeruginosa and S. bethesda from cells grown in complex media under aerobic conditions.

All figures refer to the amount in mg. per 100 mg. dry weight of cell walls.

Age of cells.	Strain	Protein (aver.)	Total - P	Lipid Total	Carbohydrate*	Total %
24	Ps. plaque	58	2.2	18	8.3	87
96	Ps. plaque	57	2.0	16.5	6.9	82
24	Ps. plaque-free	66	1.6	16.1	13.0	97
96	Ps. plaque-free	68	1.8	16.3	14.2	100
24	S. bethesda	57	0.8	12.2	18.6	89
96	S. bethesda	55	1.2	14.0	13.0	83

* total reducing sugar.

TABLE 30.

Composition of cell walls of *Ps. aeruginosa* and *S. bethesda* prepared from cells grown in complex and defined media under anaerobic conditions. (mg. per 100 mg. dry weight of cell walls).

Age of cells	Strain	Medium	Protein (aver.)	Total- P	Lipid Free	Lipid Total	Carbo-hydrate [x]	Total %
24	Ps.plaque	Broth	50	2.3	8.2	15	9.6	77
72	Ps.plaque	Broth	59	2.9	8.4	15	-	-
96	Ps.plaque	Broth	62	2.6	-	10	-	-
192	Ps.plaque	Broth	65	2.4	8.4	10	8.0	85
24	Ps.plaque	C.N.T.	54	1.7	5.2	15	8.0	80
48	Ps.plaque	C.N.T.	50	1.1	-	15	-	-
64	Ps.plaque	C.N.T.	-	1.2	-	12	-	-
96	Ps.plaque	C.N.T.	61	1.4	-	-	-	-
192	Ps.plaque	C.N.T.	64	1.6	5.4	9	6.1	81
24	Ps.pl-free	C.N.T.	70	1.6	6.3	16	12.1	100
96	Ps.pl-free	C.N.T.	72	1.4	-	14	-	100
120	Ps.pl-free	C.N.T.	69	1.0	-	11	-	-
192	Ps.pl-free	C.N.T.	70	1.0	5.8	13	13.5	97

CONTINUED OVERLEAF......

TABLE 30 (cont'd)

Age of cells	Strain	Medium	Protein (aver.)	Total- P	Lipid Free	Lipid Total	Carbo-hydrate x	Total %
48	*S.bethesda*	C.N.T.	77	1.7	8.7	12	18.5	92
96	*S.bethesda*	C.N.T.	63	1.7	6.0	11	-	-
192	*S.bethesda*	C.N.T.	63	1.6	7.2	10	15.4	90

x Total reducing sugar.

- = not done.

Ps. plaque = *Ps. aeruginosa* (plaque strain).

Ps. pl.-free = *Ps. aeruginosa* (plaque-free strain).

any trends due to ageing were too small to be detected (Tables 29 and 30). The plaque-free strain and the S. bethesda cell walls gave similar total phosphorous estimates. No nucleic acid could be detected in the cell wall preparations of young or old cells grown aerobically or anaerobically.

The total recovery of cell wall material varied from 80 to 90% usually (Tables 29 and 30). Recoveries of the plaque-free cell wall material were almost 100%, due to an unexplained increase in total protein. The incomplete recoveries for the other two organisms indicated that a number of other undetected minor cell wall components had not been included in these balance sheets.

2. Composition of Young Ps. aeruginosa Cell Walls Incubated with the Concentrated Autolysin.

The cell walls of the plaque producing strain of Ps. aeruginosa underwent a number of changes in chemical composition with ageing which were not apparent in the plaque-free strain, or in the S. bethesda preparations. These changes could apparently be correlated with the appearance of autolytic enzymes in the culture media. However, a more direct proof of the association of these enzymes with the observed cell wall changes seemed desirable.

Cell walls prepared from 24 hour old Ps. aeruginosa (plaque strain) cells were incubated in the presence of the crude enzyme concentrate prepared from 192 hour old broth culture of Ps. aeruginosa (plaque strain). The cell walls were then washed and assayed for total protein, amino acid composition, carbohydrate and hexosamine content and total lipids. The results of these assays will be detailed in the following sections.

a. Total protein was determined by the Folin-Ciocalteau method only. As was expected from the absence of significant proteolytic activity in the enzyme preparation (vide p. 85) the protein content of the cell wall was not affected by incubation with the enzyme concentrate. The protein figures of 58 to 68 mg.% were similar to those already recorded for Ps. aeruginosa cell walls (c.f. Tables 21 and 31). The individual amino acids in the cell wall, estimated before and after treatment with the autolysin were recorded in Table 32. The total amino acid recovery from the three preparations was approximately 45 mg.% of the original cell wall material. After incubation with the enzyme preparation, only a very faint spot corresponding to DAP could be observed. The heated enzyme control did not remove significant amounts of DAP from the cell walls. In addition, the hydrolysed cell walls contained very little lysine and only a portion of their initial serine and alanine. The amounts of the remaining amino acids were not greatly affected by the enzyme treatment although the relative amounts of several acids, (e.g. glutamic and aspartic acids, phenyl alanine and leucine) did increase slightly. The similarity between the cell wall changes observed in this experiment and those recorded for the ageing Ps. aeruginosa cells (vide p. 109) suggested that the mechanism was the same in both cases.

b. Total carbohydrate. The cell wall total reducing sugar content did not vary greatly after incubation with the autolysin preparation. This fraction accounted for six to nine mg.% of the dry weight of the original cell walls (Table 31). Quantitative estimation of the individual sugars following chromatography disclosed that incubation with the autolytic enzyme resulted in the virtually

TABLE 31.

Composition of *Ps. aeruginosa* (plaque strain) cell walls following hydrolysis by the crude enzyme concentrate.

Amounts expressed as mg. per 100 mg. dry weight cell wall (original)

	Control	Enzyme	Heated enzyme
Protein (Folin-C).	58	63	68
Total reducing sugar	9	6	8
Glucose	3.2	0.6	2.9
Mannose	0.4	0.3	0.4
Rhamnose	2.0	1.9	1.9
Glucosamine	0.8	Tr.	0.8
Muramic acid	1.0	0.5	0.8
Lipid (total)	15	10	13
Lipid (free)	6	7.6	-
Total	82	88	89

Tr = Trace only.

\- = not estimated.

TABLE 32.

Amino acid composition of *Ps. aeruginosa* (plaque strain) cell walls following hydrolysis by the crude enzyme concentrate.

Amounts expressed as mg. per 100 mg. dry weight of cell wall.

Amino acid	Control	Enzyme	Heated enzyme
DAP	1.9	Tr.	1.7
Lysine	6.2	1.2	7.2
Serine	4.5	2.5	4.5
Aspartic acid	6.8	9.0	6.3
Glutamic acid	8.6	9.4	9.6
Alanine	5.9	3.8	5.3
Tyrosine	1.9	1.6	-
Proline	+	+	+
Histidine	1.0	1.3	1.1
Arginine	0.8	0.8	0.9
α amino butyric acid	0.8	1.0	0.8
Valine	1.1	1.4	1.0
Methionine	0.6	1.0	0.7
Leucine + isoleucine	2.6	3.5	3.0
Phen. alanine	4.7	5.7	4.3

Tr = trace.

\- = not estimated.

\+ = spot detected but not estimated quantitatively.

complete removal of the glucose and glucosamine residues from the cell wall. The muramic acid content of the hydrolysed walls decreased by almost 50%. The mannose and rhamnose content was not affected. The heated enzyme control showed no corresponding losses of cell wall carbohydrate or amino sugar.

c. Total lipid. Incubation of the cell wall with the autolysin resulted in a decrease in the total lipid content from 15 mg.% to 10 mg.% (Table 31). The free lipid content was not apparently affected. Thus, the results of this experiment again suggest that the removal of some lipoidal complex from the cell wall is an intregral step in the attack by the autolytic enzymes on the *Ps. aeruginosa* cell wall.

The assays for protein, carbohydrate and lipid accounted for 80 to 90 mg.% of the dry weight of the cell wall (Table 31). In general, these figures agreed favourably with those already obtained for the ageing cell walls (Table 30).

Conclusions to Chapters VII, VIII and IX.

As one of the outermost structures of the bacterial cell, the integrity of the rigid cell wall appears to be necessary for the continuance of cellular growth and viability in hypotonic media. Thus, the cell wall seems to be the most likely site of attack by the autolytic enzymes demonstrated in an earlier section of this study to be present in the medium. The quantitative studies of the amino sugar, carbohydrate, amino acid and fixed lipid content of the ageing cell walls of the plaque strain of *Ps. aruginosa* suggested that the autolytic enzymes removed a definite portion of the cell wall so that the cell was then no longer osomotically stable, and

cell lysis resulted. The absence of demonstrable autolytic enzyme activity in the plaque-free or in the *S. bethesda* culture media could be correlated with the absence of extensive cell lysis in these cultures. Thus, a sequence of enzymic and chemical changes appeared to occur within the cell walls of the plaque strain of *Ps. aeruginosa*. These changes were very similar to those observed when the cell walls were incubated with the partially purified autolytic enzyme obtained from aged cultures of the plaque strain of *Ps. aeruginosa*. Thus, the demonstrated changes in cell wall structure were at least partly responsible for the sequence of events observed when the viable and total populations of ageing cultures of this strain were studied.

The overall impression gained from this study of the transition from active growth to senescence and cell death must remain almost one of surprise at the constancy in the composition of both the cell and the cell wall throughout this period. The demonstration that small variations occurred to the amino acid and carbohydrate content of the lysing cell walls suggested that other subtle and important changes may occur to the cellular composition during the ageing process. However, the demonstration of such changes will probably depend on the development of more refined assay techniques in the future.

CHAPTER X.

DISCUSSION.

The problems associated with the growth and multiplication of cells are amongst the most fascinating and complex in biology. Because of the absence of many of the complicating factors found in the higher forms of life, bacterial cells constitute some of the most useful research tools at present available for such studies. Despite the apparent simplicity of these unicellular organisms, the behaviour of the macromolecular constituents is similar in all living cells, and many of the findings for bacteria may be applied to the higher plants and animals also.

In a similar manner, bacteria should be suitable tools for the study of cell decay and death. However, while the problems associated with cell growth and multiplication have received much attention, the subsequent phases of death and decay have not been as fully examined. The sensitivity of Ps. aeruginosa to anaerobic incubation made possible the study of the processes of decay under controlled conditions. From these studies, rapid death of Ps. aeruginosa cells was found to contrast sharply with the behaviour of S. bethesda. This difference in behaviour was replicated many times, and appeared to be real. However, the possibility existed that the viable counts done on the two cultures may not have always represented a true estimate of the number of living cells present.

The direct comparison of two different bacterial populations is subject to certain assumptions, the most important of which is that the plate count is an accurate means of estimating the viable numbers of different bacteria grown under a variety of experimental conditions.

The usual definition of viability depends on the assumption that the ability of each cell to develop into a visible colony was equal when placed on the surface of a nutrient agar plate. Comparison of the *Ps. aeruginosa* counts with those of *S. bethesda* was made on the assumption that equal numbers of viable cells of both organisms would develop into the same number of colonies, irrespective of any differences in the cultural and environmental conditions of the original cultures. However, in any plate count there must be a time lag of eight to ten hours before a visible colony can develop from a single viable cell, and during this time we have no idea of the events occurring within the cell.

The problem of viable but sterile cells may be a very important one. Spoerl, *et.al.*, (1954) reported an overall increase in the dry weight of irradiated *E. coli* even after the viability of the culture had apparently been reduced to one per cent. The well know reactivation effect of visible light on cells irradiated with ultra-violet light indicates that cell sterility does not necessarily lead to the immediate death of the cell (Jagger, 1958). Even the direct visual counting of total and viable cells on an agar surface recommended by Powell (1956) failed to distinguish between dead cells and viable but sterile cells. Thus, it is doubtful whether we may validly assume that different bacteria grown under a variety of environmental conditions will all behave identically when placed on an agar plate, but at present there is no available alternative.

The question of cell viability was further complicated by the potential toxicity of many of the commonly used diluents for bacterial suspensions. Even with closely related strains, the

severity of this toxicity varied considerably (Winslow and Brooke, 1927; Straka and Stokes, 1957). Suspensions of Ps. aeruginosa were reported to be highly sensitive to the lethal effects of saline (Stokes and Osborne, 1956), a finding which could not be confirmed in the present study (vide p. 74). No obvious reason could be found for the increased toxic effect of distilled water or saline as compared with that of phosphate buffer. However, any lethal effect of the saline in the present viable counts was minimised by ensuring that the cells were in contact with the saline for as short a time as possible. The dilution procedure was therefore rigidly standardised.

Even when viable cells were introduced onto the surface of an agar medium, various nutritional factors may still interfere with the accuracy of the count. Curran and Evans (1937) reported that the recovery of irradiated B. subtilis spores was higher on complex media than on basal media, but the actual enrichment substances varied with the bacterial species under study. Similar findings have been reported by Nelson (1943) and Straka and Stokes (1959). Alper and Gillies (1958) noted higher recoveries of irradiated E. coli cells on a semi-deficient agar compared to that found on complex media. This difference was ascribed to cell lysis due to an unbalanced cell growth on the complex medium. Thus a number of errors may occur and give a completely erroneous impression of the viability of the cells. The only way by which such criticism could be overcome would be to change the methods of assessing bacterial viability.

The present difficulty revolves around the impossibility of detecting viable but sterile cells by the plate count technique. As these cells may still be of metabolic importance in the culture it may be misleading

to consider them as "dead". For instance, the increase in the size of the cell following ultra-violet irradiation indicates that a considerable amount of growth may occur after the cell has lost its ability to replicate (Spoerl, et.al., 1954). Viability has been defined as a property of any "portion of matter endowed at any one time with self-maintaining organisation" (Luria, 1960). However, this does not help greatly as it merely moves the problem one stage back to that of demonstrating the "self-maintaining organisation" in each bacterial cell. Possibly a technique which could demonstrate the continuation of chemical synthesis by the cell could be adapted to a micro-scale, and so become a useful criterion of continued cell viability. The synthesis of DNA by the cell could be one such activity. Such a method would however depend on an accurate micro-assay of the increasing intracellular DNA content of individual cells, and this is not practicable at present.

Another method of demonstrating viability might be to demonstrate the continued production or utilisation of energy within the cell. Cellular motility requires the expenditure of energy, and the demonstration of motion by an individual cell would indicate viability for that cell. However, such a criterion has a number of serious disadvantages, not the least of which would be its lack applicability for non-motile species. It is well known that motile bacteria can be rendered non-motile without affecting their ability to produce colonies, and the progressive loss of motility by ageing cultures is well known (Topley and Wilson, 1955). Finally, active motility can be shown to depend on the cultural conditions (Sherris, et.al., 1957). The strain of *Ps. aeruginosa* used in the present study was only sluggishly motile in young aerobic cultures,

while neither flagella nor motility could be demonstrated in the anaerobically incubated cultures. Therefore, motility was not a suitable criterion for the present study.

The use of vital stains has been recommended by Bishop and Smiles (1957) for the differentiation of living and dead spermatozoa. Similarly, Delaporte (1956) stained granules within living bacterial cells with dilute Neutral Red. However, Knaysi (1951) does not consider that the so-called vital dyes are, in fact, absorbed by healthy living cells, but enter only when the permeability of the cell membrane has been seriously affected by the dye. Thus the term "vital" stain may well be a misnomer.

Perhaps the most rigorous criterion of cell death would be the direct microscopic verification that protoplasmic streaming has ceased within the cell. Bacteria are not generally believed to exhibit this phenomen, which has, however, been used successfully for animal and plant cells (Murray, 1960). Evidence of protoplasmic streaming in bacteria has been only of an indirect nature (Whitfield and Murray, 1955), and little more can be done until a more direct technique has been discovered for the visible demonstration of this phenomenon. Thus the plate count still remains the best available means for estimating cell viability. The errors inherent in the method may be minimised as far as possible by rigeurous standardisation of the technique, and by a comparison of the relative changes in viability in the different cultures, rather than reliance on absolute values of viability.

The total cell count methods at present available also varied considerably in their accuracy. Turbidity measurements were rejected as unsuitable for the present study for several reasons. This method

measures the total quantity of cellular protoplasm present and makes no allowance for the extensive variations in the average cell volume during the growth cycle (Henrici, 1923). Serious errors may also be introduced if the cell suspensions are granular and non-homogeneous, or if the cells have a tendency to clump (Mitchell, 1950). As they aged, *Ps. aeruginosa* suspensions became very granular, and the older cells showed a marked tendency to aggregate into small clumps, probably because of the presence of mucoid materials released into the medium by the lysis of some of the cells. In any case, the turbidity of the culture had to be related ultimately to total cell numbers as determined microscopically, and so the tedious but more accurate method of direct microscopic counting was preferred. For much the same reasons, estimation of total cell numbers from dry weight estimations was considered to be unsatisfactory for the present purpose.

The Williams (1952) total count method was found to give the most reproducible results, especially for the ageing cultures. The use of dark ground illumination was preferred to phase contrast since the cell walls were more clearly visualised by the former method of illumination. Provided that a very intense light source was used, the intact cell walls could be distinguished from those of the disintegrating cells, and reproducible whole cell counts were possible even with eight-day old cell suspensions. Despite the possible errors associated with the viable and total count methods, the observed differences in the behaviour of the decline phase cultures of *Ps. aeruginosa* and *S. bethesda* were sufficiently large to suggest that real differences existed in the behaviour of the two organisms under strictly anaerobic conditions.

As expected, the intracellular events responsible for the death of

the cells were too subtle for detection by existing techniques. Investigation of the influence of environmental changes on viability was unsatisfactory, with one possible exception. Examination of the influence of nitrate ions on the continued viability of anaerobic cultures of Ps. aeruginosa revealed that the continuous presence of nitrate in the medium virtually inhibited the toxic effects of prolonged anaerobic incubation. Prolonged incubation under strongly reducing conditions apparently led to certain metabolic changes within the cells which ultimately affected the ability of the cells to multiply. The presence of abnormally large amounts of certain organic acids in the anaerobic medium of the decline phase Ps. aeruginosa cultures (vide p. 73) was an interesting finding, and in direct contrast with those of Kluyver and his co-workers who had been unable to detect any intermediaries of glucose metabolism by denitrifying cultures of Ps. aeruginosa (Verhoeven and Goos, 1954). The prolonged anaerobic incubation of Ps. aeruginosa in the present study apparently caused extensive changes to the normal glucose metabolism of the cell, resulting in an accumulation of these acids in the medium. The ability of Ps. aeruginosa under aerobic conditions to utilise lactic, succinic or citric acid as sole sources of carbon suggests that these acids accumulated in the anaerobic medium because the cells were able to utilise them further only by an oxidative type of metabolism. Of the acids tested, only fumaric acid was able to support the anaerobic growth of Ps. aeruginosa (vide p. 71) and it was interesting to note that fumaric acid did not accumulate to any extent in the decline phase culture medium (Table 3).

The Ps. aeruginosa cells were apparently unable to carry out

extensive linked oxido-reductions between the various glucose intermediaries and so were unable to continue growing anaerobically. The severe limitation in the energy supply under anaerobic conditions would be expected to prevent further growth and replication by the cell but, on its own should not result in the rapid and extensive mortality which was, in fact, observed. Thus, other factors besides bacteriostasis appeared to be likely lethal factors. The development of highly reducing conditions within the culture may have resulted in the simultaneous reduction of one or more vital enzyme systems or free radicals within the cell. Such strongly reducing conditions might therefore be expected to have a highly deleterious effect on the internal economy of a strictly aerobic organism.

The decreases in viability, as well as in total cell numbers, in anaerobically incubated cultures of _Ps. aeruginosa_ agreed with the earlier report of extensive lysis following anaerobic incubation of _B. subtilis_ (Nomura and Hosoda, 1956a). These workers reported that the initiation of cell lysis appeared to depend on the release of an autolytic enzyme by the disorganised cell. Kauffman and Bauer (1958) noted a similar lytic phenomenon when the cytochrome oxidase of _B. subtilis_ was poisoned. Apparently the reducing conditions activated the lytic enzymes within the cell, and extensive autolysis resulted. These workers also postulated some protective mechanism within the aerobic cell which inhibited these lytic enzymes. While such a reductive type of reaction may have been responsible for the rapid death of _Ps. aeruginosa_, the onset of cell lysis was slower than that reported for the Bacillus cultures, and other factors were therefore thought to be involved in the initiation of autolysis by

Ps. aeruginosa.

The surviving Ps. aeruginosa cells detected after prolonged anaerobic incubation did not show any increased resistance to anaerobiosis when re-cultured. Thus, the surviving cells merely represented a statistically resistant minority present in the culture (Jordan, et.al.,1947). The occasional, limited increases in the viable numbers observed during the decline phase of Ps. aeruginosa cultures were ascribed to "cannabalism" of the dead and lysing bacteria in much the same sense as was used by Steinhaus and Birkeland (1939). All attempts to isolate a mutant strain of Ps. aeruginosa able to grow vigourously under strictly anaerobic conditions were unsuccessful, although lysis-resistant strains were easily obtained.

In view of the extreme sensitivity of the Ps. aeruginosa cells to prolonged incubation under anaerobic conditions, an interesting comparison of the survival of this organism and S. bethesda in freeze dried cultures can be made. Suspended animation has been used for many years as a means of preservation of micro-organisms, but until recently very few quantitative studies of their survival under such conditions have been reported. Most workers have been content merely to demonstrate the survival of some cells after a period of months or years (Engley, 1956). Fry and Greaves (1951) demonstrated the importance of the suspending medium for high survival rates of most organisms in freeze dried cultures. Later, Annear (1958) emphasised the variability in the sensitivity of different organisms to the drying process. Thus, extensive variations in the survival of freeze dried cultures of the present strains might be expected.

However, freeze dried cultures of both Ps. aeruginosa and S. bethesda remained viable for at least two years. Quantitative viability determinations carried out after a period of one and four weeks indicated that within the limits of experimental error, the decline in viability was of the same order for both organisms. Thus, no significant difference between the two organisms was noted when the cells were preserved in the freeze dried state. The processes resulting in cell death are obviously dependent on a number of incompletely understood factors and there seems little point in discussing further this aspect of the study until more information is available on the internal organisation of the cell itself. The present discussion will therefore turn to some of the factors involved in the lytic changes associated with the ageing Ps. aeruginosa cultures.

The sensitivity of Ps. aeruginosa (plaque strain) cells to lysis, compared with the plaque-free mutant or with S. bethesda raised the important question of the mechanism of this lysis, together with the reasons for the resistance of the mutant. Earlier workers have referred, almost in passing to the occurrence of lysis-free strains of Ps. aeruginosa, but no systematic study has been made of the possible biochemical mechanism responsible for the change. The occurrence of lysogeny in Ps. aeruginosa has now been firmly established (Warner, 1950b; Holloway, et.al., 1960), and the phages have been used as a typing aid for both human and animal pathogens (Habs, 1957; Sandvik, 1960). However, phage lysis is essentially a phenomenon associated with young actively multiplying cells, but, by the time lysis of the anaerobic Ps. aeruginosa cultures was observed,

more than 99% of the cells present were dead. This does not completely exclude the possibility of continued lysis of the dead cells by phage enzymes produced during the earlier growth phases. Weidel (1951) and Weidel and Primosigh (1957) isolated cell wall degrading enzymes associated with an E. coli bacteriophage, while Murphy (1957) isolated a lytic enzyme from phage lysates of B. megaterium. Jacob and Fuerst (1958) have recently described the release of an "endolysin" from ultra-violet irradiated E. coli K12 cells. The release of this endolysin was ascribed to the activation of the phage by the irradiation. Welsch (1958), however, suggested that much of the extensive lysis which followed phage infection was due to simultaneously released autolysins, which then attacked the surrounding cell walls, producing further lysis. Thus, spontaneous lysis by the lysogenic phages of Ps. aeruginosa may well be the initiating step in the release of the autolytic enzymes in ageing cultures. The small amount of enzyme released by the initial spontaneous phage induced lysis would gradually increase as the surrounding cells lysed to release more enzyme into the medium. The effect of the autolytic enzymes would therefore become more apparent as the decline phase progressed. Autolysis of the ageing Ps. aeruginosa cultures appeared to follow a pattern consistent with the above hypothesis.

The normal architecture of the living cell is such that the hydrolytic enzymes in the cytoplasm are inactive, and lysis "from within" cannot occur while the cell is alive (Welsch, 1958). Following the death of the cell, the intracellular organisation is not maintained and autolysis may result. In the anaerobic

Ps. aeruginosa cultures almost 100% of the cells were dead within 24 hours of the exhaustion of the nitrate supply. Thus if autolysis of these cells occurred "from within" one would expect the total count would parallel the sharp fall in the viable curve. The absence of extensive autolytic activity in the plaque-free *Ps. aeruginosa* cultures could thus be due to an absence of a prophage "trigger". The absence of both "irridescent" plaques and spontaneous lysogenic phage plaques in growth of the plaque-free strain of *Ps. aeruginosa* on TSA plates could be used as supporting evidence for this theory.

Such a theory was unable to explain the limited amount of lysis (10% to 20%) observed in the anaerobic plaque-free *Ps. aeruginosa* cultures. If we assume that lysis resistance was due to a prophage mutation then the observed 20% lysis of the plaque-free cultures may be due to some unknown secondary lytic phenomenon. Irrespective of the mechanism of the observed lysis of the plaque-free cells, one would expect the destruction of these cells to result in the release of autolytic enzymes into the medium, with the ultimate lysis of most of the cells present. Thus, the absence of early lysogenic bursts would be expected to cause an increased lag before lysis was detected, but the lysis curves for the parent and mutant should thereafter be parallel, and the final percentage of lysis in both plaque and plaque-free cells would be expected to be about the same. The inability to demonstrate the presence of lytic enzymes in old cultures of the plaque-free strain suggested that the intracellular changes responsible for lysis resistance were more complex than a simple prophage mutation. The resistance of the plaque-free cells to lysis seemed rather to depend on the absence of the lytic enzymes from the culture medium. Thus, the plaque-free

cells may have gained the ability to produce an intracellular inhibitor which prevented the synthesis of the autolytic enzymes by the cell. However, as stated above, the actual factors responsible for the apparent absence of the lytic enzymes from the plaque-free cultures could not be determined.

The "irridescent" lytic phenomenon observed in Ps. aeruginosa cultures has traditionally been considered to be of non-phage origin, largely because of repeated failure to isolate a propagating strain of the hypothetical phage. The late development of the plaques on solid media has also been cited as evidence against a phage-actuated mechanism (French, 1947). However, in some of the cultures of Ps. aeruginosa examined in the present study, typical lysogenic phage and "irridescent" plaques were observed to develop at about the same time in the same culture. The claim that the "irridescent" plaques arose only in old cells (48 to 72 hours), while phage lysis occurred only in young cells was made on the assumption that all the cells in a colony were of the same age and at the same stage of biochemical development. The existence of such a uniform state was most unlikely in the slowly enlarging colony of a strictly aerobic organism. One would expect that the surface cells would continue to multiply while the deeper cells would be in the decline phase, or even dead. Thus, the surface layer of cells in a two or three day old colony may be in an active physiological state quite suitable for phage growth. While the author agrees that the "irridescent" plaque does not seem to be due to a normal type of phage action, the evidence so far tendered as proof that a phage is not involved is far from conclusive. This is particularly so in view of the relatively small number of possible propagating strains so far tested (Don and van den Ende, 1950; Warner, 1950a;

The circumscribed appearance of the "irridescent" plaques suggested that the initiation of lysis occurred in a single cell or small group of cells (vide Plate 1). Such a phenomenon was unlikely to occur if an autolytic enzyme was released by all the dead cells in the culture. Thus, the known facts seem to indicate an unusual and atypical type of phage-induced lysis, quite distinct from the usual lysogenic burst (Holloway, 1960; personal communication). The observation that the "irridescent" plaques were produced only when the growth of the colony reached a critical density may mean that a specialised local environment (e.g. a critical oxygen tension, or food concentration) was essential before the "irridescent" plaques could appear. Thus, the repeated inability in the present study to demonstrate the formation of "irridescent" plaques under anaerobic conditions led to the conclusion that this form of lytic change was unlikely to play an important role in the lysis of the anaerobic liquid cultures.

The rapid death and extensive lysis of the Ps. aeruginosa cultures suggested that a number of important cellular changes must occur as the cells age. Since S. bethesda was not subject to the lethal effect of anaerobic incubation, it seemed worth while to compare the chemical composition of the two organisms during ageing. A search of the available literature revealed several investigations of the chemical composition of Pseudomonas strains, but very little quantitative information exists on the makeup of Ps. aeruginosa (Smithies, et.al., 1955; Salton 1953a). As a rule, chemical investigations of the Gram negative bacteria concentrated mainly on the Coliform-Salmonella group (Knaysi, 1951). The S. bethesda cells were therefore useful as controls for the various assays employed in the present study.

The protein, nucleic acid, lipid and carbohydrate content of the <u>Ps. aeruginosa</u> cells constituted up to 90% of the cell dry weight, indicating that some minor components were still unaccounted for. However, the exact extent of the disparity was uncertain, since the results of some of the assays varied considerably, depending on the method used. This was particularly so with the protein and carbohydrate estimations. The protein content as estimated by the Folin-Ciocalteau method, was 10 to 20 mg.% higher than the corresponding Biuret figures. This effect was due chiefly to differences between the composition of the standard protein and that of the bacterial proteins. The colour produced by the Folin-Ciocalteau reagent was proportional to the amount of tryptophane and tyrosine in the protein (Kabat and Meyer, 1948). On the other hand, the Biuret reaction was proportional to the number of peptide linkages in the protein. In the absence of highly purified bacterial preparations, bovine albumin was used as a control for both estimations. Thus the higher protein estimates obtained with the Folin-Ciocalteau reagent were probably due to a smaller relative amount of tryosine and trytophane in the bacterial protein as compared with standard bovine albumin. The relative proportions of the aromatic amino-acids in the bacterial proteins as compared with the bovine albumin were not determined, as trytophane was destroyed during the acid hydrolysis prior to chromatographic separation of the constituent amino-acids (Polson, 1948). The total nitrogen figures could not be used to check the protein content of the cells because of the presence of a considerable amount of non-protein nitrogen. Therefore, in the absence of a pure bacterial protein standard, an average of the two protein determinations was taken as the most accurate estimate of the true

protein content of the cells available.
The total carbohydrate content of the cells was estimated using a glucose standard. Since a large proportion of the cellular carbohydrate consisted of pentoses, methyl pentoses and amino sugars, as well as the various hexoses, the colours produced by the different reagents varied considerably. The total reducing sugar content of the hydrolysed whole cells or cell walls was consistently higher than was obtained by the colourimetric methods and these figures were therefore accepted as an estimate of the maximum carbohydrate concentration in the cell. They agreed quite well with the total carbohydrate figures estimated from the chromatograms, and were therefore accepted as the closest available approximation of the total carbohydrate content of the cells or cell walls. Similarly, the differences in the reducing sugar content of the plaque and plaque-free cell walls could be correlated with the changed glucose content of the mutant cells, as estimated chromatographically.

The Dische and the Orcinol reagents reacted best with the pentoses so that the use of a glucose standard led to obvious inaccuracies in the final estimates of total carbohydrate by these methods. On the other hand, the Anthrone test depended on the reaction of this reagent with a furfural derivative produced by the dehydration of the carbohydrate by concentrated acid. The furfural reaction usually resulted in the destruction of some of the carbohydrate, and so the lower recoveries by this method were not surprising. The total reducing sugar and the Anthrone estimations were recorded for most of the preparations as representing the two extremes for the carbohydrate content.

On the whole, the Ps. aeruginosa (plaque strain) whole cells

closely resembled the corresponding _S. bethesda_ preparations in their overall chemical composition. Of the strain differences which were sufficient to deserve comment, the increase of 10 mg.% in the protein - N which occurred when the plaque strain cell mutated to the plaque-free form was the most difficult to explain. The absence of any corresponding decrease in the other cell components suggested that this was a real increase in the relative amount of protein in the cell. A similar increase in total protein content was observed in the cell walls of the plaque-free mutant. The rise averaged 15 to 20 mg.% suggesting that much of the protein increase noted in the whole cells was associated with the wall proteins.

Another change worthy of comment was the sudden decrease in cellular RNA when the cells reached the end of the logarithmic growth phase. The rapidity of the decrease prompted an investigation into the possible reasons for the change, together with the fate of the nucleic acid. The correlation between the cellular RNA peak and the end of the logarithmic growth led a number of workers to suggest a connection between the RNA and the initiation of cell division (Wade, 1952; Davidson, 1953). Wade and Morgan (1957)demonstrated the presence of two distinct RNA-containing fractions in growing cells, and suggested that the fluctuating RNA was specifically concerned with the initiation and control of cell division. The similarities in the rate of increase in cellular RNA and the viable population observed during the present investigation agreed with this hypothesis, which also explained the ability of the cells to dispense with the "fluctuating" nucleic acid once active cell proliferation ceased. The suggestion by Caldwell, _et.al._, (1950) that the RNA was concerned with protein synthesis was also compatible with the observed variations.

Conversely, the conservation of cellular DNA agrees with our present concepts of the function of this material in the cell economy (Stuy, 1958; Thompson, et.al., 1958; Horiuchi, et.al., 1959).

The virtual absence of RNA from the anaerobic Ps. aeruginosa culture media probably arose from the almost complete disappearance of this nucleic acid from the cells by the time lysis became apparent. The mechanism of this intracellular RNA degradation seems to be in some doubt. For instance, Jones, et.al., (1957) observed considerable RNase activity in A. aerogenes cultures. Other workers (including the present author) were unable to demonstrate any nuclease activity (Catlin, 1956). The rapidity of the RNA removal by the stationary phase cells strongly suggested the presence of a definite degradative pathway, resulting in the release of free nucleotides within the cell.

The absence of RNase activity in the Ps. aeruginosa cultures suggested that the intracellular hydrolysis of the RNA was due to the action of specific phosphorylases which released the constituent nucleotides for re-synthesis into other cellular components. If we assumed that the stationary phase cells were unable to produce all the purines and pyrimidines necessary to maintain even limited cell growth and multiplication, then the cells may well utilise the "fluctuating" RNA as a food reserve. The demonstration by Park and Strominger (1957) of an uridine nucleotide in the cell walls of Staph. aureus indicates a possible fate for at least part of the RNA molecule. Recently Baddiley, et.al., (1956) showed that cytidine diphosphate was concerned in the synthesis of the teichoic acids in L. arabinosus cell walls. Thus, the limitation or absence of free nucleotides in the stationary phase cells may be due to an inability of the older cells to produce all their pyrimidine requirements. Such a

deficiency may limit cell wall synthesis and ultimately prevent the growth of the cells. Toenies and Shockman (1953) and Meadow, et.al., (1957) have shown that limitation of key cell wall constituents may limit cell wall synthesis, ultimately resulting in the lysis of the cells. Thus, the stationary phase cell may be forced to utilise the ready-made pyrimidines in the "fluctuating" RNA, or be unable to continue synthesising cell wall material. The simultaneous decrease in the RNA content of ageing S. bethesda cells suggested that any such postulated limitation of cell wall synthesis due to pyrimidine deficiency was not sufficient in itself to cause extensive lysis of the cell, however.

The demonstration that the base proportions of the cellular nucleic acids of Ps. aeruginosa and S. bethesda did not change appreciably as the cell aged, means that the findings of previous workers (Wade and Morgan, 1957; Stuy, 1958; Catlin and Cunningham, 1958) has been extended into the late stationary and decline phases. The close similarity between the base composition of the intracellular and extracellular DNA confirmed the belief that the DNA release occurred only after the lysis of the cell (Takahashi and Gibbons, 1957; Newton, 1953; Catlin and Cunningham, 1958). The DNA in the medium recorded base proportions slightly different from that observed in the young cells (c.f. Tables 13 and 15), but the changes were small and no evidence was obtained that partial hydrolysis of the nucleic acid occurred prior to its excretion by the intact cell.

The nucleic acid variations in the cells, and their presence in the culture media, formed only a portion of the overall investigation of the composition of the cells. The determination of the other cellular constituents made a contribution to the available information on the

composition of the _Ps. aeruginosa_ cells. The absence of significant changes in the cellular composition (other than in RNA) over the eight day period of the experiments may simply reflect our present inability to detect the small but significant changes in molecular architecture by chemical means. Dagley and Sykes (1957) detected extensive changes in the ultracentrifuge patterns of extracts of starving _E. coli_ cells when compared with the normal controls. Such extensive macromolecular changes within the cell were too subtle for detection by ordinary chemical analysis, but may have been of the highest significance so far as the cellular economy was concerned (Mitsui, _etlal._, 1960). In addition, highly significant changes which occurred to only a small portion of the cell would not be easily detected if there was a large pool of unaffected material also present. However, extraction of the affected fraction should allow chemical or immunological detection of these changes. One of the cell structures which could be isolated readily was the cell wall, and, since cell lysis would be expected to bring about extensive changes to its chemical composition, attention was switched from the whole cells to the isolated walls.

The development of an efficient means for rupturing the cell has made the isolation of pure microbial cell walls a routine procedure. Pure cell walls washed free of intracellular materials could be obtained easily with young cells, but as the cells aged granular cytoplasmic material seemed to remain within the cell envelope (Salton and Horne, 1951a). Extended sonic disintegration broke up the cell envelope releasing the trapped cytoplasmic granules. However, such drastic treatment resulted in the complete dissolution of much of the cell wall. Marr and Cota-Robles (1957) estimated this loss to be as high as 40%

for *A. vinelandii*. For this reason, no attempt was made at quantitative recovery of cell walls in the present study. The partial solubilisation of the cell wall probably explained the low recoveries of lipopolysaccharide from the purified cell walls. However, the disadvantages of the lower yield were preferred when clean cell wall preparations could be obtained without the need for gradient sedimentation (Ribi, 1960, personal communication).

A considerable amount of data on the chemical composition of bacterial cell walls now exists, but the majority of it refers to the Gram positive bacteria and to *M. lysodeikticus* in particular (Salton, 1958). This is due, at least partly, to the extensive studies into the chemical alterations produced by various lytic factors, (particularly by lysozyme) on the Gram positive cell walls (Salton, 1960). In general, however, less work has been carried out using the cell walls of Gram negative bacteria. Much of the available information on this group of micro-organisms was obtained using coliforms, and only limited investigations have been made with other genera. These studies indicate that differences may occur in various cell wall components, and suggests the need for more systematic studies of the cell wall composition of a wide variety of Gram negative genera (Salton,1960).

The absence of extensive investigations of the composition of *Ps. aeruginosa* cell walls was one of the reasons for the present study. As well as contributions to this field of knowledge,the comparison of the cell walls of the two *Ps. aeruginosa* strains revealed that the mutation to plaque resistance was accompanied by a number of quantitative changes in cell wall composition. Thus, the mutation did not merely involve the loss of lysogenicity by the *Ps. aeruginosa* cells, but also involved a

number of other changes, the significance of which is at present unknown. One of the most puzzling of these changes was the 20 mg.% increase in total protein previously discussed (*vide* p.134). This rise was unlikely to be due to a significant decrease in the trytophane or tyrosine content of the cell wall proteins since the Biuret figures also showed a corresponding rise. The higher amino acid recoveries from the corresponding chromatograms of the cell wall hydrolysates confirmed the belief that a real increase had occurred in the relative amount of protein in the plaque-free strain cell walls. The higher protein content resulted in a 100% recovery of cell wall substance compared with the recoveries of 80 to 90% for most of the other cell wall preparations. The absence of any corresponding decreases in the other wall constituents of the plaque-free strain was puzzling, and it seemed difficult to imagine that the minor constituents represented by the missing 10 to 20 mg.% in the plaque strain cell walls disappeared following the mutation to plaque resistance. However, no obvious explanation for the observed increase could be found.

The only report of major changes in cell wall amino acid composition during ageing was made by Graziosi and Tocco (1957). However, these results have been severely criticised on technical grounds by Salton (1960). The amino acid content of the cell walls of the plaque-free strain and of *S. bethesda* increased slightly as the cells aged, and thus resembled the ageing cell walls of *Str. faecalis* described by Shockman, *et.al.*, (1958). However, the finding that the amino acids alanine, lysine, serine and DAP decreased as the plaque strain cells aged, was particularly interesting in view of their importance in the cell wall mucopeptide (Salton, 1958). The amount of these amino acids decreased at

a time when cell lysis was first noticed, and this suggested that their removal could be concerned with the lytic phenomenon.

The process of ageing did not appreciably affect the carbohydrate composition of cell walls of the plaque-free strain or of _S. bethesda_. In fact, the small decrease noted in the total carbohydrate content, combined with the increased protein content, was quite consistent with the immunological evidence for a progressive loss of smooth antigens. On the other hand, chromatographic estimation of the plaque strain cell wall sugars revealed a decrease in the glucose and, to a lesser extent, in the rhamnose content as the cells aged. Significantly, most of the decrease coincided with the initiation of cell lysis in the anaerobic culture. A similar decrease in glucosamine, but not in muramic acid suggested that the autolytic enzymes released much of the glucose and glucosamine (probably as N-acetyl glucosamine) from the wall. Experiments with a wide variety of micro-organisms have made it clear that removal of the entire cell wall was not necessary for the initiation of cell lysis(Norris, 1957; Slade and Slamp, 1960.) In fact, several workers have expressed doubts whether the cell walls of Gram negative bacteria were ever completely digested away during "protoplast" formation (McQuillen, 1960). Thus, the retention of considerable amounts of the cell wall carbohydrate by the plaque strain was not incompatible with the theory that the progressive decrease in cell wall glucose and glucosamine was at least partly instrumental in causing cell lysis.

The results of the chemical assays carried out on the ageing _Ps. aeruginosa_ cell walls showed reasonable correlation with the later studies carried out on the autolysin digested walls, suggesting that the observed lysis of the _Ps. aeruginosa_ cultures was due to the hydrolytic

action of the autolytic enzymes released by the cells into the medium. Efforts were made to determine the probable composition of the Ps. aeruginosa cell wall mucopeptide. Examination of the products of E. coli cell wall digestion by lysozyme indicated that a N-acetyl glucosamine, N-acetyl muramic acid chain linked to a peptide of alanine, glutamic acid and lysine or DAP was released (Salton, 1958). O'Brien and Zilliken (1959) reported the occurrence of peptides containing alanine, glutamic acid and aspartic acid resides in cultures of E. coli. All the available evidence so far indicates that mucopeptides of this type occur in the cell walls of most of the Gram negative bacteria. These complexes also seem to form the substrates for lysozyme and bacteriophage enzyme attack (Weidel and Primosigh, 1957; Salton, 1960). Relatively little information is available on the site of action of the autolytic enzymes on the cell walls of Gram negative bacteria although a number of reports have been published on cell wall digestion by more or less purified enzymes obtained from various sporing Bacilli (Dark and Strange, 1957; Strange, 1959). The results of the present study suggest that the autolysin of Ps. aeruginosa removes a mucopeptide from the cell wall. The autolysin differed in its action from lysozyme by removing the cell wall glucose residues as well as the glucosamine but apparently left a considerable amount of the muramic acid in the cell wall. If we assume that, as in E. coli (Brumfitt, et.al., 1958) the peptide was linked to the glucosamine: muramic acid chain through the muramic acid, then it is difficult to visualise how the autolysin could remove the peptide and the glucosamine moiety without at the same time releasing the muramic acid residue.

Several possible explanations for this puzzling phenomenon were

considered. In *Ps. aeruginosa* cell walls some of the muramic acid present may have occurred as a part of the cell wall structure which was not attacked by the autolysin which therefore removed only that portion of the total muramic acid which was associated with the mucopeptide. While the occurrence of muramic acid in other fractions of the *Ps. aeruginosa* cell wall besides the mucopeptide could not be discounted, it was not considered to be very likely in view of the known cell wall composition of all other Gram negative bacteria so far examined.

Another explanation could be based on the different characteristics of lysozyme and the Pseudomonas autolysin. Lysozyme is a single enzyme which attacks the β 1-4 links between the N-acetylmuramic acid and the N-acetyl glucosamine (Brumfitt, *et.al.*, 1958), resulting in the release of the mucopeptide complex. On the other hand, the Pseudomonas autolysin contains several enzymes which may result in the more complete hydrolysis of the mucopeptide molecule. Thus, the components of the mucopeptide may be released as separate molecules. If such hydrolysis occurred *in situ*, the muramic acid may remain attached to the cell by secondary linkages not attacked by the autolysin while the remaining components diffuse into the medium. Such a secondary attachment of the muramic acid to the cell wall structure could not account for the release of about 50% of the muramic acid from the hydrolysed cell walls however, Another possible explanation might be that some of the muramic acid was re-absorbed by the cell wall following its release from the mucopeptide molecule. In this way, the apparent release of only a portion of the muramic acid from the hydrolysed cell walls could be most readily explained.

Confirmation that treatment of the cell walls with the autolysin resulted in the complete hydrolysis of the mucopeptide to its separate

components would be possible only be demonstrating their release into the medium. Successful studies of this nature have in the past been undertaken for lysozyme digestion of E. coli cell walls (Salton, 1958). However, such studies involving the isolation of low molecular weight cell wall fragments would require the use of a highly purified enzyme preparation. In the present study, the autolytic enzyme concentrate contained considerable quantities of contaminating materials originating both from the lysed cells and the culture medium. The presence of these contaminants would make the search for the released cell wall components virtually impossible. Thus, in the absence of a highly purified autolysin preparation there seemed to be little point in pursuing the matter further.

Treatment of the Pseudomonas cell walls with the autolysin produced a considerable decrease in combined lipid content. This finding suggested that hydrolysis of one or more lipoidal complexes in the wall was an integral step in the autolysis of the cell. It was interesting to note that the rate of lysis of a number of Gram negative bacteria by lysozyme was increased if the cells were first treated with a fat solvent such as Teepol, acetone or chloroform (Warren, et.al., 1955; Colobert, 1957). In the present study, the addition of Teepol or desoxycholate to Ps. aeruginosa suspensions greatly increased the rate of autolysis. The stimulatory action by these fat solvents makes the presence of the esterase in the autolytic enzyme preparations of some significance. Sierra (1957) demonstrated the presence of several exterases in Ps. aeruginosa cells, and also noted a correlation between their appearance in the culture medium and the induction of cellular lysis. A number of workers have demonstrated the presence of lipoproteins in the cell walls and cytoplasmic membranes of various Gram negative

bacteria (Cummins, 1956; McQuillen, 1958, 1960). If we assume that the lipoproteins masked or protected certain key cell wall substrates so that the autolysin was unable to attack the intact cells (Pethica, 1958), the importance of the esterases in the initiation of the massive cellular lysis in the ageing cultures of Ps. aeruginosa became obvious. The absence of demonstrable esterase activity in the cell-free filtrates of the plaque-free cultures appeared to confirm that demonstrated esterase activity of the autolysin was primarily directed against one or more of the lipoprotein complexes within the cell wall and (perhaps) the cytoplasmic membrane of ageing cells of the plaque strain of Ps. aeruginosa, thus rendering them more sensitive to autolytic changes.

The chemical assays carried out on the ageing cell walls revealed few alterations in composition except when extensive autolytic changes also occurred. The observed changes in the composition of the plaque strain cell walls were correlated fairly closely with the induction of cell lysis in the culture. Furthermore, the activity of the lytic enzymes in the culture medium offered a logical explanation for the observed cultural and chemical changes associated with the process of cellular ageing. The absence of detailed studies into the autolytic enzyme systems of other Gram negative bacilli suggested that further studies along the lines of the present investigation may be fruitful. Such investigations may, for instance, reveal whether the enzymes of Ps. aeruginosa are typical for the autolytic enzyme of the Gram negative micro-organisms in general. If nothing else, such studies would add considerably to our present knowledge of the cell wall composition of many hitherto incompletely investigated Genera.

General conclusions.

The present study set out to determine some of the causes of the observed metabolic changes which occur in ageing cultures of Ps. aeruginosa. While the precise reasons for the rapid death of the anaerobic nitrate-free cultures were not determined, the important influence of nitrate on cell viability was demonstrated. The subsequent chemical assays carried out on the whole cells failed to detect any significant changes associated with the ageing and death of the cells. The changes associated with senescence would however, be expected to be subtle, and their demonstration by chemical means will have to await more sensitive assay techniques.

The subsequent lysis of the Ps. aeruginosa (but not S. bethesda) cells was shown to coincide with the release of lytic enzymes into the culture medium. The detection of several changes in the cell wall composition of the lysing cultures suggested that the autolytic enzymes hydrolysed away part of the cell wall matrix, which then lost its integrity and permitted the extrusion of the cell contents into the medium.

Due to the complexity of the problem it was impossible to place the observed metabolic changes in the Ps. aeruginosa cells on a chemical basis, but the present study did contribute some new information on the composition of the Ps. aeruginosa cells and cell walls. However, the peculiar and extensive lytic changes which occur in the anaerobic cultures of the organisms makes this a useful tool for the study of autolysis under controlled environmental conditions. The apparent retention of much of the cell wall muramic acid following autolysis suggested that the Pseudomonas autolysin differ

somewhat from other lytic enzymes produced, for instance, by the Gram positive Bacilli and this point deserves further examination, preferably using other Gram negative organisms similarly subject to extensive autolytic changes during ageing. Such a study could yield information ultimately leading to a more complete understanding of the underlying factors associated with this interesting phenomenon.

ACKNOWLEDGMENTS

I am very pleased to acknowledge the extensive help, criticism and guidance of Professor D. Rowley during the latter part of this study. I am also indebted to several members of the Microbiology Department for their stimulating discussion of various aspects of the work.

I am grateful to Professor R. Morton and Dr. M. Atkinson of the Department of Agricultural Chemistry, University of Adelaide for their helpful advice during the nucleic acid investigations.

I should like to thank Mrs. A. MacAskill for her excellent technical assistance during part of this investigation.

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