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A Study of the Processes Involved in the Change of the Cells of the Bacterial Species *Escherichia Coli* from Life to Death.

by

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PREFACE.

In his classical work, "Pathology of the Cell", Cameron (1952) has said that "We are urgently in need of chemical and physical investigation of cellular death, for our knowledge at the moment is crude and full of gaps". The truth of this statement becomes patent when we consider cellular death in the course of normal ageing. What causes any particular cell to die? Which chemical changes associated with death of a cell are causative and which consequential? Could interference with the chemical processes involved in the passage from life to death prevent or delay death of a cell? It is with the last and most limited of these questions that this investigation is concerned. An answer to this question might, however, give an approach to the larger problems of cellular death. If death of a cell could be prevented or delayed by a substance known to inhibit specific biochemical processes, we should have learned something of the chemical changes associated with death.

The work described in the following pages was designed to test this hypothesis, and conditions under which growth was unlikely to occur were chosen in determining the effect of the specific biochemical inhibition on the death of bacteria. The reason for adopting these conditions was that where growth was impossible, any interference with the processes leading to death of the bacterial cell could be easily distinguished.

Since it was impossible to examine all bacterial species, the Gram-negative bacterium Escherichia coli was chosen for most of this work. Some of the experiments were repeated using the Gram-positive organism, Staphylococcus aureus, which has widely different characters, in order to ~~determine whether the findings were of general application~~ test whether the findings applied also this species. The results are described in the remainder of this thesis and their significance discussed.

INTRODUCTION.

Death and dissolution is a fate which overtakes all multi-cellular organisms. Only cells of the germinal tissues possess immortality. It is less certain that this is true of single-celled organisms, and in fact many microbiologists would regard their material as potentially immortal. Growth of protoplasm and cellular division follow without interruption if nutrients are freely available and other conditions are favourable. But even under optimum ~~cellular~~ cultural conditions the growth of a bacterial population in a closed system does not proceed unchecked. The supply of nutrients runs out and the end products of metabolism exert a brake on the rate of population increase. ~~If~~ In many cases if a cell is unable to grow and divide it experiences the same fate as multicellular organisms, it ages and dies.

Is, then, a sharp distinction to be drawn between death in multi-cellular and uni-cellular organisms? Not if one regards the multi-cellular organism as an integrated association of single cells in which death of the organism is the

ultimate consequence of death of individual cells.

Nature operates by an intricate system of checks and balances in controlling the dominance of species. The causes of cellular death are manifold but may be grouped under one of two main headings.

1. Injury to the cell originating in the environment.

Since living bacterial cells consist largely of the substance in delicate equilibrium known as protoplasm, their normal life processes may be easily disrupted by certain changes in the environment. All cells die when injured provided the injury be sufficiently severe or prolonged. Individual cells vary, of course, in their susceptibility to injury. Some will survive, and even recover, under conditions which are fatal to others.

Cameron (1952) lists many forms of injury which may lead to death of the cell, but these may be grouped under three headings.

(a) Starvation.

(b) Physical damage.

(c) Chemical damage.

Throughout the animal kingdom most deaths would be explained by the first two causes, and even in man death is frequently the result of the operation of one or other of these three types of injury. But an explanation of cellular death in these three ~~cells~~ cases is sometimes unsatisfactory. Death seems to be the result of injury originating within the cell rather than stemming from the environment. The damage to the processes of the cell are a function of time and the terms "Ageing" or "Senescence" have been used to describe the deterioration in the efficiency of the cell which, in the course of time, leads to the death of the cell. This is the other main heading under which the causes of cellular death may be grouped.

2. Ageing or Senescence.

This is the process in which the cell loses efficiency with increasing age and ultimately death is the result. Relatively few

organisms ever reach a stage of advanced senescence, yet it is a process which is becoming of increasing interest and importance to man as the advances of medical science decrease the probability that he will die as a result of injury stemming from the environment in the form of infection by some invading micro-organism.

In the course of ageing we find that the chemical activities and the architecture of the cell suffer change, but we are still largely ignorant of the nature of these changes. In particular we can only guess at the extent of the chemical changes accompanying senescence. How are we to study the disturbance of architecture and function that accompany death? Tissues have the advantage of bulk, but they carry the disadvantage of a complicated organisation. If we want to study death at the cellular level we must use isolated and identical cells and thus reduce complicating conditions to a minimum. The bacterial cell is ideal for this purpose. Large quantities of identical cells can be obtained with little trouble and

their life cycle is compressed into a relatively short period. But while the study of the death of bacteria is interesting in itself, is there any reason for believing that the phenomenon of death in bacteria has anything in common with the death of cells organised in tissues? There are, in fact, good grounds for believing that any discoveries that might be made in studying bacterial death would have general application. Comparative biochemistry has demonstrated during the past 50 years, or so, that cells of different origin have far more in common, biochemically speaking, than they have apart. It is for the reasons given above that bacteria have been chosen as the object of this study of cellular death.

At the same time we must not take for granted that the process of deterioration follows exactly the same course in the cells of all species. The factors which are operating during ageing are too complex and too varied to allow of any neat biological classification. And, as mentioned in the opening paragraphs, under

some conditions single-celled organisms cannot be described as ageing at all. But if conditions are chosen so that bacteria are unable to grow and divide we have a situation similar to that found in the tissue cells of the ageing animal or plant.

Before going on to discuss ageing in bacteria it is necessary to say a little about what we mean by the term. The process which it represents cannot be simply a matter of time, it must be describable in terms of metabolic and structural changes of some sort. But in the absence of concrete answers to questions of fact discussions of ageing tend to use phrases such as "wear and tear" which carry little enlightenment. Understanding can only come when we are able to describe in biochemical terms the nature and causes of "wear and tear". We are only at the edge of this vast and exciting field of study and it is the conviction of the author that one of the most fruitful approaches lies in the study of bacterial death.

Changes in the Behaviour of Bacteria as they Age:

On studying single-celled organisms like bacteria, most workers have concerned themselves mainly with the earlier phases of growth. The events occurring in these earlier stages, under various experimental conditions, have been very carefully and fully dealt with by previous observers. The determination of growth curves under a variety of experimental conditions have thrown light on the phenomena associated with the growth cycle of bacterial populations. The conclusions of these studies show that there are four phases in the growth of a bacterial culture.

(1) An initial period during which multiplication is slight; this period varies between 1-6 hours according to the temperature, size and age of the inoculum and composition of the medium. (Lane-Claypon, 1909; Coplans, 1910; Penfold, 1914 Chesney, 1916; Hinshelwood, 1946).

(2) A period of logarithmic multiplication, the rate varying slightly at the same temperature, but differing widely for different temperatures.

For a given volume of fluid the time during which the bacteria continue to divide at a maximum, steady, rate depends among other things, upon the size of inoculum, the temperature of incubation and the nature of the medium. (Barber, 1908; Lane-Claypon, 1909; Jennison, 1935; Penfold and Norris, 1912).

(3) A period when the numbers remain more or less stationary. In this period, and after the culture has ceased growing logarithmically, the rate of growth slackens gradually but is still fairly active until the number of bacteria reaches a value determined by the amount of nutrients present or the presence of toxic products of the cell's metabolism. At this stage the number of living bacteria appears to remain fairly constant for some time, after which it begins to decrease slowly. (Bail, 1929; Fukuda, 1929; Dagley and Hinshelwood, 1938; Monod, 1942).

(4) A period when the number of living bacteria is diminishing. This means that for the particular organism the conditions are such that

the death rate exceeds the birth rate. At this stage the cells have ceased to divide and are beginning to die. Whether the cells are dying as a result of chemical injury by the products of their own metabolism or for some other reason remains largely undetermined (Buchanan and Fulmer, 1928, (Vol. I); Knaysi, 1930; Beamer and Tanner, 1939; and Rahn and Schroeder, 1941).

These findings are the result of investigations which were largely concerned with the bacterial population as a whole and in only a very few instances e.g. Graham-Smith (1920) were the experiments carried beyond 30 hours incubation. [Before studying the ageing of bacterial cells one must first decide whether in fact the bacterial cell can be regarded as showing anything equivalent to the ageing process seen in multicellular organisms, since, once a cell has divided it has ceased to exist and has been replaced by two young cells. This point has caused much debate but there is some evidence that the cells of bacteria undergo a regular

metamorphosis during the growth of a culture similar to the metamorphosis exhibited by the cells of a multicellular organism during its development, each species presenting three types of cells, a young form, an adult form and a senescent form. These variations are dependent on the metabolic rate and, as in a multicellular organism, the change from one type to another occurs at the points of inflection in the growth curve (Henrici, 1925). The young or embryonic type is maintained during the period of accelerated growth, the adult form appears with the phase of negative acceleration, and the senescent cells develop at the beginning of the death phase. The study of the bacterial growth cycle is, then, the bacteriological equivalent to the study of embryology, adolescence, maturity and senescence in the higher forms. Let us consider the phases of growth of a bacterial population in rather more detail.

After a phase of adjustment in which the cells will be characterized by relatively low

physiological activity (Martin, 1931-32; Mooney and Winslow, 1935; Huntington and Winslow, 1937); relatively small size (Bayne-Jones and Sandholzer, 1933; Henrici, 1928); low multiplication rate (Lane-Claypon, 1909; Coplans, 1910; Penfold, 1914) rather high resistance to unfavourable conditions (Elliker and Frazier, 1938) and relatively high electrophoretic mobility (Moyer, 1936), the cell passes into a phase of physiological youth, characterized by active metabolism and rapid increase in mass, but - at first - with delayed cell division.

Metabolic activity is markedly increased as measured by such indices as heat production (Wetzel, 1932) oxygen consumption (Martin, 1932) carbon dioxide and ammonia productions (Walker, Winslow, 1932; Huntington and Mooney, 1934) acid production (Stark and Stark, 1929) and nucleic acid content (Caspersson, 1947; Oginsky and Umbreit, 1954).

In parallel with the outburst of metabolic activity which was described and almost - but

not quite - simultaneous with it, come fundamental changes in size and other morphological characteristics of the bacterial cell. Measurements of bacterial cells indicate that individuals of a large number of species increase in size during the early hours of culture growth, but soon decrease to approximately the original level. (Clark and Ruehl, 1919; Henrici, 1928; Martin, 1932; Huntington and Winslow, 1937; and Hershey, 1939).

A third very important characteristic of the metabolically active cells of the early culture cycle is their markedly reduced resistance to various harmful chemical and physical conditions. (Sherman and Albus, 1923).

Finally there is a fourth characteristic of these large and metabolically active cells of the youthful phase of the bacterial culture cycle, which may again be related to their low resistance to harmful chemical agents but is demonstrated by direct physical measurements. This is the property of low susceptibility to agglutination, coupled with low electrophoretic

change. (Moyer, 1936).

When the full rhythm of its existence is established and maintained, a unicellular organism increases each component of its structure, so as to preserve constant ratios of them all. At a certain point, probably dependent on the deoxyribose nucleic acid content of the cell (Caldwell, Mackor and Hinshelwood, 1950; Chantrenne, 1952) division takes place in bacteria by binary fission. The number of cells and the various components increase at the same, constant, rate for each. Compounds containing the various essential elements are taken in by cells from the medium and initiate a complex series of reactions in which the cell material is reproduced. For the immense variety of chemical synthesis there must be many sequences of consecutive reactions which branch and interlock in a complicated scheme which constitutes the metabolic pattern of the cell (Walker and Winslow, 1932).

The process of self-duplication is probably the result of a co-ordinated interplay of

reactions in which the products of one set of enzymes or cell constituents build up the material of another.

The steady rhythm of growth and reproduction is seldom maintained for long and can only continue under rather carefully devised laboratory conditions. If one or other essential nutrients become exhausted, growth is thereby halted (Dagley and Hinshelwood, 1938). In other cases, metabolic products, some of which are potentially toxic, accumulate and growth is limited by their actions. (Chesney, 1916; Heap and Cadness, 1924; Lodge and Hinshelwood, 1939). The development of an adverse pH is one of such influences.

When growth is interrupted and the cells enter a non-proliferating phase, the balanced co-ordination which characterised the first phase is upset. There is still a lively internal interchange of material between the environment and the cell, and the compounds, which the cell has previously synthesized, break down and are resynthesized with a constant turnover. The

mechanism of the cell is, however, not perfectly efficient and some of the intermediates leak out slowly, the time scale varying from several hours to several weeks. In these conditions an exact balance between synthesis and degradation is not easy to maintain. Eventually unless the cell advances it must go back and a phase of decline ensues. This decline, does not go unopposed but is accompanied by a vigorous struggle for regeneration. Nevertheless, the co-ordination which maintained a harmonious ratio, between all the constituents is lost; enzyme activities fall, (Gale, 1940; Wooldridge and Glass, 1937; Woods and Trim, 1942) various intermediates of low molecular weight are increasingly lost from the cell by diffusion. (Hinshelwood, 1946; Bayne-Jones and Rhees, 1929; Mooney and Winslow, 1935). The first effect changes the nature of the actual sites of reaction, while the second completely alters the concentrations and concentration gradients which had been established in the steady state. Lytic processes supervene and death of the cell is the final result. There

is an initial period during which the death rate is quite low and it rises only after the adverse environment has had time to bring about progressive changes in the cells.

The Death Processes of the Cell.

In the last paragraph some of the changes which accompany the death of the cell have been described. But how are these changes initiated? It is difficult to conceive of a cell dying as the result of multiple and simultaneous chemical changes. One tends to think of primary changes which, once they have taken place, allow all the secondary processes of cellular dissolution to occur. If this crude picture of the death of the cell has any validity, it might be possible to prolong life by inhibiting these primary changes and so gain information as to the nature of the first steps in the processes leading to cellular death. For such inhibition to be informative it must be specific. Immobilising cell metabolic processes by chemical means tells us no more about cellular death than does immobilising by physical means, such as freezing,

unless the particular processes inhibited are known. This involves the use of a specific inhibitory agent with a known mode of action. If by the use of a highly specific inhibitor, known to interfere with certain biochemical processes, we could delay or prevent cellular death, we should have learned something about the biochemical processes leading to death of the cell.

Is it possible to prevent or delay the death of the cell by interfering with the metabolism of the cell by chemical means? Two findings had suggested that this might be possible. Investigations carried out on the human Hela cells and using a series of enzyme inhibitors including iodo-acetic acid had shown marked prolongation of life by the above inhibitor (Morrison and McLaughlin, unpublished). In addition it had been found that cells of E. coli seemed to die more slowly in the presence of certain concentrations of the bacteriostatic antibiotic, chloramphenicol. (Fletcher, 1956).

Choice of Chloramphenicol as Inhibitor of Bacterial Death.

I have already referred to the desirability of using a highly specific enzyme inhibitor in order to pursue a detailed study of the effect of inhibiting specific biochemical processes. Unfortunately enzyme inhibitors are on the whole not highly specific. In a later section of the thesis the results of the use of enzyme inhibitors of partial specificity (sulphydryl inhibiting compounds) and of high specificity (esterase inhibitor) will be described. But for most of the experiments chloramphenicol was used to prevent bacterial death. There were several reasons for choosing this bacteriostatic antibiotic, the most important of which was, perhaps, the finding that it could delay cellular death. Other considerations involved in the use of this substance are as follows. It is a relatively simple, stable compound, whose chemical structure is known in detail (Rebstock et al, 1949; Controulis et al, 1949). Moreover much information has accumulated concerning the

effect of structural alterations on its anti-bacterial action (Collins et al, 1952; Carrara et al, 1950-51). Lastly, its inhibitory effect on the growth of widely differing micro-organisms suggests that it may interfere with a process of fundamental importance among micro-organisms.

An indication of the nature of an important metabolic process affected by chloramphenicol was found in the earlier observations that this antibiotic inhibited the formation of adaptive enzymes in E. coli (Hahn and Wisseman, 1951). This effect was interpreted as indicating a possible interference with protein synthesis as was later confirmed (Wisseman et al, 1952, 1953; Hahn et al, 1952; Gale and Folkes, 1953; Wisseman et al, 1954).

Why should the known mode of action of chloramphenicol in inhibiting protein synthesis make it a suitable compound to use in preventing the death of bacteria? One would imagine that this property would be more likely to increase cellular death as indeed it does in the treatment

of bacterial infection.

The mechanisms of protein synthesis is poorly understood at present, and interference with any one of a number of different contributing processes could result in the failure of an organism to synthesize proteins. On the other hand, many of the reactions of the cell are reversible, and it was thought that chloramphenicol might, at a suitable concentration and under the special conditions of the experiments, also prevent the break-down of proteins. It might be possible, then, so to choose the experimental conditions that the effect in inhibiting break-down of protein outweighed the inhibitory action on protein synthesis.

A living organism, being a graded structure of open systems, maintains itself in accordance with its inherent principles with its constituent elements constantly undergoing change. It seemed reasonable to assume that the rate of renewal and breakdown of these elements should have some influence on the life

span, and that chloramphenicol might, by altering this rate, affect the life span.

The fact that the effect of an antibiotic depends on a complex system comprising pathogen and drug makes it very responsive to slight changes in environmental conditions. Valentine (1956) noticed that the tendency of bacteria to die under conditions of bacteriostasis induced by chloramphenicol varies from species to species.

Gray (1952) noticed that there is a difference in the anti-bacterial effect of chloramphenicol when it is added to a 16-hour culture, causing a slight reduction of the viable counts after 6-7 days, and the effect when an inoculum of the same or an old culture is added to chloramphenicol. In the latter case death of all organisms ensues in less than 24 hours. He gave two explanations for this difference, one was that an optimal concentration of antibiotic may be required in relation to the density of a bacterial population for most efficient anti-bacterial results. As a

second explanation he stated that another influence may operate, as indicated by:
(1) the marked increase in the 24 hour bacterial survival rate in a water-chloramphenicol mixture as compared to a broth-chloramphenicol mixture.
(2) the "lag period" when aliquots of an 8-day culture are added to fresh chloramphenicol broth.
(3) the similarity in the hourly fall of the viable counts when a 16-hour and an 8-day culture are added to fresh chloramphenicol broth. These findings may indicate that the antibiotic did not interfere with bacterial life except at or immediately after the reproductive phase.

Gray's result gave additional weight to our hypothesis that under certain conditions, the interference of chloramphenicol on the metabolism of the cell might, predominantly, be an interference with metabolic processes involved in the death of the cell. Were this so, chloramphenicol would prolong, rather than decrease the viability of the cell.

Just before embarking on this work, some results had already been obtained on the effect

of chloramphenicol in prolonging the life of E. coli cells under some conditions of bacteriostasis. It was noticed (Fletcher, 1956) that growth, or at least conditions known to benefit growth, might in some circumstances be a prerequisite for death. He also found that by choosing suitable conditions, chloramphenicol was able to inhibit bacterial death.

The Effect of the Density of the Populations on Experimental results.

Early in the course of experiments, it was noted that the bacterial number in the suspension had a great influence on the results obtained. The general phenomenon of increased survival when a heavy inoculum is used is of course by no means a new one having been noted or implied by Abbot, (1891), Jordan et al (1904), Wright (1917), Bigelow and Esty (1920), Smith (1921), Weiss (1921) and Graham Smith (1920-21).

Gray (1952) in investigating the possibility that rising concentrations of chloramphenicol might be required for maximal anti-bacterial

action against rising bacterial population noticed that when the initial viable counts were 29×10^7 and 94×10^8 organism/ml. sterility was reached on the 5th. day and was reached on the 7th. day when the initial count was 30×10^9 organism ml. This showed that there was an optimal antibiotic concentration which was related to the density of the bacterial population.

Effect of pH of the medium on the growth, death and enzymic activities of Bacteria.

In our work on the death of E. coli it became apparent that the production of alkaline metabolites played an important role in the processes leading to death of the cell and it is therefore necessary to discuss in some detail the effect of pH on the well-being of the bacterial cell.

There are many factors which may influence the growth, maintenance and death of bacteria. Omitting from consideration the obviously large part played by the food supply, there are certain predominating physical and physicochemical factors,

chief among which are temperature and the concentration of H-ion (Cohen, 1922; Porter, 1947). There is an extensive literature upon the effects of hydrogen -ion concentration on many biological processes as a glance at the references quoted by Clark (1920) will show. In the field of bacteriology its importance as a controlling factor is well established. The control is exercised in many subtle and unexpected ways upon the activity of specific enzymes, upon toxin production, upon the dissociation of essential food stuffs and upon the state of aggregation of cellular protoplasm. Our present meagre knowledge permits us usually to see only the end result in growth, metabolism or death. (Shohl and Janney, 1917; Dernby and Avery, 1918; Clark, 1920).

Although the literature of bacteriology is replete with statements that the "reaction" of a culture medium is important, its importance for which particular phases of growth or for which specific process of metabolism or favouring which rapid causes of death is not yet known in

much detail (Cohen and Clark, 1919).

The influence of the H-ion on cellular multiplication is probably far from being one of those simple matters which we call fundamental, yet it is obvious that we must seek detailed information concerning this phase before studying intensively the metabolism of a culture as a whole over long or short periods and at various pH reactions.

The effect of H-ion concentration of the medium on bacteria suspended in it is rather complex (Topley and Wilson, 1955). There is first of all an optimum concentration for growth; for E. coli this is about 7.6. There is secondly an optimum concentration for survival; for E. coli this is about 6.0. Thirdly there is a point at which the acid and alkali tolerance of the organism fails; the values for E. coli are 4.6 and 8.5 respectively. (Buchanan and Fulmer, 1928). There is considerable variation in the minimum, optimum and maximum pH values for growth from one genus to another, but for the majority of bacteria the figures range from a minimum pH of 4.5-5 to a

maximum pH of 8.0-8.5. The optimum pH is generally within 0.5 unit of neutrality - that is 6.5-7.5. (Oginsky and Umbreit, 1954). E. coli can grow at any pH between 4.5 and 9 (Gale and Epps, 1942) although the yield of organisms towards the limits of this range is small. Sherman and Hohn (1922) also pointed to the fact that beyond the range of optimum growth, there seems to be a decided retardation for each small change of H-ion concentration.

There are some points of special interest to be noticed in the fact that, while organisms can multiply rapidly for short periods in media having a considerable range of pH, a slight change at the border of this zone determines a definite decline in numbers. It was noticed (Cohen and Clark, 1919) that with E. coli the time of maximum rate of growth was delayed until a late hour in the more alkaline media. The period of maximum rate of increase occurred as follows -

pH	5.0	5.5	6.1	7.0	8.1	8.7	8.9
Period in hours.	3-5	3-5	2-4	2-4	2-4	8-10	10-12

In other words the generation time is unaffected by growth pH as long as the latter falls between the approximate limits 5.8 - 8 (Gale & Epps, 1942) but the period of lag or latency is very distinct and prolonged in the more alkaline media.

The pH limits for the growth of any particular organism are a reflection of the pH limits for the activity of the enzymes with which that organism synthesizes new protoplasm and divides into daughter cells. (Gale, 1940-1941, Silverman and Werkman, 1941). Bacteria react to an alteration in their external environment by an alteration in the enzymic constitution. The changes appear to involve two principles (1) an attempt to counter the adverse external change and (2) an attempt to maintain essential activities at constant value. Adaptability of the bacterial cells to the changes in pH of the medium varies with the physiological age of the cell. Hegarty (1939) records that young cells adapt more readily than older cells, and Pinsky and Stokes (1952)

that the adaptability of E. coli decreased during active growth and was restored as the culture entered the stationary phase.

As for growth, there is an optimum for enzymic activity and various studies have been unable to show any significant shift of the optimum pH of any enzyme with the alteration of the growth pH and it seems that this factor is a characteristic of the enzyme rather than of the culture in which it is produced. Gale (1942) divided the enzymes into two groups according to their variations with growth pH:

Group I: those enzymes whose formation undergoes a variation so that their activity/cell is constant whatever the medium pH. This means that the potential activity of the cells in respect of these enzymes increases as the growth pH deviates from their optimum pH.

Group II: those enzymes whose formation is greatest when the growth pH approaches their optimum activity pH, but falls off rapidly towards extremes. There is evidence, however, that H-ion concentration most suitable for

certain fermentation process is different from the optimum pH for growth. (Topley and Wilson, 1955). The above two groups of enzymes contribute to the stability of cellular equilibrium, the first group by neutralisation of inhibiting products of metabolism and the second group by acting as ~~adaptive enzymes in respect to pH and not substrates~~ though they were adaptive in respect to pH. Thus in an acid medium amino-acid decarboxylases, and consequently amines, were produced, on the other hand, amino acid deaminases and consequently hydroxy acids, were produced in alkaline medium.

As well as having a marked effect on growth and metabolism the pH of the medium has a marked effect on the death rate of bacteria. (Cohen, 1922; Cohen & Clark, 1919; Friedenthal, 1919; Clark & Lubs, 1917a; Pasteur, 1879). Referring to the death process Clark and Lubs (1917) have said: " ---- in cellular destruction temperature is to be considered as an accelerating condition ------. Among the active agents concerned the concentration of H-ions may be of great significance". Indeed in bacterial death

the role of pH may become paramount (Cohen, 1922). As for growth, there exists for each kind of organism, for every definite set of environmental conditions (exclusive of H-ion concentrations) an optimum concentration, a maximum concentration and a minimum concentration of H-ion for the death rate (Buchanan and Fulmer, 1928, Vol. II). The pH zone of tolerance or minimum death rate of E. coli is wider than of ~~Bact. typhosum~~ Salmonella typhi and is centred near absolute neutrality (Cohen, 1922).

The metabolic activity of the bacterial culture may produce from the components of the medium, a variety of end products, some acidic, some basic. Even if the medium were initially at pH 7.0, the release of such substances during growth would shift the pH above or below the outside limits for growth and thus cell reproduction would become impossible (Stephenson, 1949; Ravin, 1952; Oginsky and Umbreit, 1954). Sierakowski (1924) concluded that there were two phases of pH changes in bacterial cultures. First the acid media became more alkaline and

the alkaline media more acid approaching a common point (7.6 for E. coli). In the second phase all became alkaline. While bacteria have long been differentiated on the basis of their ability to produce acid from carbohydrates and change thereby the colour of indicators present, more recently it has been suggested that in a given environment a particular kind of organism will produce a definite H-ion concentration which will constitute one of the factors inhibiting growth. Michaelis & Marcora (1912) were the first to demonstrate this. Working with E. coli, it was found that H-ion concentration above 7 or below pH 6 gives a much more rapid death rate than occurs when the pH is maintained within these limits (Falk, 1920). An uncontrolled H-ion concentration may affect the death rate variably in unbuffered surroundings, so much as to obscure the effects of wide ranges of environmental conditions (Clark, 1915). When the pH is controlled, however, by means of dilute buffer solutions the death rate become stabilized. (Buchanan and Fulmer, 1928, Vol. II, Van Slyke, 1922; Clark,

1915; and Kolthoff, 1925). It is therefore advisable to include as a component of a culture medium a chemical that will act as a hydrogen -ion buffer over the pH range at which the organism can grow.

The importance of this point emerged in our studies of the effect of chloramphenicol on the death of bacteria, since this antibiotic appeared to suppress the production of metabolic products of the E. coli which caused a rise in the pH. Such alterations caused an increased death rate and so chloramphenicol's action in prolonging the life of the cell might be, by some means, akin to the action of buffers.

These, then, were some of the considerations which influenced the choice of this particular approach to the causes of cellular death or which emerged in the course of the experimental work.

The first part of the thesis describes studies of the death of bacterial cells, mainly E. coli cells, in the presence of certain chemical interfering agents, chiefly chloramphenicol.

Some success was achieved both in delaying cellular death and in elucidating the manner in which this delaying effect operated. But the pace of the investigations was greatly slowed by the very tedious and time consuming method used to follow cellular death - repeated viable counts. It was therefore of considerable importance to find some simpler alternative method which could be used to follow the death of bacterial cells.

The loss of antigens appeared to be a possible indicator of cellular death and the second part of the thesis describes attempts to discover a simple technique which would enable the death of cells to be followed by measurement of antigenic loss. These attempts were successful and a method has been worked out in which the loss of antigens is followed by means of a gel diffusion technique.

SECTION I.

The Effect of Chemical Interference with *E. coli* cells on viability.

EXPERIMENTAL METHODS.

Methods used in experiments in which the death of bacteria was followed by viable counts.

The experimental work fell in the following groups of experiments:

I : The effect of chloramphenicol on the death of Bacteria.

(a) In the first instance death of bacteria in a heavy suspension was studied and the variables were the temperature of incubation and the concentration of chloramphenicol.

(b) The death of bacteria in more dilute suspensions at different temperatures and with different concentrations of chloramphenicol was studied.

(c) The death of bacteria in a medium without nutritive value such as saline and using as variable both the temperature of incubation and the concentration of the chloramphenicol was investigated.

II : The second group of experiments was designed to show whether the chloramphenicol effect could be explained in terms of pH changes.

III : The third group of experiments was concerned with the relation of drug sensitivity to the prevention of death by chloramphenicol.

IV : The fourth group of experiments used enzyme inhibitors, ~~to provide a different means of bacteriostasis~~ and the results obtained were compared with those in which chloramphenicol was used.

MATERIALS AND METHODS.

Strains:

Most of the experiments in the present work were done using E. coli I (N.C.T.C. 8196).

Some experiments were repeated using Staphylococcus aureus (5 S).

The organisms were maintained as stock cultures at room temperature on meat extract agar slopes after incubation at 37°C. for 24 hours. Sub-inoculations of stock cultures were done every fortnight, and their purity confirmed from time to time by plating and testing sugar fermentation reactions etc.

The cells were harvested from 24 hours cultures in meat extract broth and resuspended in the different media used.

Media:

1. Meat extract broth (M.E. broth) was used for growth of the initial cultures and for the dilutions of the viable counts as well as for suspending the cells in the controls of the experiments in which chloramphenicol broth was used.

2. M.E. broth to which was added variable concentrations of chloramphenicol (B.P.) in saline was used for resuspending the harvested organisms except where otherwise indicated.

3. Physiological saline was used as a suspending vehicle in some of the experiments.

4. M.E. agar was used in the plates for the viable counts.

5. Varying concentrations of glucose in M.E. broth from 2% concentration to 0.1%.

6. 0.0006 per cent solution of phenol red indicator was added to M.E. broth and used instead of M.E. broth in some of group II experiments to try and control the pH of the medium colorimetrically.

7. $\frac{N}{5}$ HCl and $\frac{N}{5}$ NaOH were used to bring the pH

of the medium back to the original value.

8. Buffers:

a. Buffer indicator broth: This is Fisher (1957) buffer to which was added 0.0006% phenol red indicator.

The Fisher buffer contains:

0.4% (W/V) NaCl.

0.02% (W/V) $MgSO_4$ 7 H_2O.

0.68% (W/V~~/20~~) KH_2PO_4.

These are dissolved in M.E. broth, pH adjusted with $\frac{N}{5}$ NaOH to 7.4 and sterilised through sintered glass filters.

b. Sørensen phosphate buffer pH 7.38

This buffer was added to M.E. broth to which was added 0.0006% phenol red indicator. In order to attain pH 7.3 the following proportions of $\frac{M}{15}$ NaH_2PO_4 (x) and $\frac{M}{15}$ Na_2HPO_4 (Y) were added:

X 2.0 and Y 8.0

The buffer was filtered through sterilising sintered glass filters.

After the addition of cells and indicator the final pH was 7.

c. Veronal Buffer: This buffer was also added to M.E. broth containing 0.0006% phenol red indicator.

The Buffer contains:

Sodium Barbitone : 2.06 gm/100 mls. M.E. broth (pH 8.8)

Barbitone : 0.368 gm/100 mls. M.E. broth (pH 7.33)

20 mls. of the sodium barbitone solution were added to 100 mls. of the barbitone solution and the 0.0006% phenol red indicator. The medium was then sterilised by filtering through a sterilising sintered glass filter. The pH of the medium was 7.4 and after addition of cells 7.2. This buffer is a modification of a veronal buffer of pH 8.6 in which:

Sodium Barbitone = 10.3 gm/litre.

Barbitone = 1.84 gm/litre.

d. Palitzsch Borate Buffer pH 7.36.

Again M.E. broth containing 0.0006% phenol red indicator was used to which was added:

Boric acid 0.2 M (X)

Borax 0.05 M (Y)

these were added in the proportion:

X 9.0 and Y 1.0 to give a pH of 7.35.

The buffer was sterilised through sintered glass filters. Final pH after the addition of the cells was 7.2.

9. Glucose phosphate medium (Dagley et al, 1953) ~~(Harrington, 1958).~~

$K H_2 PO_4$	:	5.4 g.
$(NH_4)_2 SO_4$	:	1.2 g.
$Mg SO_4 . 7 H_2O$	:	0.4 g.
Glucose		12 g.

made to 1 litre with dionised water.

The pH was adjusted to 7.1 by 1 N NaOH and the medium sterilized through sintered glass filters.

10. M.E. agar slopes containing 50 ug. ml. chloramphenicol were used for weekly subculturing the artificially induced resistant strains of the E. coli to maintain their resistance.

11. Chloramphenicol containing plates of M.E. agar for raising the resistance.

The method used to raise the resistance of the cells was by the use of the wedge plate technique. Petri dishes 4 inches in diameter

were used. The dishes were placed in a sloping position, one side being raised higher than the other. 10 mls. of melted M.E. agar containing the required amount of chloramphenicol/ml. was poured in these plates and left to set, giving a wedge of M.E. agar with the required amount of chloramphenicol/ml. at the deep end. The plates were then put flat and another 10 mls. of melted agar was poured in order to attain a flat surface and a graded strength of chloramphenicol throughout the whole plate with the required maximum chloramphenicol concentration over the thick wedge of chloramphenicol agar.

12. Enzyme Inhibitors.

A - Sulphydryl inhibitors:-

1. Iodoacetic acid: Iodoacetic acid solution was prepared by adding 0.1 gm. to 10 mls. M.E. broth. It was sterilised by filtering through sintered glass filter and from this 10^{-4}M and 10^{-5}M solutions were prepared. When these were added to the suspension of cells the final concentrations obtained were 10^{-4}, 10^{-5} and 10^{-6}M.

2. Iodosobenzoic acid: Iodosobenzoic solution was prepared in the same way as the iodoacetic. From this 10^{-4}, 10^{-5} M strengths were prepared which when added in appropriate amount to the cell suspension gave final concentration of 10^{-4}, 10^{-5} and 10^{-6} M.

3. p-Chloromercuribenzoate: p-Chloromercuribenzoate was prepared in the same way as the above two inhibitors and gave a final concentration of 10^{-3} M, 10^{-4} M and 10^{-5} M when added to the cell suspension.

4. Mixtures of the enzyme inhibitors: Mixtures of the various inhibitors were prepared from the above mother solutions according to the requirements of the various experiments.

Also mixtures of the inhibitors with the various buffers, M.E. broth and chloramphenicol were prepared from the above mother solutions in different strengths as specified in the different experiments.

B. Esterase inhibitors: 10^{-2} M solution of the Isopestox (N.N. di-isopropyl phosphorodiamidic fluoride) was prepared in

phosphate buffered broth and filtered through sintered glass filter. From this 10^{-3} M, 10^{-4} M and 10^{-5} M were prepared. When these were added to the cell suspensions they gave the final concentrations of 10^{-4} M, 10^{-5} M and 10^{-6} M.

13. Antibiotic solutions. These were prepared by dissolving 100 mgm. (0.1 gm) of chloramphenicol (B.P.) in 100 mls. saline i.e. 1000 ug/ml and sterilised by filteration through sterilising sintered glass filters. Different concentrations were prepared from this mother solution. It was kept in the refrigerator at 5°C. for not more than one month to ensure that no loss of value occurred.

Except when otherwise indicated the usual final strengths of chloramphenicol used were 100 ug/ml and 10 ug/ml.

Terms:

M. Concentration: This term, after Bail (1929) was used to describe the population of bacteria attained in unaerated fluid cultures after 24 hours of incubation. Multiples and submultiples

of this M. concentration prepared by centrifugation and resuspension in fresh media, were given different names according to the relative quantities of the resuspending medium to original. Thus 10 M is ten times the 24 hours population; 5 M is 5 times the 24 hour population; ½ M is only half the original 24 hour population and so on.

Death of the Cell: By this term is meant the inability of the cell to reproduce itself after being left under inimical conditions.

EXPERIMENTAL DETAILS:

A. Temperature of incubation:

1. The cells were always harvested from 24 hour culture at 37° C.

2. The various temperatures of incubation used for the different experiments were 5°C, 22°C and 37°C.

3. The M.E. agar plates for viable counts were tested for sterility, dried and incubated for the counts at 37°C.

B. Preparation of cell suspension:

1. For initial harvesting of cells 250 ml.

pyrex conical flasks containing 100 ml. amounts of M.E. broth were inoculated with a loopful of a 24 hour culture of the organism and incubated overnight at 37°C.

2. Centrifugation of the organisms was carried out in 250 ml. sterile centrifuge bottles. They were spun for 30 minutes at 2600 r.p.m.

3. Varying concentrations of the organisms were made by resuspending centrifuged cells in the vehicle to be used.

C. Total Counts:

Estimations of the total counts were done by the Spekker absorptiometer calibrated by direct counts in a Helber chamber. Using a series of carefully prepared dilutions from 10 M down to 0.78 M in 8 steps, i.e. doubling serial dilutions of the cells, a series of Spekker readings and their corresponding counts by Helber chamber was done. The method used for counting the cells is a modification of the method for counting bacteria in vaccine standardisation (Kolmer, 1952) in which 80 squares instead of 20 squares were counted and the average/square

calculated.

A graph was drawn of the Spekker readings against the corresponding number of organism/ml. for future direct readings of the number of organisms corresponding to the Spekker reading.

In experiments of more than 1 M concentration, 10^{-1} dilution of the suspension was used for Spekker readings using the same vehicle as control for the reading. While in experiments with less than 1 M concentration the undiluted suspension was used.

D. Viable Counts:

The viable counts were determined by a modification of the method of Miles and Misra (1938) in which 5 plates each with 20 drops, i.e. 100 drops for each dilution instead of 6 drops were used giving about a six-fold reduction in the error of estimation, that is from about ± 20% down to ± 3½%. A series of tenfold dilutions of the suspensions were made in M.E. broth and the dilutions were always made in 6 x ⅝" sterile test tubes with 9 mls. of broth + 1 ml. of suspension.

For a 5 M suspension 10^{-7} dilution was usually made initially and for $\frac{1}{2}$ M suspension 10^{-6} dilution in order to get the most clearly enumerable number of colonies in each plaque (between 10 and 20 colonies/plaque was the best number to aim at with large colonies like E. coli. Final dilutions of older suspensions depended on age and the presence of bacteriostatic agents. (Photograph No. 1).

The plates were left on the bench slightly open until the liquid drops had dried. They were then closed and incubated overnight at 37°C. (If they are not dried, the colonies will spread and inaccurate counts would result).

Calculations - After incubation the total number of colonies on each set of five plates was counted (using a hand tally counter), i.e. the number of live organisms present in 100 drops of the dilute suspension. If total colonies from 100 drops = X, and dilution was 1 in 10^7 then the number of live organisms in 1 ml. of 5 M suspension = $\frac{X}{100}$ (average/drop) x 50 (number of drops in 1 ml.) x 10^7 (dilution).

E. Estimation of pH.

In the first group of experiments the pH values were determined by using B.D.H. narrow range indicator papers.

For more accurate determination of pH values for the second group of experiments a pH meter was used.

F. Assessment of the strength of chloramphenicol solutions kept at various temperatures.

The object of this experiment was to assess the strength of a solution of 100 ug., 50 ug. and 10 ug. chloramphenicol/ml. kept at 5°C, 22°C and 37°C for extended periods of time to see if there was any destruction of chloramphenicol by prolonged incubation. The greatest dilution giving inhibition of growth was measured.

The results showed that:

At 37°C the chloramphenicol solution came to:

$\frac{1}{2}$ its value after 1 week.

$\frac{1}{4}$ its value after 2 weeks.

$\frac{1}{8}$ its value after 3 weeks.

At 22°C the chloramphenicol solution came to:

½ its value after 1 week.

Remained at half its value for 2 weeks and came to ¼ its value after 3 weeks.

At 5°C. the chloramphenicol solution came to:

½ its value after 1 week and remained constantly at this value to the end of the third week.

G. Assessing the sensitivity of E. coli (8196):

The dilution technique was carried out on various colonies of E. coli (8196) after the bacteria had been in contact with 100 ug/ml and 10 ug/ml for various periods, one week and two weeks at 22°C, and compared with control cells kept in the absence of chloramphenicol. The object was to find out if the original sensitivity of the cells (3 ug/ml) to chloramphenicol changed with such contact in such a period.

It was observed that no change in the sensitivity of the organisms occurred after incubation with both the 10 ug/ml and 100 ug/ml

chloramphenicol solution after as long a contact with the drug as 2 weeks at 22°C.

H. Method of Raising the resistance of E. coli (8196) to chloramphenicol:

The resistance of the cells to chloramphenicol was induced by the following modification of the wedge plate method. Subcultures on the chloramphenicol containing plates were done every 2 days and the following strengths of chloramphenicol were used successively, 5 ug/ml, 5 ug/ml , 10 ug/ml , 10 ug/ml 20 ug/ml, 50 ug/ml, 50 ug/ml, 75 ug/ml, 75 ug/ml, at 2 daily intervals. (Photographs 2 and 3).

A dilution technique was again used to ascertain the exact sensitivity and the 50 ug/ml resistant strain was used in the experiments and was called E. coli R.

The strain was tested for loss of resistance by subculturing on plain agar for 10 days. The results obtained from the sensitivity test performed proved that the cells kept their resistance even after 10 days' incubation away from chloramphenicol.

The strain was maintained by weekly subcultures on 50 ug/ml chloramphenicol containing slopes of M.E. agar.

RESULTS.

I. Viability of *E. coli*.

Table 1 : Changes in the total and viable counts of 5M suspension of E. coli in M.E. broth at 5°C in the presence and absence of chloramphenicol.

Period of Storage (days).	Total Count cells/ml x ~~10~~ 10^{-7} *			Viable Count cells/ml x ~~10~~ 10^{-7} *		
	Concentration of Chloramphenicol ug per ml.					
	0	10	100	0	10	100.
0	780	700	700	410	400	390
1	720	630	630	400	385	375
2	800	650	600	420	395	375
5	750	550	510	390	395	405
8	850	650	650	410	410	370
12	820	630	570	395	405	365
21	660	580	550	225	420	415
35	400	550	550	57	310	350

* In this and all subsequent tables the figures shown are multiplied by 10^7 (or 10^6 where indicated) to give the actual number of bacteria per ml. Thus 780 represents 780×10^7 cells/ml.

Group I Experiments.

The Effect of Chloramphenicol on the death of Bacteria.

a. Using a heavy suspension of cells.

In the first group of experiments, a heavy suspension of E. coli cells (5M) was used and the death of bacteria at different temperatures of incubation was determined. ~~Various~~ The concentrations of chloramphenicol ~~were~~ used ~~ranging from~~ were 10 ug/ml ~~to~~ and 100 ug/ml for every temperature.* The different temperatures used were: (1) 5°C to make the conditions as inimical as possible to cell multiplication. (2) 22°C and (3) occasionally 37°C.

The Effect of Chloramphenicol on the total and viable counts of 5M suspension of E. coli in M.E. broth kept at 5°C.

It can be seen from Table 1 that the viable count of thecontrol suspension stored at 5°C fell to about half of its original value by the end of three weeks, whereas in the presence of 10 or 100 ug/ml of chloramphenicol, it remained unchanged. On the other hand the total counts

* Below 10µg/ml the effect of chloramphenicol was slight; above 100µg/ml it became lethal to the cell.

of the control increased in the first two weeks and then dropped to a slightly higher level than suspensions kept in the presence of both concentrations of chloramphenicol by the end of the third week; the total counts of the chloramphenicol suspension remained steady during the whole three weeks. Thus although a temperature of 5°C was chosen as inimical to cell multiplication, some did occur.

The Effect of Chloramphenicol on the total and viable counts of 5M suspension of E. coli in M.E. broth kept at 22°C.

When similar suspensions were stored at 22°C, the viable count of the control suspension fell to about one eighth of its original value within one week, but with 100 ug/ml chloramphenicol, it showed only a slight decrease and with 10 ug/ml it was still more than half the original value.

The total counts showed the same but a much accelerated pattern in both the control and the chloramphenicol containing suspensions as those at 5°C. The control counts rose during the first two days to double the original counts

Table 2 : Changes in the total and viable counts of 5M suspension of E. coli in M.E. broth stored at 22°C in the presence and absence of chloramphenicol.

Period of Storage (days).	Total Count cells/ml x 10^{-7}			Viable Count cells/ml x 10^{-7}			pH		
	Concentration of Chloramphenicol ug per ml.								
	0	10	100	0	10	100	0	10	100
0	680	640	660	470	400	430	7.00	6.86	6.97
1	1050	580	570	530	410	435	7.55	7.06	7.07
2	1220	660	620	535	425	435	7.97	7.24	7.12
4	960	520	500	220	390	415	8.42	7.68	7.23
7	510	550	520	60	250	350	8.61	7.75	7.59
11	480	350	550	20	60	250	8.90	8.10	7.72

and then fell dramatically from then onwards to the end of the experiment. The chloramphenicol containing suspensions however, remained constant during the first week and dropped slightly afterwards, and the drop was more with the 10 ug/ml chloramphenicol than with the 100 ug/ml. (Table 2).

The Effect of Chloramphenicol on the total and viable counts of 5M suspension of E. coli in M.E. broth kept at 37°C.

When a 5M suspension of E. coli was incubated at 37°C, the changes obtained in the total and viable counts were so rapid that by the seventh day, the viable counts were very low, but always the control viable counts were higher than the chloramphenicol containing ones. When higher concentrations of chloramphenicol were used, they were lethal.

It can be seen from Table 1 and 2, that instead of hastening the death of bacteria, chloramphenicol may, under the conditions of the experiments, act in such a way that the viability of the culture is maintained. This conclusion

is even more apparent with the suspensions incubated at 22°C than at 5°C. A large number of repeat experiments gave a similar pattern of results to those described in the above tables. Some of these results are already published (Morrison, Bagoury, Fletcher, 1956).

It was seen from the pH records in these experiments that the control readings increased as the experiment progressed, while in the presence of chloramphenicol the pH rose much less rapidly, especially with 100 ug/ml. By comparing the viable counts with the rise in pH in the different suspensions, the lowest viable counts occurred when the pH of the control exceeded pH 8, and the steady viable counts in the chloramphenicol containing suspensions is associated with a more or less steady pH reading.

b. Using a small population of cells.

This group of experiments is essentially a repetition of the first group but using a small concentration of the cells. A $\frac{1}{2}$M suspension of E. coli was used in the absence and presence of 10 ug/ml and 100 ug/ml of chloramphenicol and the temperatures used were 5°C and 22°C.

Table 3 : Changes in the total and viable counts of ½M suspension of E. coli in M.E. broth stored at 5°C in the presence and absence of Chloramphenicol.

Period of Storage (days).	Total Counts cells/ml x ~~10⁶~~ 10^{-6}			Viable Counts cells/ml x ~~10⁶~~ 10^{-6}		
	Concentration of Chloramphenicol ug per ml.					
	0	10	100	0	10	100
0	550	540	550	520	490	495
1	980	600	580	530	510	555
2	1280	550	520	640	460	460
7	2400	620	620	1095	465	410
21	5750	520	550	1820	235	200
42	1340	620	660	200	245	195
84	200	550	500	27	253	160

The Effect of Chloramphenicol on the total and viable counts of ½M suspension of E. coli in M.E. broth at 5°C.

It can be seen from Table 3 that during the first 6 weeks of the experiment, the level of viable cells in the control was always much higher than the chloramphenicol containing suspensions due to multiplication of the control cells even at this low temperature.

This was not the case during the later stages of the experiment. In the latter 6 weeks of the experiment the effect of chloramphenicol was in favour of maintaining the viability of the cells instead of hastening their death.

The Effect of Chloramphenicol on the total and viable counts of ½M suspension of E. coli in M.E. broth at 22°C.

The beneficial effect of chloramphenicol in helping the viability of the E. coli cells was still noticed here with the 10 ug/ml chloramphenicol, and this was specially noticed after the first two weeks of the experiment.

Table 4 : Changes in the total and viable counts of ½M suspension of E. coli in M.E. broth stored at 22°C in the presence and absence of Chloramphenicol.

Period of Storage (days)	Total Count cells/ml x ~~10⁶~~ 10^{-6}			Viable Count cells/ml x ~~10⁶~~ 10^{-6}		
	Concentration of Chloramphenicol ug per ml.					
	0	10	100	0	10	100
0	400	350	350	140	120	130
1	1500	370	350	605	130	65
2	2650	350	340	1210	135	25
9	4160	340	470	520	100	1.6
16	1950	460	540	85	170	1.1
23	1750	400	500	70	145	0.98

The level of viable cells in the control was higher during the first two weeks due to multiplication of the cells. The 100 ug/ml chloramphenicol was lethal to the number of cells in suspension. ($\frac{1}{2}$M) (Table 4).

The results obtained in the above two experiments confirmed what we concluded about the effect of chloramphenicol in favouring the viability of the E. coli cells instead of hastening their death, but suggested that there might be an optimal concentration of chloramphenicol per cell at which this effect was manifested. (Gray, 1952).

c. Using different nutritive conditions for ageing of cells.

Saline:

In this experiment a medium with no nutritive value was used for suspending either a heavy concentration of cells (5M) or a more dilute suspension of cells ($\frac{1}{2}$M) thus making conditions quite inimical to cell multiplication. The variables in this experiment were the temperature of incubation, 5°C and 22°C, and the

Table 5 : Changes in the total and viable counts of 5M suspension of E. coli in saline stored at 5°C in the presence and absence of Chloramphenicol.

Period of Storage (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}			Viable Count cells/ml x ~~10⁷~~ 10^{-7}			pH		
	Concentration of Chloramphenicol ug per ml.								
	0	10	100	0	10	100	0	10	100
0	1080	950	930	455	465	500	6.71	6.72	6.69
2	640	600	700	390	465	490	6.70	6.71	6.70
7	600	570	500	370	510	485	6.72	6.70	6.70
21	500	530	565	355	470	433	6.75	6.70	6.71
35	400	565	565	171	226	302	6.75	6.72	6.75
56	350	300	530	33	64	68	6.77	6.60	6.80

Table 6 : Changes in the total and viable counts of 5M suspension of E. coli in saline stored at 22°C in the presence and absence of Chloramphenicol, pH records.

Period of Storage. (days).	Total Counts cells/ml x 10^{-7}			Viable Counts cells/ml x 10^{-7}			pH		
	Concentration of Chloramphenicol ug per ml.								
	0	10	100	0	10	100	0	10	100
0	500	500	475	321	340	325	6.86	6.92	7.15
1	340	305	305	315	298	301	7.37	7.53	7.44
2	390	345	350	288	284	294	7.10	7.50	7.38
6	405	390	390	108	155	157	7.92	7.85	7.85
10	250	240	240	51	47	57	7.92	7.85	7.86
15	250	250	230	26	25	14	7.95	7.97	7.88

concentration of the antibiotic, 10 ug/ml and 100 ug/ml, and the population of cells suspended, 5M and ½M.

The results are shown in Tables 5, 6 and 7.

At 5°C the results (Table 5) showed that in the presence of chloramphenicol the viable counts kept steady for the first three weeks and then dropped gradually in the following five weeks. The control viable counts, however, showed a gradual drop during the whole 8 weeks and the levels were always higher with the chloramphenicol containing suspensions than the control in all the stages of the experiment.

The total count in both the control and the chloramphenicol containing suspensions showed a gradual drop during the whole eight weeks of the experiment.

At 22°C with 5M suspension in saline (Table 6) the chloramphenicol did not have any beneficial effect on prolonging the life of the cells.

When a ½M suspension of E. coli was suspended in saline and stored at 22°C in the

Table 7 : Changes in the total and viable counts of ½M suspension of E. coli in saline stored at 22°C in the presence and absence of Chloramphenicol with pH records.

Period of Storage (days)	Total Counts cells/ml x 10⁶			Viable Counts cells/ml x 10⁶			pH		
	Concentration of Chloramphenicol ug per ml								
	0	10	100	0	10	100	0	10	100
0	560	550	550	307	298	311	6.30	6.08	5.79
1	560	525	525	296	271	267	6.28	6.24	6.10
2	530	490	525	246	258	260	6.22	6.21	6.15
7	460	460	375	160	143	115	6.28	6.28	6.20
11	375	390	390	74	66	40	6.26	6.26	6.16
15	350	345	370	30	24	7	6.30	6.26	6.10

presence and absence of chloramphenicol, the pH of the medium did not vary much in the whole experiment. The viable counts of the cells in the control was always a little higher than in the chloramphenicol containing ones especially the 100 ug/ml one. (Table 7).

It can be seen that the pH in the control suspensions and in the chloramphenicol suspension were much the same when saline was used as suspending medium, and where there was no rise in pH there was little difference between the death-rate in the presence and absence of chloramphenicol.

Glucose phosphate medium:

In this experiment the suspending medium consisted of inorganic salts and glucose, a medium able to support the growth of E. coli. Heavy suspensions (5M) were used. In such a medium the end products of metabolism are acidic and the pH falls. Table 8 gives the results of a typical experiment.

The results indicate that in a medium with the pH reaching values on the acid side of

Table 8 : Changes in the total and viable counts of 5M suspension of E. coli in glucose phosphate medium stored at 22°C in the presence and absence of Chloramphenicol with pH records.

Period of Storage. (days).	Total Count cells/ml x 10^{-7}			Viable Count cells/ml x 10^{-7}			pH		
	Concentration of Chloramphenicol ug per ml.								
	0	10	100	0	10	100	0	10	100
0	490	400	410	221	268	251	6.94	6.90	6.90
1	530	450	450	256	198	205	4.43	4.43	4.50
2	890	480	425	226	177	184	5.15	5.15	5.15
7	410	410	410	81	53	60	5.70	5.70	5.90
10	440	400	390	75	42	75	5.85	5.75	5.95

neutrality there is little difference in the death rate of cells aged in the presence and absence of chloramphenicol.

The drop in the viability was greater than one would expect from the changes in pH. This phenomenon was noticed and commented upon by Gale and Epps (1942), who found that the presence of glucose in the medium during growth of E. coli suppresses the formation of certain enzymes, and that the degree of inhibition is greater than the effect produced by growth in a medium adjusted to the final pH produced in the glucose medium by fermentation.

When Staphylococcus aureus, which has a widely different metabolism from E. coli was used in the above group of experiments, the results obtained were not very constant. However, with low concentrations of chloramphenicol and under the previously mentioned conditions of the experiments the viable counts remained steadier in the chloramphenicol containing suspensions (5 ug/ml, and 10 ug/ml) than the control ones, while with higher chloramphenicol

Table 9 : Changes in the total and viable counts of 5M suspensions of Staphylococcus aureus at 22°C in the presence and absence of Chloramphenicol. Cells suspended in M.E. broth.

Period of Storage. (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7} Concentration of Chloramphenicol ug/ml						Viable Count cells/ml x ~~10⁷~~ 10^{-7} Concentration of Chloramphenicol ug/ml					
	0	5	10	20	40	100	0	5	10	20	40	100
0	660	530	530	570	530	440	265	229	227	227	226	116
7	590	520	520	590	575	620	237	195	168	145	171	133
13	550	495	550	550	620	550	221	204	199	142	150	201
19	490	475	430	490	530	495	199	225	190	159	122	135
27	440	440	410	440	460	440	126	185	254	180	78	55
33	600	355	520	475	530	475	61	102	139	103	54	32

concentrations (20 ug/ml, 40 ug/ml and 100 ug/ml) we got the opposite results. Due to clumping of the organism the counts obtained were not very accurate (Table 9).

Table 10 : Changes in the total and viable counts of 5M suspension of E. coli in buffered (Fishers) indicator broth stored at 22°C in the presence and absence of Chloramphenicol.

Period of Storage. (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}			Viable Count cells/ml x ~~10⁷~~ 10^{-7}			pH		
	Concentration of Chloramphenicol. ug per ml.								
	0	10	100	0	10	100	0	10	100
0	655	640	640	254	256	260	7.39	7.37	7.37
1	590	440	425	299	257	263	7.41	7.38	7.37
2	950	620	440	300	252	271	7.67	7.41	7.39
5	575	390	320	130	219	230	7.92	7.42	7.39
8	470	310	300	59	190	200	8.12	7.71	7.41

Group II Experiments.

Attempts to control the pH of suspensions.

The results of the earlier experiments show clearly the marked effect upon mortality which the pH of the medium has.

In the first experiment the cells were suspended in solutions of phosphate buffer in meat extract broth.

Use of Phosphate Buffer:

From Table 10 it can be seen that the buffer was ineffective in holding the pH. The use of higher concentrations of the buffer led to rapid death of cells.

Use of Glucose broth:

It was thought that the acidity produced by the addition of glucose to the M.E. broth suspensions of E. coli might help to keep the pH of the suspension from rising. So glucose in varying concentrations ranging from 2% to .01% was made and added to 5M E. coli suspension incubated at 37°C. The pH of each concentration was measured after 24 hours and after various periods of incubation. The

viable counts were also made for each reading.

The results obtained showed that the higher concentrations of glucose, although they kept the pH of the medium on the acid side, had a deleterious effect on the viability of the cells. On the other hand, the lower concentrations of the glucose were not able to keep the pH from rising for more than 2 days. So this method of controlling the pH was abandoned.

Use of Saline:

It was then thought that a saline suspension of cells would not allow much change in the pH value of the medium and so a repeat of the saline experiments at 22°C was done and records of pH at every viable count reading were taken.

The results showed that although the pH kept nearly steady ranging between 7 and 8, this range was still too wide to get a very clear idea about the effect of the changes in viable count produced by the change in the pH of the medium.

The total and viable counts of both the control and chloramphenicol containing suspensions

dropped during the whole experiment but the control levels were always lower than the chloramphenicol ones (see Table 6).

Adjustment of pH:

In this experiment phenol red indicator was added to the M.E. broth of both the control and the chloramphenicol containing suspensions. The pH was kept constant by the addition of $\frac{N}{5}$ acid or alkali (HCl or NaOH) judging the pH by the colour of indicator and checking with readings of pH meter. A 5M suspension was used and incubated at 22°C for three weeks.

The results obtained were still not very satisfactory, because although the pH was brought back to the original level twice every day, the control pH rose during the night to higher readings which were thought to be deleterious to the cells.

Nevertheless, the viable count readings of the control were higher than the corresponding chloramphenicol ones after the first week.

Table 11 : Changes in the total and viable counts of 5M suspension of E. coli stored at 22°C in the presence and absence of Chloramphenicol, changing the medium daily.

Period of Storage. (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}			Viable Count cells/ml x ~~10⁷~~ 10^{-7}		
	Concentration of Chloramphenicol ug per ml.					
	0	10	100	0	10	100
0	750	655	655	428	466	435
1	1100	740	560	416	408	417
2	1275	600	480	418	341	355
4	1550	500	410	446	274	256
7	1350	525	300	368	185	108
10	1970	345	175	319	101	36
15	1850	150	120	325	11	0

Removal of medium daily to prevent pH rise:

It was then thought that if the vehicle used for suspending the cells was changed every day, the organisms would not have a long enough time to split the amino acids of the M.E. broth to a degree that would raise the pH of the medium to deleterious levels. So, in this experiment centrifugation of the suspensions was done daily in 250 ml centrifuge flasks for 30 minutes at 2600 r.p.m. and the vehicle used for suspending the cells was replaced every day. The pH was recorded every day to ensure that it did not rise during the 24 hours. The suspensions were 5M ones and they were incubated at 22°C.

The results obtained proved that the pH did not change much during the whole experiments and ranged between 6.9 and 7.5. As for the total counts of the control, they kept rising during the whole two weeks of the experiment reaching three times the original population by the end of the second week, while the viable count remained steady for the first week and diminished gradually during the following week.

Table 12 : Changes in the total and viable counts of 5M suspensions of E. coli in Veronal Buffer Indicator Broth (pH 7.2) stored at 22°C in the presence and absence of Chloramphenicol.

Period of Storage. (days).	Total Count cells/ml x ~~10^{7}~~ 10^{-7}			Viable Count cells/ml x ~~10^{7}~~ 10^{-7}		
	Concentration of Chloramphenicol ug/ml					
	0	10	100	0	10	100
0	655	640	600	345	381	368
1	850	600	500	380	392	374
2	700	410	525	411	359	347
5	640	400	375	293	219	199
8	620	320	300	100	70	77
11	530	345	270	86	51	20

The total counts of the chloramphenicol containing suspensions kept steady during the first week and dropped gradually during the whole experiment.

The level of both the total and viable counts of the chloramphenicol containing suspensions were lower than the corresponding control ones during the whole experiment and the difference in the viable count was appreciable falling to ~~half~~ one third of the control level at the end of 10 days and ~~1/200~~ 1/30 of the control level at the end of 15 days with 10 µg/ml. chloramphenicol (Table 11).

It was decided to confirm the results of the centrifugation experiment by attempting to find a buffer which could be effective in holding the pH of the suspensions without exerting a lethal action on the cells. Sørensen phosphate buffer as well as veronal buffer appear to fulfil this requirement.

Use of Veronal Buffer:

It can be seen from Table 12 that when the pH of the medium was kept constant in the control and in the chloramphenicol containing suspensions, the chloramphenicol had no effect whatsoever in maintaining viability.

Table 13 : Changes in the total and viable counts of 5M suspension of E. coli in Sørensen phosphate buffered indicator broth (pH 7) stored at 22°C in the presence and absence of Chloramphenicol.

Period of Storage. (Days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}			Viable Count cells/ml x ~~10⁷~~ 10^{-7}		
	Concentration of Chloramphenicol ug/ml.					
	0	10	100	0	10	100
0	550	550	530	306	292	264
1	640	450	440	315	297	334
2	890	390	410	309	240	284
7	890	375	320	154	91	107
10	575	300	230	151	95	66
14	550	270	250	161	63	23

Sørensen Phosphate Buffer:

The results seen in Table 13 confirms those obtained with the veronal buffer.

These results obtained in the above groups of experiments suggest that the effect of chloramphenicol in maintaining viability was due to its suppression of the formation of alkaline products of metabolism.

We therefore can reach the following conclusions:-

Under chosen conditions, chloramphenicol may delay the death of E. coli cells. This effect of chloramphenicol in delaying cellular death is related to its ability to prevent the formation of alkaline products of metabolism.

Table 14 : Changes in the Total Counts, Viable Counts and pH of the medium in a 5M suspension of E. coli R in M.E. broth stored at 22°C in the presence and absence of Chloramphenicol.

Period of Storage. (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}				Viable Count cells/ml x ~~10⁷~~ 10^{-7}				pH			
	Concentration of Chloramphenicol ug/ml.											
	0	10	50	100	0	10	50	100	0	10	50	100
0	480	450	450	450	283	256	267	278	6.92	6.98	6.97	6.91
1	900	780	700	640	285	292	271	225	7.18	6.95	6.85	7.00
2	1020	890	800	750	342	324	301	281	7.50	7.34	7.19	7.10
6	335	425	345	355	62	67	71	75	8.75	8.58	8.15	8.30
9	250	350	220	250	23	39	31	38	9.19	8.85	8.32	8.24
11	230	210	200	200	18	15	15	17	9.06	9.06	8.70	8.59

Group III Experiment.

Relation of drug sensitivity to the prevention of death by Chloramphenicol.

The next question that arose was, could we by raising the resistance to chloramphenicol of the originally sensitive strains (3 ug/ml) change the metabolism of the cells in such a way that it would give us a further proof as to the effect of the changes in the pH of the medium on the viability of E. coli in the presence and absence of chloramphenicol?

The strain E. coli R was used in these experiments and it was resistant to 50 ug/ml chloramphenicol.

As can be seen from Table 14, raising the resistance of the cells did change their metabolism in such a way that incubating them in presence of 100 ug/ml, 50 ug/ml or 10 ug/ml chloramphenicol did not have the previous effect it had on the sensitive strains both on the viability and on the ~~maintainance~~ maintenance of a steady pH. As can be seen, there was not much to choose between the control and the chloramphenicol-containing

suspensions in these two points. The pH started to become lethal after 48 hours of incubation especially with the lower concentrations of chloramphenicol and the cells did not show any preferential viability in the presence or absence of chloramphenicol. This adds a further proof to the conclusion reached that any beneficial effect that chloramphenicol had during the earlier experiments on maintaining the viability of E. coli cells was mainly directed towards keeping the pH of the medium in these suspensions from becoming inimical to the cell's growth and multiplication as was the case in the control experiments.

Group IV Experiments.

The Use of Enzyme Inhibitors.

In this group of experiments an attempt was made to prolong the life of E. coli cells by using enzyme inhibitors to inhibit the primary changes leading towards cellular dissolution. Three enzyme inhibitors known to interfere with enzymes possessing sulphydryl groups were chosen. These were, iodoacetic acid, iodosobenzoic acid and p-chloromercuribenzoate; in addition, the highly specific esterase inhibitor, Isopestox (N.N di-isopropyl phosphorodiamidic fluoride) was used. These inhibitors were used at concentrations ranging from 10^{-3} to 10^{-6}M; they were tried in mixtures of sublethal concentrations, they were used in different buffers and in the same buffer with different pH values. In every experiment a total count, a viable count and pH of the medium was determined at various intervals (Tables 15, 16, 17, 18, 19, 20 and 21). However, as can be seen from these tables, under none of the conditions used did these inhibitors prove successful in prolonging the life of the E. coli

cells.

Table 15 : Changes in the total and viable counts of 5M suspension of E. coli in M.E. broth stored at 22°C in the presence and absence of iodoacetic acid.

Period of Incubation at 22°C (days).	Total Count cells/ml x ~~10⁷~~ 10^{-7}				Viable Count cells/ml x ~~10⁷~~ 10^{-7}				pH			
	Concentration of Iodoacetic Acid.											
	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M
0	750	730	730	750	369	368	375	374	6.46	6.56	6.76	6.46
1	1420	550	1100	1375	432	298	408	416	7.18	6.82	7.25	7.15
2	1375	530	1250	1375	343	286	309	346	8.00	6.86	8.26	8.20
6	545	500	450	475	22	37	24	24	8.26	8.00	8.36	8.18
8	345	390	345	345	21	22	22	20	8.50	8.69	8.7	8.59
14	320	335	320	320	17	20	20	16	8.72	8.70	8.72	8.73

Table 16 : Changes in the total and viable counts of 5M suspension of *E. coli* in M.E. broth stored at 22°C in the presence and absence of iodosobenzoic acid.

Period of Storage (days).	Total Count. cells/ml x ~~10^{7}~~ 10^{-7}				Viable Count. cells/ml x ~~10^{7}~~ 10^{-7}				pH			
	Concentration of iodosobenzoic acid.											
	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M
0	760	730	750	750	370	374	366	368	6.43	6.35	6.44	6.43
1	1075	1100	1160	1110	484	464	446	479	7.19	7.25	7.39	7.32
2	1306	1275	1275	1275	353	361	255	368	8.20	8.21	8.33	8.19
6	435	440	410	410	21	25	22	16	8.22	8.33	8.30	8.00
8	400	390	320	335	12	16	17	16	8.65	8.69	8.72	8.63
16	270	335	300	280	11	11	8	8	8.74	8.74	8.76	8.76

Table 17: Changes in the total and viable counts of 5M suspension of E. coli in M.E. broth stored at 22°C in the presence and absence of P-chloromercuri-benzoate.

Period of Storage (days).	Total Count cells/ml x 10^7				Viable Count cells/ml x 10^7				pH			
	Concentration of parachloromercuri-benzoate.											
	0	10^{-3}M	10^{-4}M	10^{-5}M	0	10^{-3}M	10^{-4}M	10^{-5}M	0	10^{-3}M	10^{-4}M	10^{-5}M
0	600	600	600	570	330	296	284	311	6.16	6.6	6.30	6.17
1	1000	490	460	1000	383	25	35	382	7.34	6.71	6.41	7.31
2	1400	450	440	1375	289	0	0	357	7.92	6.45	6.19	8.08
5	440	400	410	460	14	0	0	18	8.59	6.75	6.30	8.95
12	345	275	330	335	9	0	0	5	8.77	6.80	6.44	9.00

Table 18: Changes in the total and viable counts of 5M suspension of E. coli in M.E. broth stored at 22°C in the presence and absence of isopestox.

Period of Storage (days).	Total Count cells/ml x ~~10-7~~ 10^{-7}				Viable Count cells/ml x ~~10-7~~ 10^{-7}				pH			
	Concentration of isopestox											
	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M	0	10^{-4}M	10^{-5}M	10^{-6}M.
0	1100	1040	1100	1100	276	309	332	316	6.90	6.90	6.90	6.90
1	1840	1540	1540	1860	446	461	416	416	6.96	6.92	6.92	6.92
2	1400	1400	1440	1440	443	451	517	508	7.87	7.79	7.86	7.87
5	655	730	730	760	35	36	36	33	8.58	8.49	8.43	8.42
14	550	490	525	550	26	26	25	27	8.41	8.54	8.64	8.64

Table 19: The effect of mixtures of the different enzyme inhibitors used previously in their sublethal doses on the Total and Viable cell counts of 5M concentrations of E. coli in M.E. broth at 22°C.

Period of Storage. (days).	Total Count cells/ml x ~~10^7~~ 10^{-7}				Viable Count cells/ml x ~~10^7~~ 10^{-7}				pH			
	Control.	Iodo-acet. 10^{-4}M Iodo-zob. 10^{-4}M	Iodo-acet. 10^{-4}M P-chloro 10^{-5}M	Iodo-zob. 10^{-4}M P-chloro 10^{-5}M	Control.	Iodo-acet. 10^{-4}M Iodo-zob. 10^{-4}M	Iodo-acet. 10^{-4}M P-chloro 10^{-5}M	Iodo-zob. 10^{-4}M P-chloro 10^{-5}M	Control.	Iodo-acet. 10^{-4}M Iodo-zob. 10^{-4}M	Iodo-acet. 10^{-4}M P-chloro 10^{-5}M	Iodo-zob. 10^{-4}M P-chloro 10^{-5}M
0	550	525	525	525	360	306	303	306	6.15	6.41	6.61	6.64
1	1250	460	490	460	523	27	333	60	6.93	6.60	6.69	6.58
2	1250	390	555	460	185	0	63	0	8.14	6.43	8.12	6.31
5	640	400	420	440	23	0	25	0	8.69	6.41	8.18	6.39
7	440	270	425	325	22	0	10	0	9.21	6.46	9.16	6.96

Table 20: The effect of a mixture of iodoacetic acid (10^{-4}M) and parachloromercuribenzoate (10^{-5}M) on the total and viable cell count of 5M E.coli in borate buffer with different pHs at 22°C.

Period of Storage. (days).	Borate Buffer pH 7.6						Borate Buffer pH 8.						Borate Buffer pH 8.4.					
	+Enzyme inhibitor mix.			Control.			+Enzyme inhibitor mix.			Control.			+Enzyme inhibitor mix.			Control.		
	T.C.	V.C.	pH	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.
0	220	187	7.57	230	208	7.5	250	209	7.9	230	127	7.87	300	280	8.36	320	268	8.39
1	270	65	7.57	230	185	7.53	280	68	7.9	230	124	7.87	260	109	8.36	360	195	8.36
3	180	10	7.57	335	84	7.52	200	10	7.9	300	47	7.9	270	6	8.36	320	41	8.4
6	130	0	7.57	280	25	7.6	200	0	7.9	250	9	7.9	250	1	8.36	180	13	8.4

T.C. = Total Count, cells/ml x ~~10^{7}~~ 10^{-7}.
V.C. = Viable Count, cells/ml x ~~10^{7}~~ 10^{-7}.

Table 21: The effect of a mixture of iodoacetic acid (10-4M) and parachloromercuri-benzoate (10-5M) on the total and viable cell count of 5M E. coli in varying vehicles. with nearly the same pH at 22°C.

Period of Storage (days)	Phosphate Buffer.						Borate Buffer.						Saline.						M.E. Broth.					
	+Enzyme inhibitor mix.			Control.			+Enzyme inhibitor mix.			Control.			+Enzyme inhibitor mix.			Control.			+Enzyme inhibitor mix.			Control.		
	T.C.	V.C.	pH.	T.C.	V.C.	pH	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.	T.C.	V.C.	pH.
0	360	220	7.08	390	190	6.92	305	204	7.00	360	212	7.00	370	270	6.94	390	289	6.99	360	239	7.00	390	217	6.95
1	320	54	7.2	850	214	7.07	350	104	7.10	450	251	7.03	405	204	7.11	760	329	7.44	400	143	7.30	970	296	7.67
2	350	53	7.15	895	214	7.12	290	23	7.00	440	174	7.02	420	35	7.04	700	201	7.77	340	71	7.20	1120	290	8.10
6	300	26	7.67	760	118	7.42	220	25	7.09	340	80	7.05	360	32	7.95	360	38	7.93	700	69	8.10	930	42	8.80
9	360	23	8.68	490	31	7.76	230	7	7.00	320	30	7.09	390	14	7.94	360	27	7.98	620	31	8.60	410	14	8.90
13		6	8.92	270	12	7.92	150	2	7.00	270	7	7.05	390	9	8.18	340	18	8.28	440	10	8.80	390	9	9.20

T.C. = Total Count cells/ml x ~~10⁷~~ 10^{-7}

V.C. = Viable Count cells/ml x ~~10⁷~~ 10^{-7}

SECTION II.

The Use of Antigenic loss as an indicator of cellular death.

INTRODUCTION

We referred in the first section to the way in which the investigations were limited by the slowness of our method of measuring the number of viable cells. It became imperative to find some quicker and simpler technique for indicating cellular death.

When a mammalian cell passes from the living to the non-living state it undergoes morphological changes which are, presumably a reflection of degradative changes in the material forming the structure of the cell. Morphological changes are less obvious in the rigid-walled bacterial cell, but degradation of cell substance must nevertheless occur since life involves a continuous replacement of the components of the cell. It therefore seemed of interest to follow the decay of one group of proteins, the cell antigens. So, at this point, the emphasis of our studies was switched to the degenerative changes in the constituents of dying cells particularly of a specific group of cell proteins, the antigens. The specificity of an antigen

reflects its structural form, and comparatively slight modifications in structure may lead to a modification or loss of antigenicity. It was therefore thought that the antigens might reflect any changes occurring in the living bacterial cell as it aged, and that these changes might provide an easily detectable indicator of the change of the cell from life to death.

In attempting to find out whether agglutinogens were lost by ageing E. coli cells, the first method of investigation used was a simple tube agglutination technique in which cells of varying ages were titrated against a specific antigen. However, the naked eye reading of the end titre proved extremely difficult to read and the method was finally abandoned.

The next approach was to apply the method of quantitative agglutination (Pereira and Travassos, 1952).

Lastly the Ouchterlony gel-diffusion technique was used to measure loss of antigens.

(a) Quantitative Agglutination: Method of the Study of Specific Agglutination of Micro organisms.

The study of the specific agglutination of bacteria, rickettsias and viruses by immune sera is usually made by the serial dilution method, and the results are expressed as the highest dilution of serum which causes a standard degree of agglutination. Agglutination methods, however, give only very approximate ideas of the strength of the serum or antigen. By letting one single tube determine the titre, the determination may be subject to a large error and the degree of agglutination in the surrounding tubes may not reflect alterations in antigen or antibody in a clear-cut fashion. Moreover, the dilution interval between the tubes is great if the two-fold serial method is used. This error can naturally be reduced by repeating the agglutination with a smaller dilution factor, but there it must not be overlooked that the difference in degree of agglutination between tubes is small, a fact which renders the determination of the end-point

much more difficult. In practice it was found that it is not easy to express the titre by the final end point i.e. the dilution in the last tube where agglutination is perceptible.

In view of the limitation of the customary methods of estimating serological end-points and in order to obtain greater uniformity and reproducibility of the results of such tests, Reden (1946) described a method for the determination of 50% end-point based on the method of Reed and Muench (1938) in animal tests. This method has the advantage of taking into consideration a number of dilutions for the evaluation of the titre but it suffers from the influence of the personal bias which may be considerable when agglutination tests are read for different workers.

Quantitative analysis using 50% end-point dates back to Leschly (1914) who had titrated complement quantitatively, Morse (1916), who recommended the point of 50% haemolysis for the more accurate titration of complement and Brooks (1919-1920) who wrote "------ in choosing

complete haemolysis, as a criterion, immunologists have chosen to use not the most accurately, but precisely the least accurately, determinable end-point." Morse (1921-22) later advised the use of 50% haemolysis as the end point. The most systematic studies employing 50% haemolytic unit of complement were by Wadsworth, Maltaner & Maltaner (1938) and Kent et al (1946).

The use of quantitative titration was also used by Dreyer and Inman (1917) who used a certain standard agglutination of salmonella bacteria by determining and interpolation reading from the degree of agglutinations in the tube on each side of the end point. The average reading of the titre in these two tubes was taken as the dilutions giving standard agglutinations.

For the titration of therapeutic and toxic substances in animals Gaddum (1933) has pointed out the suitability of choosing as end point the dilution at which 50% of the animals react. An end point in biological procedures such as

titration of sera or viruses is usually taken as the dilution at which a certain proportion of test animals react or die. The advantage of using the dilution at which half the animals are affected have been set forth by Gaddum on the basis of pharmacological tests. This end point is less affected by small chance variations than is any other, the worst in the report being the 100% point so frequently used.

The 50% point was used in quantitative complement fixation tests by Wolfe et al (1949) and Rice (1946).

In studies of the agglutination reaction due to influenza virus (Hirst et al 1942), the optical determination was found important in several respects. By this method the measurement of haemoagglutinin and antibody titres can be made more accurate and reproducible. In addition, the readings were more objective than formerly and accuracy is not so dependent on the experience of the operator as with the former visual method.

Compared with the technique usually used, and from the results obtained by Pereira and Travassos (1952), it could be expected that the application of the 50% point in sedimentation-centrifugation serological experiments would be of value in providing evidence not only in inter-group antigenic differences but also in the study of the antigenic structure of bacteria.

(b) Antigenic analysis by diffusion in gels:

The employment of the usual dilution technique is not particularly suitable for antigenic analysis. In dealing with a substance of complex antigenic structure, e.g. normal serum, addition of the corresponding immune serum gives in the test tube a precipitate which is apparently homogenous but really consists of various precipitates such as albumin + antibody, globulin + antibody etc. Only by fractionation of the antigen and absorption of the immune serum is it practicable to separate the individual antigenic components.

Similarly, qualitative analysis of the naturally occurring bacterial cell or any complex of antigens by such serological tests is at best rough and incomplete.

During the last few years a versatile technique involving specific antigen-antibody precipitation in gels has been studied (Ouchterlony, 1948a; Becker et al, 1949 a-b; Oudin, 1948, Petrie, 1932). There are two ways of carrying out the technique; simple diffusion and double diffusion.

Historical review of the diffusion in gels:

The fact that antigens and antibodies diffusing in gel react in this way was observed many years ago. In (1905) Bechhold described a reaction of this kind in 1% gelatine between goat serum and anti-goat serum obtained from the rabbit. A similar observation was made by Nicolle et al (1920) for diphtheria toxin and anti-toxin from horse. Diphtheria immune serum as a surface layer in a tube was allowed to react with toxin mixed with an equal part of 10% gelatine. They then observed a disk-like precipitation of toxin-antitoxin character. Between 1932-34 Petrie (1932) Sia and Chung (1932) and Kirkbride and Cohen (1934) described specific and non specific (halo) phenomenon around bacteria colonies growing on substrates containing immune serum. Their investigations concern meningococci, pneumococci and shiga dysentry bacilli. The method was later worked out for the serological typing of meningococci and pneumococci. In 1948 toxin-antitoxin reactions of similar character were described by Ouchterlony (1948b). Tetanus

bacilli, toxin and toxoid, gas gangrene bacilli and toxin, pneumococci and ~~chick~~ toxin from streptococci have been tested by the method and with similar results.

Agar precipitation techniques (Oudin, 1946, 1952, Ouchterlony, 1948, 1953, Oakley and Fulthorpe, 1953) have recently been applied to the analysis of complex antigen systems. Using the method of Ouchterlony, the antigens of C. diphtheriae were examined by Ouchterlony, Ericsson and Neumüller (1950) and those of Bacterium tularense by Ormsbee and Larson (1955). Tunevall (1953) has studied the antigens of H. influenzae and Bjorkland and Berengo (1954), have characterised the toxins of clostridia. Halbert, Swick and Sonn (1955 a, b) have also employed these methods to study the antibodies produced in human streptococcal infections and Schiott (1953) and Wodehouse (1954) have investigated human antibodies in case of allergy.

The effect of a number of inhibitors of assimilation and protein synthesis was tested with the P. pestis antigen system. The effect

of Diazouracil, 2, 4 dinitrophenol (10^{-4}M) and chloramphenicol on antigen synthesis and on respiration was followed by Fox and Higuchi (1958). Olitzki and Sulitzeanu (1959), using a slight modification of the technique described by Ouchterlony (1948), in their studies of the antigenic structure of Haemophilus aegypteus and H. influenzae. Dumbell and Nizamuddin (1959) described a simple and rapid laboratory test for diagnosis of smallpox.

Wilson and Pringle (1954) undertook an experimental evaluation of Ouchterlony's test with the purpose of establishing additional criteria for a standardised technique. They tackled the effects of the following factors on the agar plate precipitin test: initial concentration of agar, size of petri-dish, initial sodium chloride concentration in agar, pH of agar, temperature of incubation, arrangement of basins, absolute and relative concentrations of reagents and discontinuous diffusion of reagents.

The entire subject of specific precipitation

in gels has been reviewed by Oudin (1952).

As already mentioned there are two methods of carrying out the gel diffusion technique. In "simple diffusion" one reactant, usually antigen diffuses through a gel containing a constant amount of the other reactant, usually antibody. (Oudin, 1946, 1952; Oakley and Fulthorpe, 1953). In "double diffusion", two or more reactants diffuse towards each other through a gel which originally contained neither reactants. (Ouchterlony 1948 a & b, 1953). The result by either method is the formation of specific antigen-antibody precipitation zones visible as lines or rings in the medium.

The method of double diffusion, first developed by Ouchterlony (1948 a, b), Elek (1948, 1949) and later by Bjorklund (1952) for studies of toxin production by living cultures of bacteria, is based on the principle that specific antigen and antibody diffusing towards each other through a gel will precipitate when they reach a state of equivalence or optimal proportions, assuming the presence of sufficient

antigen and antibody to cause precipitation. The numbers of precipitation zones should be equal to the minimum number of antigens in the system and not greater than the maximum. The method of Ouchterlony, unlike that of Oudin, and Oakley and Fulthorpe, has the advantage that a number of antigen or antibody mixtures can be compared directly with one another and common components thus recognised.

The technique of double diffusion in gels appears to have considerable value as a highly precise method for qualitative analysis of antigens, it appears to be an excellent method for comparative studies of not only heterologous but closely related organisms, particularly in connection with cross immunity.

Through Ouchterlony's method, the gel precipitation was developed into an analytical procedure of wide applicability in the study of antigen-antibody systems. The analysis of complicated reaction systems was further prompted by Bjorklund (1952) who could eliminate certain precipitates by pre-treatment of the

medium with some of the reactants or by partial neutralization of the antiserum in one basin.

Advantages of the gel-diffusion technique: Analysis of the antigenic composition by the application of recent techniques in which antigens and antibodies are allowed to react in an agar gel is revealing advantages and complexities which could not be demonstrated by the classical methods of serology when the reaction takes place in liquid medium (Bjorklund et al, 1954; Halbert et al, 1955; Ransom et al, 1955; Ormsbee et al, 1955; and Chen et al 1955).

Ouchterlony's modification of the agar plate precipitin test appears to be valuable for the qualitative analysis of immunologic systems, especially when comparative studies of antigens or antibodies are desired. It seems to yield a resolving power and a specificity of the high order which is necessary in work of this kind. With this method it is possible to compare directly the occurrence of single components in antigenic preparations. Besides

the above mentioned advantages offered by the gel diffusion method, an additional feature appears also to render it suitable for particular studies. If for instance we are dealing with weak sera, that is sera with a slight precipitation content, it will often happen that the usual precipitation test fails to give any visible precipitate while the actual presence of antibody may be demonstrated by the above mentioned method.

Points of importance in interpretation of diffusion results. As shown in previous papers, concerning immuno-precipitation in a gel, there are some general principles regarding the appearance and site of the precipitates.

Appearance: It is possible in comparative immunological analysis to prove serological identity on non identity between antigens or antibodies by means of the gel diffusion technique with interference phenomena. By this is meant, that the adjacent reactions between reacting substances of the same kind affect each other in such a way that the streak like

precipitations interfere with one another. That is not the case in antigen-antibody reactions of different kinds, where so called cross phenomena appear instead. It is possible with the help of these interference and cross reactions to demonstrate serological differences or identity between different precipitating or flocculating substances.

A phenomenon resembling a spur can also appear when an antigen solution is compared with an excessively concentrated solution of the same antigen. In this case the precipitate formed by the more concentrated antigen will migrate towards the serum basin giving a picture of a partial identity reaction. The observation of the "partial identity reaction" may contribute to the possibilities of analysing with the gel diffusion method even more complicated immunological systems. For instance Ouchterlony, Ericsson and Neumüller, 1950 analysed the antigens of C. diphtheriae in different stages of purification.

Site: The position of the precipitation

streak in the diffusion medium is probably influenced by such factors as where optimal proportions between antigens and antibodies appear, the time necessary for the formation of a visible precipitate and the concentration threshold value of the reaction.

The concentration of the reacting substances are of decisive importance in determining whether and where the reaction will appear. Furthermore, if the concentration of one of the substances is known, it is even possible under certain conditions to estimate the other substance quantitatively.

It must also be remembered that antigens that are released or that diffuse very slowly form only faint zones even if the plates are examined after 2-3 weeks of incubation.

In conclusion it may be said that there is every reason to adopt the gel diffusion method, not only because of its obvious advantages in antigenic analysis, but furthermore because it perhaps may also offer a possibility of observing antigen-antibody

reactions that cannot be produced with the ordinary technique (Schiott, 1953). For these reasons we have attempted to employ Ouchterlony's agar-plate precipitation test in the study of E. coli antigens as the cells age. The literature, although very rich in references to applications of this technique to a wide variety of problems is surprisingly bare where the species E. coli is concerned.

EXPERIMENTAL METHODS.

Methods used in Experiments in which the death of bacteria was followed by determining antigenic loss.

The experimental work fell in the following groups of experiments.

I : Antigen-antibody agglutination experiments:

The effect of chloramphenicol on the antigenicity of E. coli cells left to age in its presence and in its absence and comparing the antigenic and the viability sequence in these cells. Two procedures of agglutination were followed.

a. The serial doubling dilution method.

b. The use of close intervals of dilution.

II : Agglutination sedimentation experiments:

In these experiments the T_{50} or the length of time required to centrifuge out enough cells from the agglutination system to bring the turbidity in the supernatant to 50% of the original value was measured.

The effect of chloramphenicol was also tested here under different environmental conditions.

a. Different temperature of incubation of the cells.

b. Suspending media of different nutritive value.

III : Gel diffusion experiments.

In these experiments the loss of antigen was followed by ageing E. coli cells under various conditions, and a relationship was established between the viability and antigenicity of the cells and the pH of the medium used for their suspension.

The various conditions used were :

a. The nutritive value of the medium.

b. The temperature of incubation of the cells during the process of ageing.

c. The pH of the medium.

Materials and Methods.

Strains:

All of the experiments described below used *E. coli* (026 B 6). The reason for choosing this strain was that a specific antiserum was already available at the time the experiments were started.

The organisms were maintained at room temperature on meat extract agar slopes after having been incubated at 37°C for 24 hours. Sub-inoculations were done fortnightly on to fresh slopes and the purity of the culture confirmed from time to time by plating and by biochemical reactions.

The cells were harvested from 24 hours' cultures in meat extract broth and resuspended in the different media used.

Media:

1, 2, 3, 4 and 9. The same as in the first section.

5. Buffered M.E. broth with different pH values:

The buffer used to give different pHs to M.E. broth was Fisher buffer. The pH was adjusted with $\frac{N}{5}$ NaOH to give pHs 7.5, 8 and 8.4.

The medium was sterilized through sintered glass filters.

Terms:

T_{50} : The period of centrifugation needed to reduce the number of cells in the supernatant of a serum/cells mixture to half the initial value as measured by the absorptiometer.

Antigenicity index:

This term is defined as follows -

$$\text{Antigenicity index} = \frac{\text{half sedimentation time in saline}}{\text{half sedimentation time in anti-serum.}}$$

Antigen Complex:

The pattern of E. coli precipitation lines obtained in gel diffusion plates and resulting from the various antigens diffusing from cell suspensions or supernatants and giving lines of precipitation with specific antibodies in the zone of optimal proportion.

EXPERIMENTAL DETAILS.

A. Temperatures of incubation:

1. The cells were always harvested from 24 hours cultures at 37°C.

2. The various temperatures used for incubation

in different experiments were 5°C and 22°C.

3. The M.E. agar plates for viable counts were tested for sterility, dried and incubated for the counts at 37°C.

4. The gel diffusion plates were incubated at 22°C for one week and at 5°C for another week.

5. Antigen-antibody agglutination reactions were put in water baths at 56°C for 4 hours and then overnight at room temperature.

B, C, D, E and F, that is preparation of cell suspensions, total counts, viable counts, estimation of pH and assay of chloramphenicol were as described in the first part of the thesis.

G. Recovery of the organisms:

After harvesting, the cells were resuspended in the vehicle required for the various experiments in 5M concentrations in universal containers. The containers held 10 ml each of the batch of cells used and were incubated at the temperature required. The specific container was taken out of the incubator after the required interval and treated as follows:

1. For the antigen-antibody agglutination experiments the cells were centrifuged for ½ hour at 2000 r.p.m. washed twice in saline, resuspended in saline and heat killed by boiling.

The cells were standardised to the same turbidity by the use of the Spekker absorptiometer and then kept at 5°C until needed for agglutination.

2. For the gel diffusion experiments the cells were collected from the universals by centrifuging. The supernatant was kept whenever needed. The cells were washed twice in saline and resuspended in 1 ml saline to get a thick suspension. Both the cells and the supernatant were boiled for 1 hour (Macpherson, 1956) after which they were kept at 5°C until required for gel diffusion.

H. Production of antisera against Bact. coli 026 B6:

The inoculum was prepared by growing the organisms overnight on thick 0.1% glucose agar plate. The plates were of soft 1% agar. A suspension of about 500 millions organisms/ml

in ¼ strength Ringer solution (using Brown's tubes for estimating the number of organisms) was employed as rabbit inoculum. Fresh living suspensions were made up not more than ½ hour before each inoculation.

The rabbit was given 5 intravenous injections at 4-day intervals with the following increasing quantities of inoculum: - 0.25 ml., 0.5 ml., 0.5 ml., 1.0 ml., and 1.0 ml. The rabbit was bled from the ear on the 5th. and 7th. day for preliminary test of agglutination titre and bled out on the 9th. day after the last injection. The rabbits were injected in batches of three. The serum should give a K titre of 1 : 800 - 1 : 1600. When small rabbits were used it was found necessary to give a further inoculation of 1 ml. if the test titre was not satisfactory. Sera giving a titre of less than 1 : 400 were discarded.

Serological analysis.

Agglutination Test:

1. Serial doubling dilutions.

~~A rack containing~~ To a set of " x " 3 x ½ test tubes ~~in which~~ each containing

0.4 ml of serially two fold diluted serum ~~was prepared~~ in saline, ~~and~~ 0.4 ml of the standardised E. coli suspension was added. The mixture was recovered from the 3 x ½ tubes and put in agglutination tubes. The tests were incubated at 56°C in the water bath for 4 hours after which the agglutination titre was read, and then they were left overnight at room temperature, and another reading was done. For each experiment a control tube containing saline instead of serum was included.

The results of agglutination were interpreted as:- ++ = clear supernatant + maximal precipitate; + = Turbid supernatant + precipitate which is very visible; ± = Turbid supernatant + suspended definite precipitate after 4 hours and very little deposits after overnight at room temperature.

- = negative agglutination giving no deposits and the same turbidity as the control tube.

All the positive agglutinations were read macroscopically by the naked eye or by the use of a hand lens.

2. The use of smaller ranges of dilution of the serum.

The various serum dilutions were prepared in the ranges required and placed in 3 x ½ tubes in 0.4 ml quantities. An equal volume of the cells was added. The cell serum mixture was removed to agglutination tubes and incubated at 56°C in a water bath for 4 hours and overnight at room temperature, after which the final macroscopic end titre was recorded. A control tube in which serum was replaced by saline was included in every test.

Quantitative agglutination test(T 50):

The method used in obtaining the T 50 and the Antigenicity Index of a serum suspension mixture is the one described by Pereira and Travassos (1952). The authors were interested in an accurate measurement of agglutinating antibodies in sera, and used a standard suspension of cells against a range of dilutions of serum. We, being interested in agglutinogens in the E. coli cells subjected to different treatments, used these cells against a fixed dilution of the

specific antiserum.

A preliminary two-fold dilution agglutination reaction ~~in~~ with the serum was carried out and the titre of the serum determined. A dilution of the serum which was not the end titre but in which the agglutination reaction was ++ was used (e.g. 1/400 dilution of the serum for an end titre serum strength of 1/1600). E. coli cells which had aged under various conditions were mixed with this suitable dilution of the specific anti-serum, incubated for 4 hours at 56°C and left overnight at room temperature. The turbidity of the suspension of cells in serum was read by the Spekker and this reading was given the term "original reading". Another reading was taken of the well mixed mixture of cells and serum after being left over-night at room temperature and this was called 0-minute reading. The suspension of cells in serum was then centrifuged at 1000 r.p.m. for successive periods of 5 minutes, the turbidity of the supernatant being measured in the Spekker photometer at the end of each 5 minute period.

The results were plotted on a graph and the period of centrifugation needed to reduce the number of cells in the supernatant to half the initial value was read from this graph (the half-sedimentation time, or T 50). The half sedimentation time in the absence of specific agglutination was found by substituting saline for serum. The ratio - $\frac{\text{half sedimentation time in saline}}{\text{half sedimentation time in serum}}$ gives a measure of the degree of agglutination and was called "antigenicity index".

If, at the dilution of serum used, no specific agglutination had occurred then the ratio would be 1.0. The greater the degree of agglutination, the shorter would be the half-sedimentation time in the presence of serum as compared with saline and the higher would be the ratio. Thus if young cells give a ratio of, say, 2.0 and an ageing cell shows a steady drop towards 1.0, using a fixed amount of anti-serum then the fall in the value of the ratio is a measure of loss of antigens. Hence

the use of the term "antigenicity index".

Serological analysis by diffusion in agar:

Diffusion of antigens and antibodies in agar was carried out by the method of Ouchterlony (1948 a, 1953) modified as described below to fit the requirements of the present work. The medium used was 1% (w/v) agar dissolved in 0.5% sodium chloride solution and containing merthiolate 1/10,000 to inhibit mould and bacterial growth. The medium was poured into petri dishes to a depth of 2 mm. approximately (10 mls. of the melted agar in the 3½ inch petri dish). The agar was left to set and then holes 8 mm in diameter were punched out at 13 mm. distance from each other using a cork-borer of that diameter. Different arrangements of the reservoirs were used for various purposes. The plates were put in the refrigerator for 2 hours to help in removing the discs of punched agar.

The reservoirs were then filled with the antigens (Cell suspension or supernatant fluid) or antisera soon after the punched discs were

removed and the plates were re-covered with their lids. The plates were then put inside polythene bags with a piece of moist cotton wool inside in order to prevent evaporation. The bags were then closed by rubber bands and the plates were put in the 22°C incubator.

The lines of precipitation which formed between the antigen and antibody reservoirs after being left in the incubator for 5-6 days were recorded and then the plates were removed to 5°C for another week after which they were re-examined for additional data.

Preservation of the gel diffusion plates by fixing them on slides and staining them:

After antigen/antibody precipitation lines had been developed by gel diffusion the lines were stained by a modification of the technique of Uriel and Grabar (1956). The gels were washed thoroughly with saline and then with distilled water. They were then transferred to lantern slides and dried down to a thin film at 56°C. The films were stained with a saturated solution of Naphthalene Black in

methyl alcohol containing 10% acetic acid for 10 minutes. Stained films were finally decolourised with methyl alcohol containing 10% acetic acid.

RESULTS.

II. Antigenicity of E. coli.

Table 22: Agglutination titre in presence and absence of Chloramphenicol.

Age of Susp. used. (days).	Control Suspension. Titre of agglut. after 20 hrs. at 50°C.							Chloramphenicol-containing Suspensions (50 ug/ml). Titre of agglut. after 20 hrs. at 50°C.						
	1/50	1/100	1/200	1/400	1/800	1/1600	C.	1/50	1/100	1/200	1/400	1/800	1/1600	C.
0	++	++	++	++	+	-	-	++	++	++	++	+	-	-
1	++	++	++	+	±	-	-	++	++	++	++	++	-	-
2	++	++	++	++	+	-	-	++	++	++	++	++	±	-
4	++	++	++	++	+	-	-	++	++	++	++	+	-	-
6	++	++	++	++	+	-	-	++	++	++	++	-	-	-

- negative. ++ clear susp. + max precipitate. + Turbid susp. + precipitate
± turbid suspension + ? precipitate.

1. Agglutination:

5M suspensions of E. coli were prepared as usual, with and without chloramphenicol, using M.E. broth for the cell suspension. They were incubated at 22°C for various periods, 0, 1 day, 2 days, 4 days, 6 days etc. After incubation the cells were washed in saline, killed by heating, standardised by the Spekker and used for agglutination.

Preliminary agglutination reactions were carried out by the serial two fold dilution method. It was noticed (Table 22) that starting from the fourth hour of incubation in the water bath, the 2-day old suspensions started to show a higher titre of agglutination in the chloramphenicol-containing suspensions, than the zero-hour ones and that this titre started to diminish in the suspensions of older cells reaching the zero hour level or even less by the end of the 6th day.

The difference in titre obtained in the above experiment was maximally one tube or at most 2 tubes difference, so it was thought

Table 23: Agglutination titre in presence and absence of Chloramphenicol using nearer ranges of dilutions.

Age of Suspensions. (days).	Agglutination Titre after twenty hours at 50°C.																							
	1/400	1/450	1/500	1/550	1/600	1/650	1/700	1/750	1/800	1/850	1/900	1/950	1/1000	1/1050	1/1100	1/1150	1/1200	1/1250	1/1300	1/1350	1/1400	1/1450	1/1500	C
0	++	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
0	++	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
1	++	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
1	++	++	++	++	++	++	++	++	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
2	++	++	++	++	++	++	++	++	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
2	++	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+	-	-	-	-	-	-	-
4	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
4	++	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	-
6	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	-	-	-	-	-	-	-
6	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	-	-	-	-	-	-	-	-	-

++) Control suspension.
+)
-)

++) Chloramphenicol treated suspension.
+) 50 ug per ml
-)

better to repeat the experiment with nearer ranges of dilution (Table 23).

As can be seen from the table, at 0 hour both the chloramphenicol-treated suspensions and the control ones gave the same end titre. After 1 day, the end titre in the chloramphenicol free suspension tubes was much higher. This was reversed in the 2 days' suspension. This preserving effect of antigenicity seemed to continue up to the fourth day, after which the effect wore off and the titre came down again.

When these results were compared with the viable counts of the corresponding suspensions it was concluded that chloramphenicol preserves the antigenicity of the cells as well as the viability. The ordinary, non-chloramphenicol-treated ageing cells, exhausted themselves more rapidly.

When a higher concentration of chloramphenicol (100 ug/ml) was used it seemed to give the same results in the first couple of days after which the high concentration of chloramphenicol was more harmful than useful in preserving the

antigenicity of E. coli cells.

When lower concentrations of chloramphenicol were used (20 ug/ml) no marked difference was seen between these suspensions and the chloramphenicol free ones.

These results were rather inconclusive since the end point was difficult to read. In addition the result for the 2 day suspensions is anomalous. A more accurate measure of loss of agglutinogens was therefore sought.

2. Quantitative agglutination test:

This method depends on measuring the length of time required to centrifuge out enough cells to bring the concentration of cells in the supernatant to 50% of the original value. This of course depends on the proportion of cells which have been agglutinated by the specific anti-serum.

In these experiments E. coli cells which had aged in the presence and absence of chloramphenicol were mixed with a suitable dilution of the specific anti-serum, incubated at 56°C for four hours and left overnight at

Table 24: Determination of the T50 of ageing *E. coli* in M.E. broth.

Age of Cells. (days).	Viable Count. x ~~10^{7}~~ 10^{-7}	Original Spekker Reading.	Readings after 4 hours incubation at 56°C and overnight at room temperature.									
			0Min.	5Min.	10Min.	15Min.	20Min.	25Min.	30Min.	35Min.	T50	Antig. Index.
0	299	0.935	.73	.503	.295	.215	-	-	-	-	$5\frac{3}{4}$	3.82
2	331	0.982	.765	.445	.285	.195	-	-	-	-	$4\frac{1}{4}$	5.17
4	306	.982	.83	.495	.305	.205	-	-	-	-	$4\frac{3}{4}$	4.63
6	353	.925	.76	.51	.355	.313	.235	-	-	-	$6\frac{1}{4}$	3.52
8	193	.96	.792	.52	.39	.34	.25	-	-	-	6	3.66
10	82	.92	.735	.59	.495	.41	.357	-	-	-	$10\frac{1}{2}$	2.09
Control	353	.905	.879	.79	.702	.6	.495	.398	.33	-	22	

room temperature. The T50 and the antigenicity index were then determined.

The results obtained, concerning the effect of chloramphenicol on preserving the antigenicity of E. coli were not always constant and reproducible in the different experiments done under variable environmental conditions of growth such as variation in the temperature of incubation or the amount of nutrients given to the cells in the process of ageing.

One fact did, however, emerge namely, that by leaving the cells to age, especially in a nutrient medium such as M.E. broth, the cells lost some of ~~its~~ their agglutinating power as shown by the doubling in the T50 readings during the period 0-10 days. (Table 24).

The results obtained from the antigenicity index experiments lead to this next question. Since a young cell differs antigenically from an old cell, is the difference a quantitative one or a qualitative one? Was the doubling in the T50 obtained in all the experiments a true quantitative difference in each cell, or was it

a difference induced by the fact that we standardized all our suspensions to the same Spekker reading before using them in agglutination tests, thus leaving more of young cells in the early suspensions and more of dead cells in the older ones?

Two experiments were designed to try and reach an answer to these questions:

1. By comparing the T50 of saline suspensions and M.E. broth ones. The suspensions were taken out of the 22°C incubator at daily intervals starting from 0 to 6 days. They were washed, standardised and used for agglutination as usual. The idea of using saline suspensions was to avoid multiplication of the cells and thus avoid undue dilution of the live cells in the suspension with dead ones (Table 25).

2. The second experiment was to use:

   a. a young suspension of cells which are mostly live.
   b. an old suspension of cells which are mostly dead.
   c. a mixture of 50% young and 50% dead cells. (Table 26).

Table 25: Comparison of the T50 of saline suspensions and M.E. broth suspensions of *E. coli* left to age for one week and prepared daily.

Age of Suspension. (days).	Viable Count. x 10^7	Original Spekker reading.	Readings after incubation for 4 hours at 56°C and overnight at room temperature.								
			0Min.	5Min.	10Min.	15Min.	20Min.	25Min.	30Min.	T50	Antig. Index.
0	201	.96	.735	.472	.302	.201	.155	-	-	$4\frac{3}{4}$	4.42
0	301	.969	.79	.44	.267	.2	.165	-	-	$4\frac{1}{2}$	4.66
1	204	.945	.795	.519	.35	.272	.235	-	-	$6\frac{1}{4}$	3.36
1	268	.985	.779	.489	.302	.243	.185	-	-	5	4.2
2	210	.93	.735	.519	.387	.26	.205	-	-	$6\frac{1}{2}$	3.23
2	280	.975	.778	.5	.324	.236	.178	-	-	$5\frac{1}{4}$	4
3	166	.964	.815	.54	.351	.25	.21	-	-	6	3.5
3	284	.93	.77	.478	.352	.254	.2	-	-	5	4.2
4	141	.965	.824	.575	.382	.289	.255	-	-	$6\frac{3}{4}$	3.11
4	211	.95	.79	.509	.37	.259	.213	-	-	$5\frac{1}{2}$	3.82
5	148	.965	.725	.552	.398	.301	.262	-	-	$6\frac{1}{2}$	3.23
5	130	.957	.335	.59	.457	.329	.29	-	-	$7\frac{1}{2}$	2.8
6	121	.99	.805	.58	.427	.345	.304	-	-	$6\frac{3}{4}$	3.11
6	83	.948	.815	.609	.47	.348	.275	-	-	9	2.33
Controls. C	-	.97	.87	.74	.653	.552	.502	.425	.367	21M	
C	-	.97	.9	.805	.72	.625	.535	.45	.409	22M	

Saline Suspensions.

M. E. Broth Suspensions.

1. The results obtained from this experiment showed that with the saline suspensions the turbidities in the suspensions taken daily from incubation at 22°C did not alter much from day to day and that the amount of saline needed to dilute the suspensions to standard was nearly the same. The T50 readings in these suspensions were nearly steady throughout the whole week of the experiment.

When these results were compared with those for the M.E. broth suspensions it was seen that the amount of saline needed to bring the suspension to standard varied from day to day because of the big difference in the turbidity every day. As to the T50, it can be seen from Table 25 that with daily readings up to the 6th. day there is ~~nearly~~ a rise to almost twice ~~a difference of double~~ the original T50. Does this confirm the conclusion that an old cell differs antigenically from a young one?

The results of experiment 2 (Table 26) are a partial confirmation.

2. When the average T50 was calculated from the young cell figure and the old cell figure which we

Table 26 : Comparing a young suspension, old suspension and 50% mixture of each.

Age of Cells. (days)	Viable Count x 10^7.	Original reading.	Spinning after incubation for 4 hours at 50°C and overnight at room temperature.								
			0Min.	5Min.	10Min	15Min.	20Min.	25Min.	30Min.	T50	Antig. Index.
0d young	278	.94	.8	.475	.302	.215	.155	-	-	5	4.4
10d old	55	.925	.78	.635	.51	.395	.305	-	-	11½M	1.82
Mix.	-	.91	.785	.59	.452	.345	.272	-	-	9½	2.31
Control.	-	.952	.855	.76	.68	.58	.525	.43	.35	22	

would expect as the T50 figure for the 50% mixture if the dead cells did not interfere with the agglutination results, the reading would be 8¼ M. The figure we got from the spinning experiment and Spekker readings after doing agglutination with the 50% mixture suspension was 9½ M, that is to say, 1¼ minutes retardation in the T50 with the actual mixture as compared with what we would expect from the calculation. This suggests that the dead cells present in the suspension did have a definite masking effect on the T50 readings obtained from the older suspensions. (Table 26).

3. Gel diffusion experiments:

It is obvious that the classical serological techniques such as were used in the previous list of work restrict the investigation to a study of the surface antigens, a factor which might well limit the scope and purpose of this type of work. The bacterial cell contains a complex of antigens of many different types and in this piece of work an attempt is made to study the behaviour of diffusible antigens and the factors

Table 27 : Viable Counts of *E. coli* cells at 22°C.

Age of cells (days).	0	2	4	6	10
Viable Count x ~~10^{7}~~ 10^{-7}.	278	332	315	222	57

which affects these antigens in an ageing cell; it was also hoped to establish a relationship between the changes occurring and the changes in the viable counts of the cell suspensions.

Relationship between the antigen loss and decrease of viability in an ageing suspension of E. coli cells.

A 5M suspension of E. coli cells was used in M.E. broth. The cells were left to age in the 22°C incubator for 10 days. A sample was taken out at various intervals. A viable count was done on it and then the cells were washed twice and resuspended in saline, boiled for one hour, after which they were kept at 5°C until used for gel diffusion in plates. The results are shown in Table 27, and plate 4.

As can be seen from Table 27 and from photograph No. 4 of the gel diffusion patterns, the results showed that E. coli cells started to lose an antigen as they aged. This loss started to be apparent from the second day of the cells' incubation at 22°C. If we compare the results obtained with the viable cell

counts (Table 27) we notice that the cells did not start to show an appreciable reduction in their count until after the sixth day. If we put these two observations together we can conclude that the E. coli cells started to lose part of their antigens before any marked death had occurred. In other words, one of the earliest components to be lost by an ageing E. coli cell is an antigen.

As earlier work had shown that the pH of the medium had a lot to do with the viability of E. coli cells, it was of interest to follow the effect of pH changes, especially to the alkaline side, on both viability and antigenic loss.

In the following experiment, the E. coli cells were suspended in buffered M.E. broth at different pHs: 7.5, 8 and 8.4. They were incubated at 22°C for various intervals, after which a viable count was done. The cells were washed twice and resuspended in saline, boiled for one hour and kept at 5°C until they were used for gel diffusion experiments. It was

also of interest to follow the appearance of any antigenic components in the suspending M.E. broth as the suspension aged and try to correlate this to the loss of antigens from the cells so a sample of the supernatant M.E. broth in which the cells had been suspended was collected every time the cells were washed. The sample was boiled for one hour and then kept at 5°C until needed. Gel diffusion plates were set up in duplicates for the cells and the supernatant fluids of each pH.

The results obtained are represented in Table 28 as well as photographs number (5,6,7,8, 9,10). It can be seen that in the plates with cells, the pH affected the rapidity by which the E. coli cells lost one of their antigens.

At pH 7.5 there was little antigenic loss until the tenth day, while at pH 8 the loss started to be apparent after the sixth day, and at pH 8.4 the loss started even earlier, beginning before the sixth day. The loss is apparent in the disappearance of the sharp line which was present in the first couple of days

Table 28 : Changes in the total and viable counts of E. coli at different pHs in buffered M.E. broth.

Period of Storage. (days).	Total Count cells/ml x ~~10~~ 10^{-7}			Viable Count cells/ml x ~~10~~ 10^{-7}		
	pH			pH		
	7.5	8.0	8.4	7.5	8.0	8.4.
0	280	280	280	125	112	116
1	890	740	530	170	124	77
2	440	335	250	102	80	61
6	405	275	140	92	70	32
8	250	170	130	60	29	22
10	200	125	120	33	17	12

at pH 8.4 and its replacement by a diffuse hazy area in pH 8 and its persistance to the eighth day at pH 7.5.

If we compare the results obtained from the supernatant fluid diffusion plates, we find that the gain in antigenic components gets more apparent as the cell aged, and more so in the higher pH than the lower one. And now if we compare these two gel diffusion results with the viability of the suspensions (Table 28) we find that these also coincide with the rate of mortality at the different pHs, showing that the pH had a very definite effect on both the antigenic loss of the E. coli cells as well as on their viability.

It was now of interest to try and establish the above conclusion. So M.E. broth suspensions of E. coli cells were used again at 22°C, as well as at 5°C where there was very little loss in the viability of the cells and very little metabolic activities of the cells themselves leading to a change in the pH of the medium. The supernatant fluid and

Table 29 : Changes in the viable cell counts of E. coli at 22°C and 5°C.

Period of Storage. (days)	0	1	2	5	7	10
Viable Count at 22°C. cells/ml x ~~10~~ 10^{-7}	430	530	535	220	60	20
Viable Count at 5°C. cells/ml x ~~10~~ 10^{-7}	410	400	420	390	410	395

the E. coli cells were collected and treated as before.

It can be seen from the patterns of gel diffusion that at 22°C the cells started to lose antigens from the second day as seen by the loss of one line. (Photograph 11). As to the pattern obtained from the supernatant fluid we notice a progressive increase in the pattern obtained from day to day as the suspension aged. (Photograph 12).

If this is compared with the results obtained at 5°C we find that there is neither any loss in the pattern of the cells' antigen complex (Photograph 13) nor any gain in the supernatant fluid gel diffusion plates (Photograph 14), which means that at this temperature there was no antigenic loss from the cell into the suspending medium.

If this is compared with the rate of death of the cells (Table 29) we find that the same relationship as before still exists. In other words the cells showed an appreciable reduction in their number from day to day at 22°C and this

Table 30 : Changes in the pH and viable counts of 5M suspension of E. coli in saline kept at 5°C and at 22°C.

Period of Storage. (days).	Temperature 22°C.		Temperature 5°C.	
	Viable Count. x ~~10^{7}~~ 10^{-7}	pH	Viable Count. x ~~10^{7}~~ 10^{-7}	pH
0	111	6.86	118	6.71
1	95	7.37	90	6.73
2	80	7.10	85	6.70
5	57	7.72	82	6.72
7	56	7.75	80	6.75
10	50	7.79	65	6.75

was absent at 5°C.

Two other experiments were set up in order to try and prove this point still further:

In the first one, a medium without nutritive value, saline, was used for suspending the E. coli cells so that they could not multiply nor should the pH alter in the absence of active metabolism. The cells were incubated in two batches: one at 22°C and the other at 5°C to emphasize the bacteriostatic conditions intended still more. The cells were left for various periods and then taken out and treated as usual. The supernatant fluid from each sample was collected and treated as usual. A viable cell count was done for every sample of E. coli cells before it was treated. (Table 30).

Gel diffusion plates were set up in duplicate for the E. coli cells and the supernatant fluid of each batch and the results were recorded after one week at 22°C and one week at 5°C.

As can be seen from the photographs (Nos. 15 and 16) of the patterns obtained from the cell

diffusion plates both at 22°C and at 5°C, there is no loss in the antigen complex as the cell ages. Both the numbers of precipitation lines and the continuity of the lines was kept without any loss or change in the pattern from both temperatures. The supernatant fluid from each temperature shows no precipitation lines, that is to say the cells do not lose in the surrounding medium any of ~~its~~ their antigen complex during the whole ten days of incubation in saline whether at 22°C or at 5°C. (Photographs 17 and 18).

From the viable count table we notice that although there is a 50% reduction in count over the period of the experiment this, with the lower initial count than usual represented a comparatively small degree of death.

In the other experiment the cells were suspended in a glucose - phosphate medium and two batches were used. One was incubated at 22°C and the other at 5°C. The cells were collected at various intervals during the 10 days incubation and treated as usual for the gel

diffusion experiments.

The results obtained were nearly the same as those obtained with the saline experiments. Both the number of precipitation lines and the pattern of arrangements remained constant during the whole period of incubation (Photographs Nos. 19 and 20) indicating the absence of an apparent loss of antigens by the E. coli cells kept in this medium. The pH did not rise to a value known to be lethal to the cells.

Two other extracts of the E.coli antigens were used instead of those obtained by boiling the cells; a trichloracetic acid extract according to the method of Boivin et al (1933) and a mechanical extract after cellular disintegration. They did not prove to be superior to the method used either in the number of precipitation lines obtained or in their clarity.

From the results of the experiments described in the above section it seems clear that the loss of a particular antigen, as measured by a gel diffusion technique can provide

an indicator of the death or of the impending death of cells. Using this technique the effect of alkaline pHs on viability and the role of chloramphenicol in maintaining viability was confirmed.

DISCUSSION.

When bacterial cells pass through their physiological youth, they enter a phase of rest. Sooner or later they begin to die, unless all metabolic processes are suspended by some means such as refrigeration or freeze drying, two methods of retaining the viability of laboratory cultures over a long period.

The first stage in the death process is the loss of essential materials from the still living cell (Hinshelwood, 1957). This is followed by a series of degradative changes which overbalance the synthetic processes, leading ultimately to the death of the cell. However, we are still largely ignorant of the way in which this process begins.

In this work, an attempt was made to discover something of the way in which the change from life to the death of the cell begins. Conditions were chosen which would discourage ~~Resting cells were chosen for this purpose.~~ active cellular proliferation. ~~They are simply cells in a state of non-proliferation, the stage in the life-cycle of all cells which usually precedes death.~~ In

general, actively multiplying cells do not show a high death-rate although, even in the logarithmic phase of growth of bacteria there is a certain amount of cellular death. (Topley and Wilson, 1955).

The concept of the occurrence of a few primary changes which start the different processes leading to the ultimate death of the cell provides us with a hypothesis for experimental attack. If the enzymic activity of the cell be considered as due to a series of chains of linked reactions there is no need to regard the chemical changes associated with cellular death as consisting of a wide array of processes operating simultaneously. The application of Occam's razor to this situation leads to the simpler explanation that multiple changes found in dying cells stem from a few primary changes, just as in the energy yielding processes of the cell the multiple end products of glucose breakdown are the consequence of a single, initial, step - the phosphorylation of glucose by A.T.P.

If, then, chemical interference with the cell prevented or delayed death it would be reasonable to assume that the interference had been with the more important primary reactions rather than with one of the many secondary reactions associated with death. The best means for such an approach would be to use an agent known to stop one specific process. Most known enzymic inhibitors, the nearest approach to such an agent, inhibit more than one chemical process, especially if used in the higher concentrations. When considering this problem we were faced with this difficulty of finding truly specific enzyme inhibitors. As a compromise between the desirable and the practicable we chose to investigate the effect of three enzyme inhibitors of partial specificity- the sulphydryl-inhibiting compounds. We also investigated the effect of a truly specific esterase inhibitor. But in no case was there found to be any prolongation of the life of E. coli. The same enzyme inhibitors when used on human Hela cells gave promising results with

one compound (iodoacetic acid). The cause of this failure with bacterial cells might be attributed to such factors as an unsuitable choice of concentration or to differences in cell permeability. The major factor however was probably the fact that the time-consuming nature of the method of viable counting limited the range of our investigations.

As an alternative approach to the problem, the bacteriostatic antibiotic chloramphenicol was used. Already some experiments had been carried out on the drug and suggested that it might be an effective agent in prolonging the life of E. coli cells (Fletcher, 1956). Among the reasons for its choice was its known inhibitory action on protein synthesis. As it is well known that many of the reactions in the cell are reversible, it was thought that it might equally inhibit protein breakdown. It was therefore decided to investigate the effect of the drug in greater detail in the hope that by the use of a certain concentration of the drug and special conditions of growth we

might reach an answer to our hypothesis.

It was noticed that the presence of the antibiotic in experiments carried out at 37°C caused death of the organisms and it was of interest to see whether broth suspensions of high cell concentrations in the presence of the antibiotic could be maintained if we changed the temperature of growth to such a low degree as 5°C, where all metabolic processes are slowed down and the cells are in a quiescent state. The idea of using a high cell concentration was to diminish multiplication in the controls as much as possible, so that the effect on the death-rate of added chemicals would be apparent. The results of these experiments showed that not only was death in all suspensions slowed down, but that the antibiotic containing suspensions showed an even slower death rate than in the controls. A surprising finding was that, even under the conditions used, the control system maintained its viable population by continual multiplication, the old cells dying and new ones

replacing them; this as well as the diminished lysis at 5°C may account for the rise in the total counts obtained. On the other hand the antibiotic containing systems maintained their viable population by apparently limiting or inhibiting death rather than by balancing multiplication and death as in the control.

When a higher temperature of growth was used, e.g. 22°C, but a temperature still lower than the optimum of 37°C, the effects produced were not only of a similar nature to those at 5°C, but were even more accelerated and dramatic, probably due to the enhancement that such a temperature has on the life processes of the organism.

The results obtained in these experiments seem to confirm some of Bail's findings (1929). In the first place and considering the use of a large population of cells, we find that the viable counts did not exceed the same high level under the various conditions used, these conditions being a change in the temperature of incubation or a change in the amount of nutrients

supplied to the organism. This is very evident in the experiments in which the suspending medium was changed daily. Here we find that although the total number of organisms in the controls doubles the original count, yet the viable number never exceeds the same maximum level. This result confirms Bail's finding that growth of the cells does not cease with the attainment of the M-concentration, but that fresh organisms continue to be produced, an equivalent number dying off so that the M-value remains constant. It also shows that the cessation of growth after reaching the maximum number is not due to exhaustion of the medium, because although the turbidity in the broth in these experiments i.e. the total count is much higher than in the same experiments in which the medium was not changed, yet the maximum concentration of the viable organisms remains unchanged.

On the other hand when a small inoculum of cells was used under the same previous conditions of incubation i.e. temperature and nutrients, the cells continued to multiply reaching higher viable

levels than the original inoculum introduced in the medium but never exceeding the maximum which was reached in the experiments in which a large inoculum was used. The total count, however, reached a much higher level indicating that the cells are continuously multiplying and that the growth does not depend on the total number of bacteria in a given volume of medium but on the maximum number of living cells within the medium.

A more dilute suspension of cells when used at both temperatures of incubation and in the presence and absence of the antibiotic showed that when the conditions are more in favour of the growth of the organisms, that is during the earlier stages of the experiments in which there is a relative excess of nutrients, the results differed from those obtained with heavy suspensions. In the earlier stages of the experiment the control cultures showed massive multiplication which tended to mask any concurrent death. Nevertheless, the much greater rise in total than in viable count for

the control showed that very considerable death of cells was taking place.

However, as time passed and multiplication in the controls ceased, the number of viable cells fell much more quickly than in the chloramphenicol containing suspensions. Thus the results with heavy suspensions of E. coli (5M) were confirmed when using the more dilute suspensions ($\frac{1}{2}$M) corresponding to the conditions normally attained when a bacterial culture reaches the stationary phase.

From these points we could conclude, that under the conditions of the experiments used, the bacteriostatic agent chloramphenicol instead of increasing the tendency of bacteria to die, had helped them to survive.

When artificially induced chloramphenicol-resistant variants of E. coli were produced and subjected to the same tests it was found that chloramphenicol showed no effect in prolonging viability. As will be discussed below, in the case of chloramphenicol-sensitive cells the death delaying effect could be attributed to

the suppression of the formation of alkaline metabolites - which raised the pH to lethal levels in the controls. In the case of chloramphenicol-resistant variants the inability of the drug to delay death was associated with an inability to suppress the rise in pH.

Thus we have the paradox that the chloramphenicol-sensitive cells which would be destroyed by chloramphenicol therapy in a clinical infection are prevented from dying by exposure to the antibiotic. On the other hand, chloramphenicol-resistant cells which would not respond in a clinical infection to chloramphenicol therapy die as fast as untreated cells when exposed to the antibiotic. In the first case we might almost say that we have an example of auto-~~b~~anti biosis prevented by hetero-antibiosis.

When the same experiments were carried out on another species, Staphylococcus aureus, in order to extend the observations, they failed to show any marked prolongation of the life of the cells. Whether the reason for this failure

was a fault in the counts, due to the clumping action of the cells, or whether it was due to the failure of the agent used to inhibit the necessary processes in the metabolism of the staphylococcal cells, requires further investigation.

Whether death is encouraged or prevented probably depends on the interplay of a number of factors, such as species of bacterium, temperature, concentrations of inhibiting agent, available nutrient, cell numbers as well as changes in the pH of the medium. This last factor was brought to our attention when it was seen that the pH of the chloramphenicol suspensions kept a steadier level than the controls, which had a tendency to rise as the experiments progressed. It was also noticed that the higher the pH in the control the lower was its viable count. Thus this action of chloramphenicol might be regarded as analogous to the suspension of metabolism by some such procedure as storing cells at low temperature, with the difference that with chloramphenicol

most metabolic activities continue.

The next step in the problem was to determine what occurred when a suspension of E. coli was put under defined conditions in order to control the inimical shift of pH to the alkaline side. E. coli cells were subjected to several treatments in order to control the alkaline rise in pH. In the first place glucose was used as a means to impart acidity to the medium by the products of fermentation. But the concentration of glucose to produce the right acidity to neutralise the alkaline products could not be achieved. When small concentrations were used, the acidity produced could not buffer the alkalinity in the controls for more than two days. On the other hand the use of high concentrations of glucose inhibited the growth of the cells and this inhibition was greater than, or bore no relation to, the effect produced by growth in a medium adjusted to the same final pH. So apparently one can alter and suppress the enzymic constitution of bacteria apart from any pH

effect involved in its fermentation. (Gale & Epps, 1942).

When a buffer was used as controlling agent it was noticed that the buffer tested showed a lethal activity which tended to obscure the differences we were looking for. As with the use of glucose, the buffer was either not powerful enough to neutralize the alkali produced by these heavy suspensions or if used at a sufficiently high concentration to display powerful buffering action, it was lethal. Adjusting of the pH regularly by the addition of acid proved difficult too, because after the cultures had been standing 2-3 days they tended to produce alkali at such a rate that large ranges in pH occurred within a few hours of adjusting to neutrality.

The next technique used in controlling the pH was daily changes of media. Apparently the M.E. broth has sufficient buffering power which keeps the pH down for about two days. With this method it was evident that in the control suspension where the medium was changed daily the

viable count did not fall much in 11 days ($428 \times 10^7 - 319 \times 10^7$). The fall in viable count is much more marked in the suspensions containing 10 ugm chloramphenicol/ml and still more marked in the suspensions containing 100 ugm chloramphenicol/ml. However, it was noticed that the death rate was higher in the presence of chloramphenicol in this experiment than with earlier experiments. This could be explained by the fact that there is a loss of chloramphenicol in the course of incubation so that in the earlier experiments the cells were in fact exposed to a much lower average concentration of chloramphenicol in the later days than was actually mentioned.

The results obtained by daily change of medium prompted a further search for a buffer which would show no lethal activity in itself and would at the same time be effective in holding the pH of heavy suspensions. Sørensen's phosphate buffer and veronal buffer fulfilled these requirements. In experiments in which these two buffers were added to the suspending

vehicle, M.E. broth, further confirmation was obtained that chloramphenicol acts by preventing the formation of alkaline products of metabolism which cause a lethal rise in pH.

Thus we may conclude that although the steadier and lower pH alone may not be the sole factor in keeping the viable counts of chloramphenicol - containing suspensions steadier and higher than the controls, yet it played a very important role in this direction.

We referred before to the way in which our investigations were limited by the slowness of our method of measuring the number of viable cells. It became imperative to find some quicker and simpler technique for indicating cellular death. There is no doubt that the power of the cell to proliferate is dependent upon the intactness of, among other things, its cellular structure. This is to be anticipated for it cannot be expected that a cell will survive whose cellular or intracellular structure has suffered extensive alteration. It therefore occurred to us that we might look

for changes in cellular components resulting from changes in cellular structure in the dying cell as an indicator of death or impending death. If these changes could be measured by a simpler and less time-consuming technique than viable-counting, the pace of our investigations could be markedly increased.

Our first thought was to investigate changes in cell agglutinogens since the agglutinating antigens are associated with the surface structure of the cell. The first technique used was a simple tube agglutination in which cells of varying age were titrated against a specific antiserum. However, the end point proved extremely difficult to read and the method was finally abandoned.

The next approach was to apply the method of Pereira and Travassos (1952) in which the amount of agglutinating antibodies in the serum were measured by measuring the time taken for a standard suspension of bacterial cells mixed with a fixed amount of antiserum to reach half the original turbidity when centrifuged at a

given speed. This was compared with the time taken by a control suspension in the absence of specific antibody, to reach 50% turbidity under the same conditions. It seemed to us that by using a fixed amount of a suitable dilution of antiserum and standard suspensions of different origin brought to the same turbidity in the Spekker absorptiometer we might have a measure of the agglutinogens in these different suspensions. Preliminary investigations indicated that this indeed was possible. But in a series of experiments it proved impossible to obtain reproducibility of results and this method too was abandoned. Nevertheless the fact emerged that a suspension of bacteria as it grows older, will gradually change its antigenic power.

As a third resort, the gel diffusion technique of Ouchterlony was used to measure the loss of antigens from cells. This technique proved a valuable and successful test for the qualitative analysis of the immunologic system of the cell as it ages. In fact we found that,

using this technique, the loss of a particular antigen from the cells into the suspending medium provided an indicator of cellular death in the strain of E. coli we were using. This provided us with a technique with which we have begun to extend our investigations in the field of causes of cellular death. E. coli was submitted to a variety of different treatments, for example different temperatures, different hydrogen ion concentrations or to a variety of nutritive conditions, and changes in the antigenic complex of the cells were followed step by step as the cell aged. From the results obtained the fact was ascertained that different treatments of the cell leading to acceleration or retardation of its death had the effect of altering, qualitatively, the relation and position of the lines produced by the antigenic complex of the E. coli cells. For here, the antigen has a definite specificity and if it be attacked by chemical or physical means two events may happen (a) a particular grouping may be destroyed with the result that a particular component of

the antigen complex can no longer react with the antiserum or (b) the surface structure may become modified with a consequent alteration in the results obtained.

Several points of interest have emerged from this investigation:

(1) No correlation could be found between the agglutinating titre of an antiserum and its diffusible patterns in agar gel. The presence of high titre agglutinating antibodies was not correlated with the ability to produce lines in agar. Sera showing strong agglutinating properties gave feeble diffusion patterns thus indicating that antigens and antibodies involved in agglutination took little or no part in the production of lines. Some of this can also be explained by a superficial or deep situation in the wall of the organism.

(2) Sera from different animals, although they were immunised by exactly the same procedure gave a different pattern in gel diffusion plates, so that although the main losses were apparent in each, yet the position of the

different lines varied with the different batches of sera used. This may be explained by the fact that the position of the antigen-antibody streak in the diffusion medium is influenced by such factors as where optimal proportions between antigens and antibodies appear, the time necessary for the formation of a visible precipitate and the concentration - threshold value - of the reaction. The differences in the position of the line lost, illustrated in the different photographs proves that the concentration of the reacting substances are of decisive importance for and where the reaction will appear.

(3) Boiling removes practically all the extractable antigens as compared with trichloracetic acid extraction or mechanical extraction after cellular disintegration. Heat extraction may offer an explanation for Kauffmann's observation (Kauffmann, 1951), that when working with a non flagellated and capsular strain, a strong O serum is obtained if the strain is killed with 0.5% formaline and not by heating.

(4) By correlating the effect of temperature

of incubation, hydrogen -ion concentration and nutritives in the gel diffusion patterns and on viability of cell we can conclude that we have now a technique which will enable us to state that, on such lines as these, the study of ageing in other bacteria may be followed.

Thus we have achieved initial success in studying the chemical processes involved in the death of the cells of the bacterial species E. coli. In addition we have found a technique which will enable the change from life to death of the cell to be followed in a convenient way.

This represents a very modest contribution to the relatively neglected field of cellular death and it might be fitting to conclude with the opening lines of this thesis "We are urgently in need of chemical and physical investigation of cellular death, for our knowledge at the moment is crude and full of gaps". (Cameron, 1952).

SUMMARY.

1. Instead of hastening the death of *E. coli* cells, chloramphenicol may, under the conditions of the experiments act in such a way that the viability of the culture is maintained. Thus the viable counts of the control suspensions stored at 5°C fell to about half the original value by the end of three weeks, whereas in the presence of 10 ug/ml or 100 ug/ml chloramphenicol it remained unchanged.

2. When similar suspensions were stored at 22°C the effect was even more marked. The viable count of the control suspension fell to about one eighth of its original value within one week, but with 100 ug/ml of chloramphenicol it showed only a slight decrease, and with 10 ug/ml it was still more than half the original.

3. When *Staphylococcus aureus*, which has different metabolic processes from *E. coli*, was used the results did not coincide with the above results. Due to clumping of the bacteria, the total counts were not very exact.

4. The E. coli cells did not acquire any increase in resistance to 10 ug/ml and 100 ug/ml chloramphenicol, although they were kept in contact with it for two weeks.

5. Chloramphenicol solution fell to half its potency in one week, $\frac{1}{4}$ its potency in 2 weeks and $\frac{1}{8}$th its potency in 3 weeks incubation at 37°C. It came to $\frac{1}{2}$ its potency in one week, remained there during the second week and came to $\frac{1}{4}$ its potency after 3 weeks at 22°C.

It came to $\frac{1}{2}$ its potency after 1 week and remained there to the end of three weeks at 5°C. Thus although the chloramphenicol content diminished during the course of the experiments, the rate of destruction was such that sufficient antibiotic to ensure a bacteriostatic effect and hence an inhibitory effect on the processes of metabolism, was present throughout the course of our experiments.

6. Noticing that the pH of the controls in most of the experiments rose faster than in the chloramphenicol-containing suspensions, and noticing also that the viable counts were lowest

with pHs more than 8, a set of experiments designed to control the pH of the media used for suspending cells were done in which, saline instead of M.E. broth, glucose to produce acidity and lowering of pH, indicator M.E. broth media controlled by the addition of $\frac{N}{5}$ HCl to lower the raised pH, replacement of the medium by daily spinning, or the use of buffered indicator broth aided by addition of $\frac{N}{5}$ HCl, were used as means of keeping the pH steady.

7. The results proved that although the steadier and lower pH alone may not be the sole factor in keeping the viable counts of the chloramphenicol-containing suspensions steadier and higher than the control ones, yet it played a very important role in this direction as indicated by a lower death-rate in the controls with the steadier pH than in the earlier experiments.

8. A set of enzyme inhibitors was used instead of chloramphenicol in order to compare the results with those of chloramphenicol, but they proved

ineffective in prolonging the life of the cells.

9. The search of an indicator which would give a simple means of detecting death of the cell was undertaken and the loss of antigens from the cell was selected as a change which could be detected by relatively simple techniques and which might accompany or precede death.

10. Simple tube agglutinations proved a failure and was abandoned as did the quantitative agglutination procedure using the half-agglutination period. In this procedure the ratio of the time required to centrifuge the control suspensions of cell to 50% turbidity, to the period of time required by mixtures of cells of different ages with specific antiserum gave a measure of the agglutinogens present on the cells and is referred to as the antigenicity index of the cells.

11. The use of the gel diffusion technique enabled us to detect loss of an antigen from the cell and its appearance in the suspending medium. The loss of this antigen could be correlated with

the change from life to death of the cell under various conditions of the environment used.

12. It is suggested that antigenic loss might be used as an indicator of the death of the cell in experiments designed to elucidate the causes of cellular death in other bacterial species.

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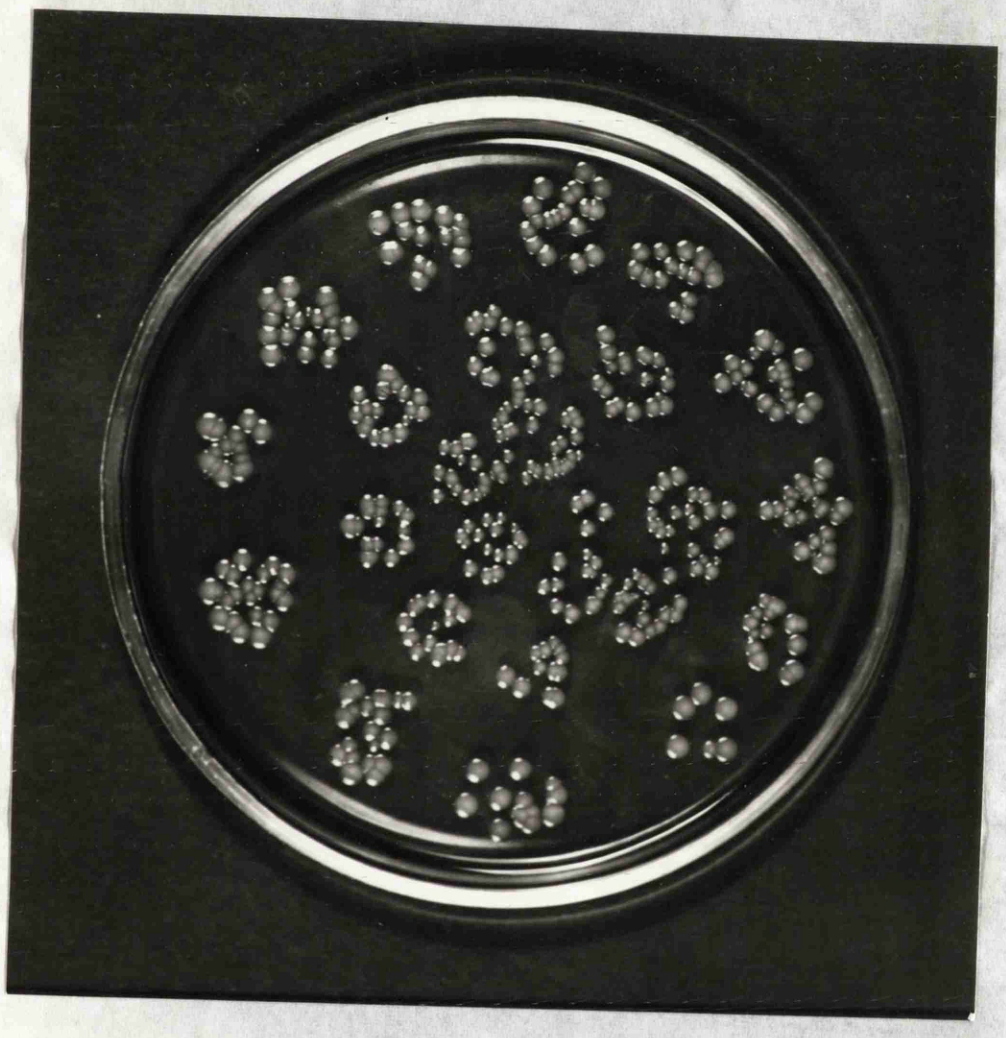
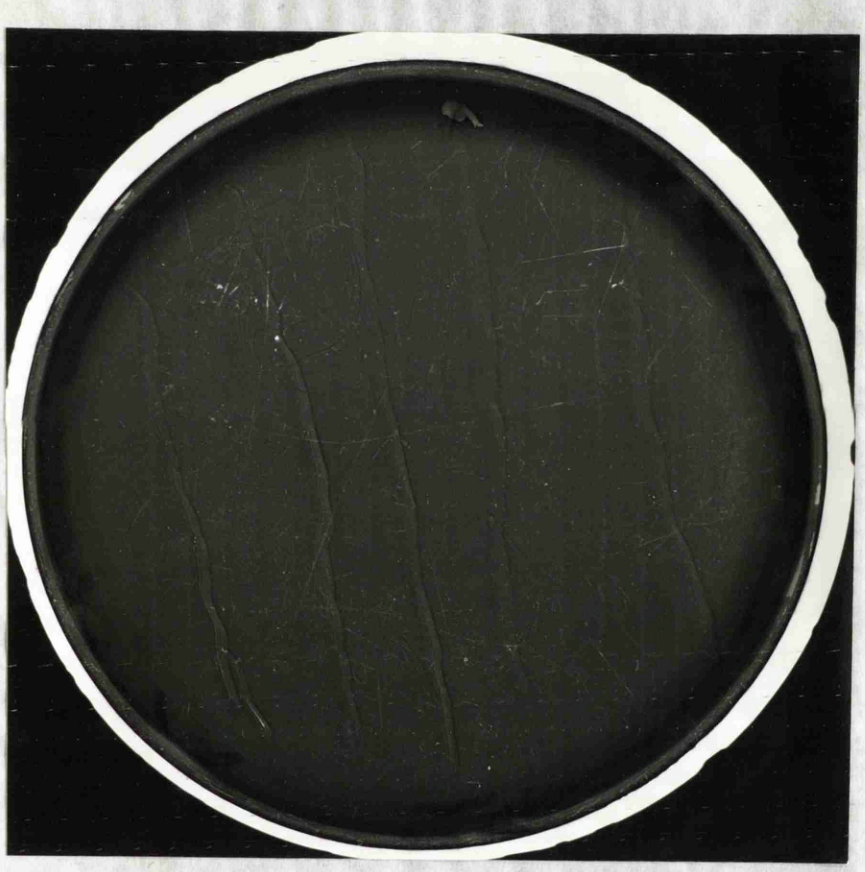


Photograph (1)

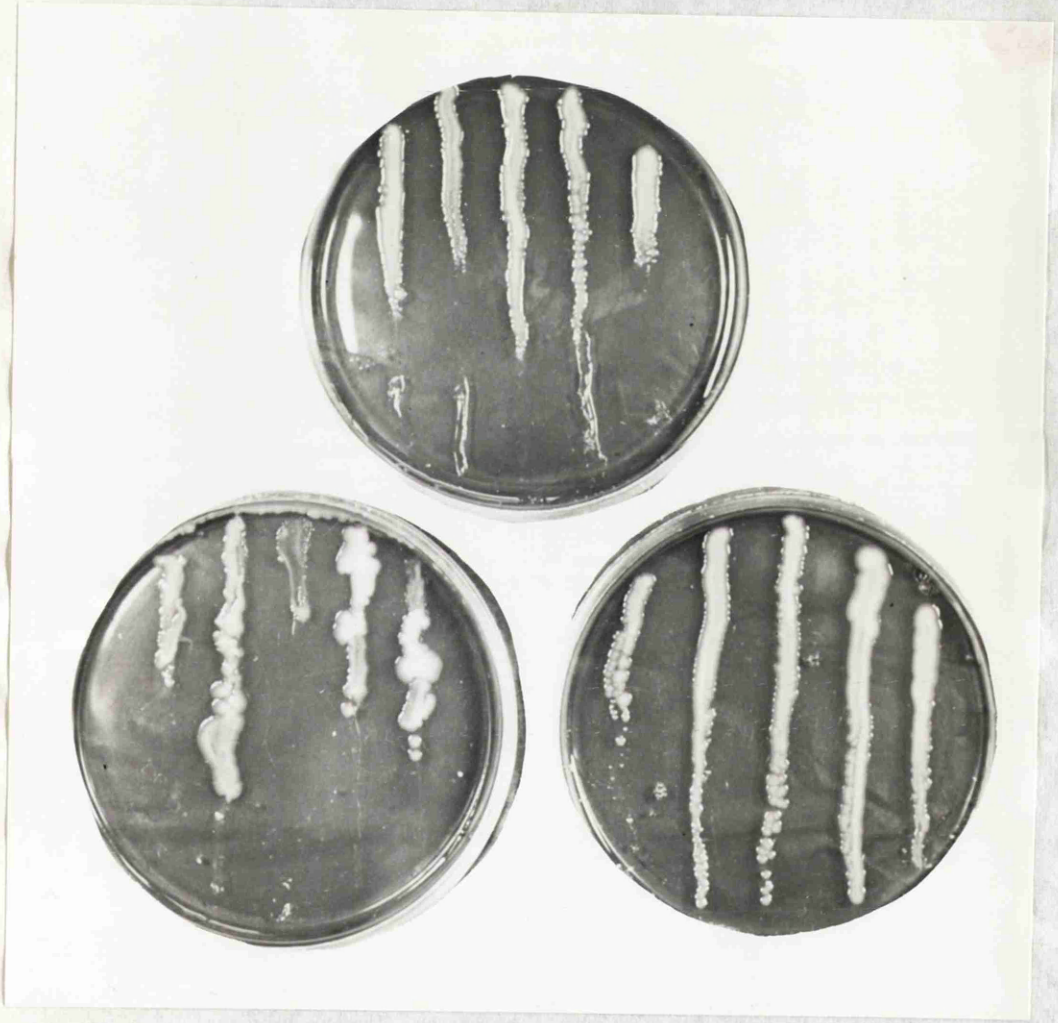
Viable Count Plate
Showing 20 Drops.

? 24 drops



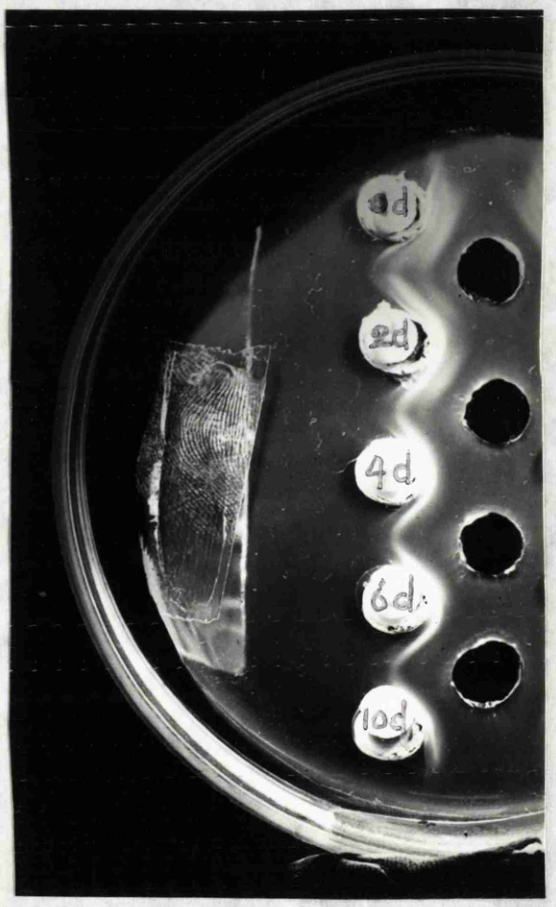
Photograph (2)

Sensitive strain of E. Coli
on Wedge plate of M.E. Agar
containing 50 mg/ml chloramphenicol.



Photograph (3)

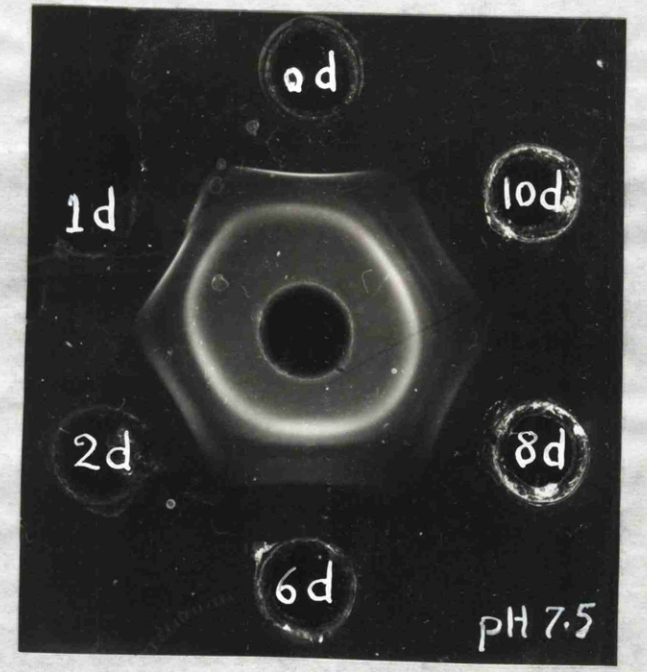
Raising the resistance of E. Coli. to chloramphenicol by Wedge plate technique.



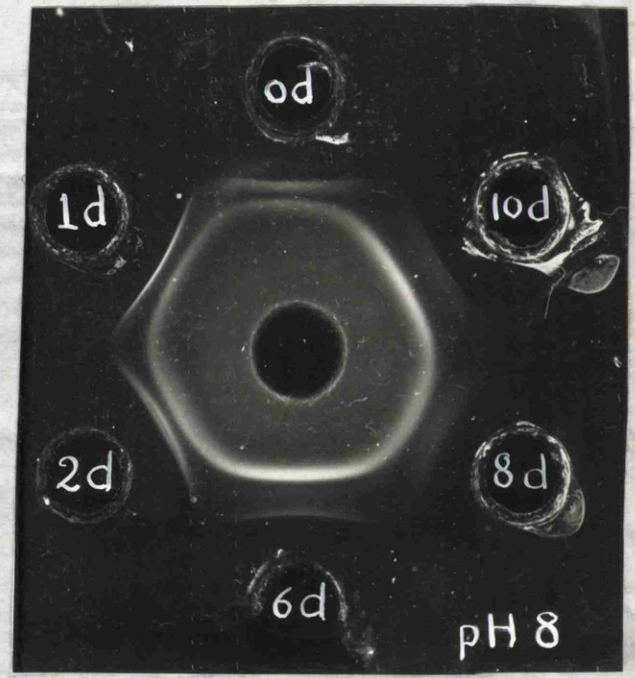
Photograph (4)

Gel Diffusion pattern of E. Coli. cells stored at 22°C for 10 days.

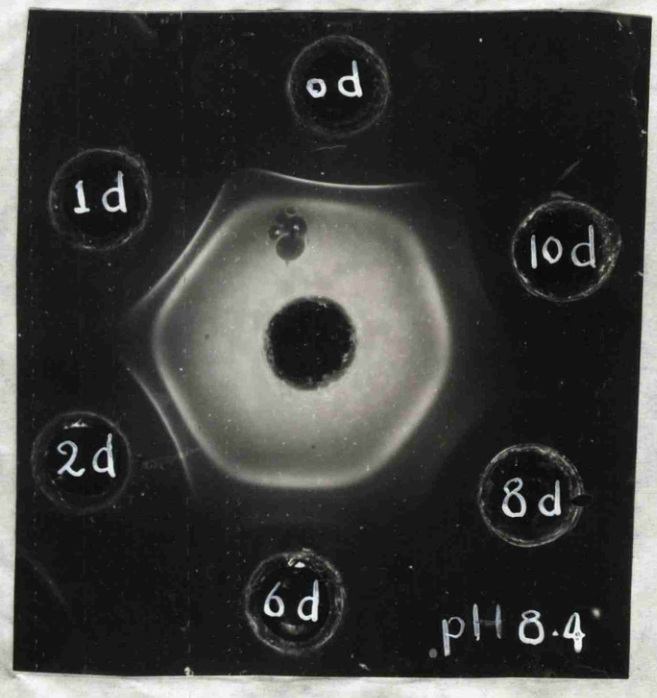
Photograph (5) pH 7·5



Photograph (6) pH 8.

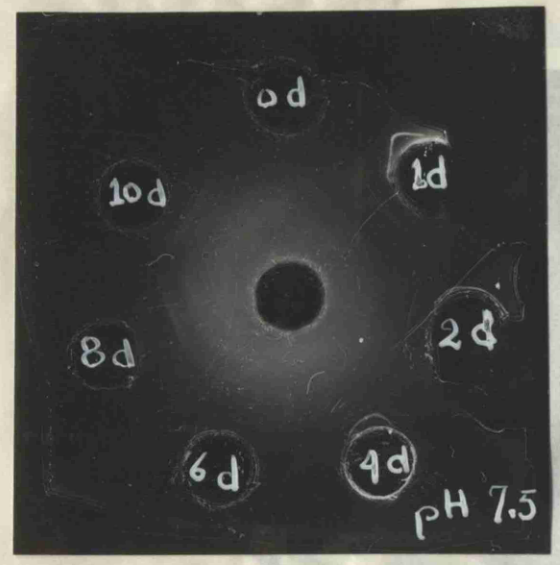


Photograph (7) pH 8·4

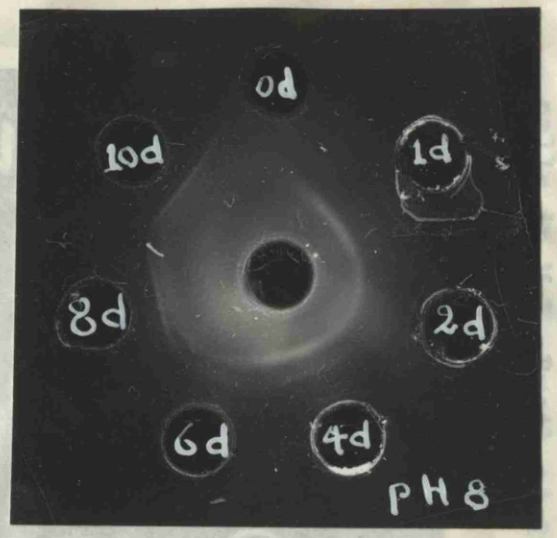


The effect of pH changes on Antigenic loss from E.Coli. Cells.

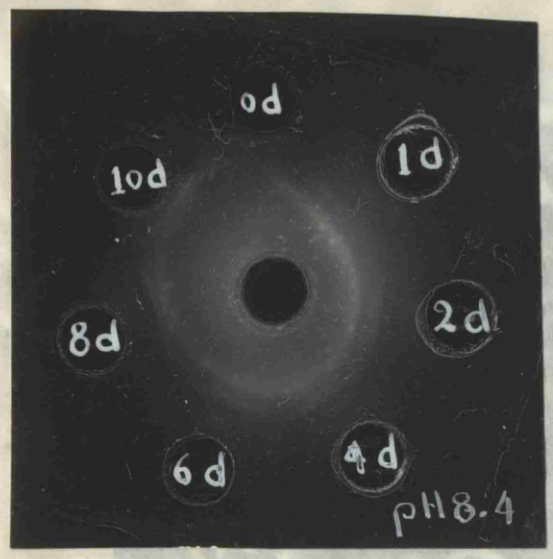
Photograph (8) pH 7.5

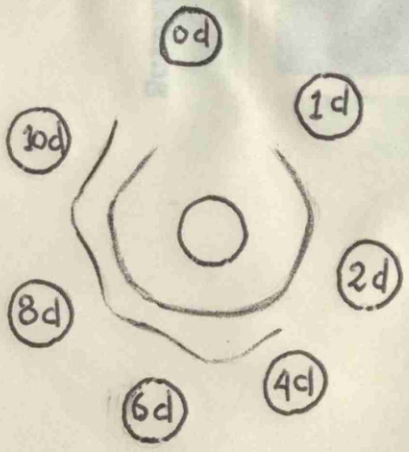


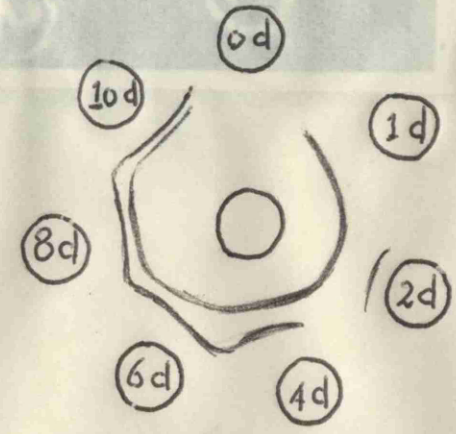
Photograph (9) pH 8

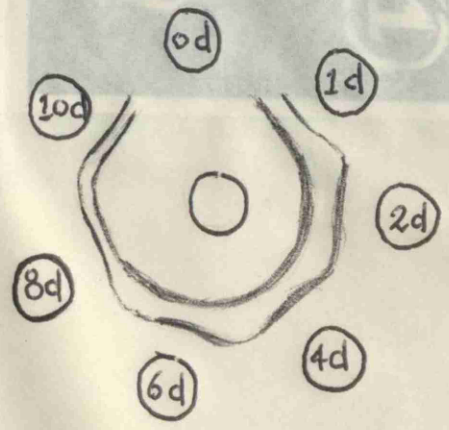


Photograph (10) pH 8·4

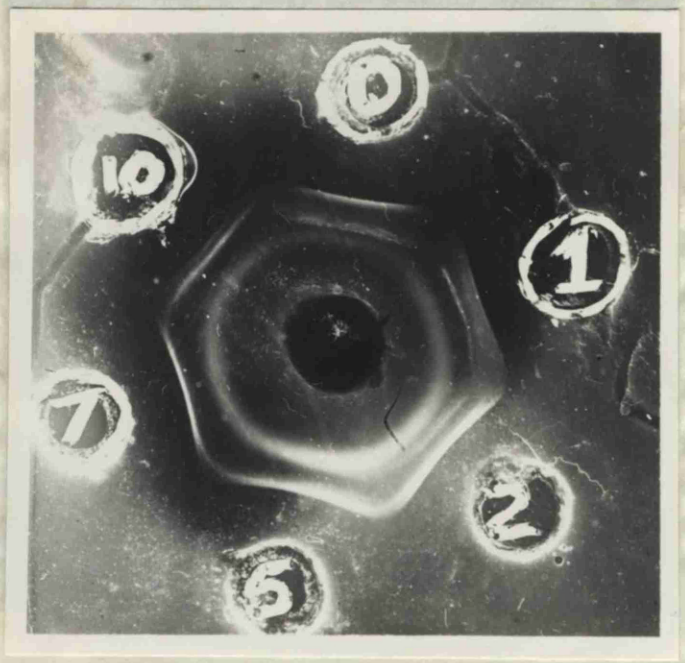






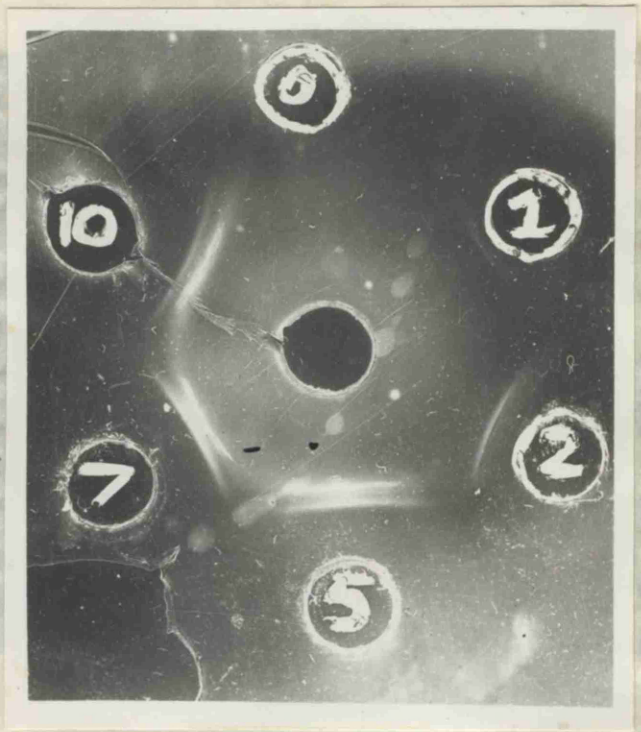


The Effect of pH Changes on Antigenic Gain in Supernatant suspending-fluid. With Illustrative Diagrams.



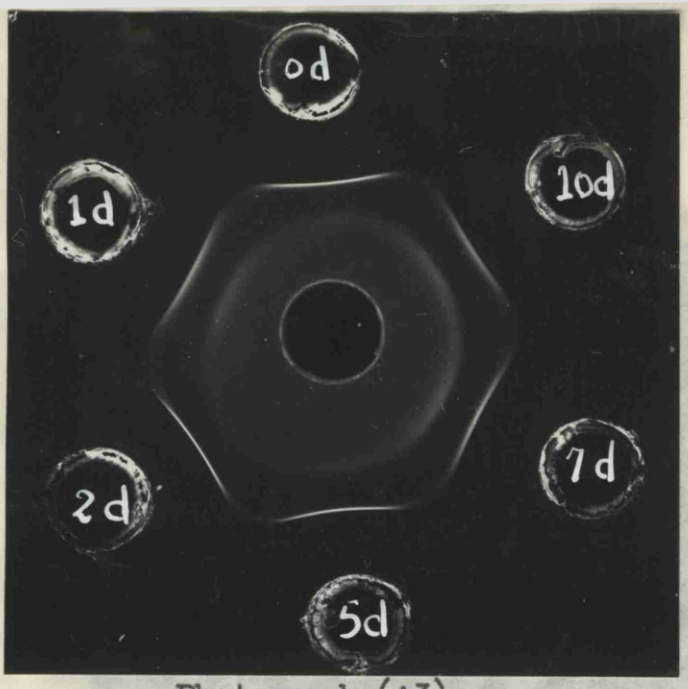
Photograph (11)

Diffusion patterns of
cells in M.E. Broth at 22°C.



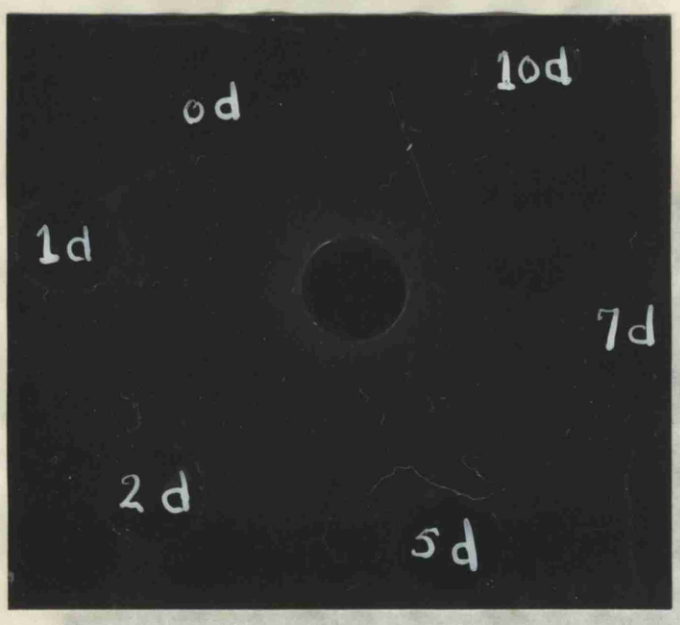
Photograph (12)

Diffusion patterns of suspending
M.E. Broth at 22°C.



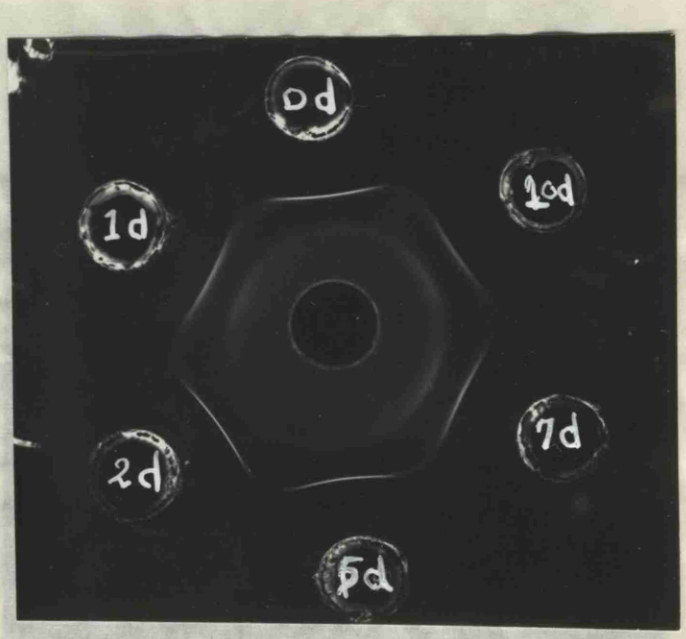
Photograph (13)

Diffusion patterns of cells in M.E. Broth at 5°C.



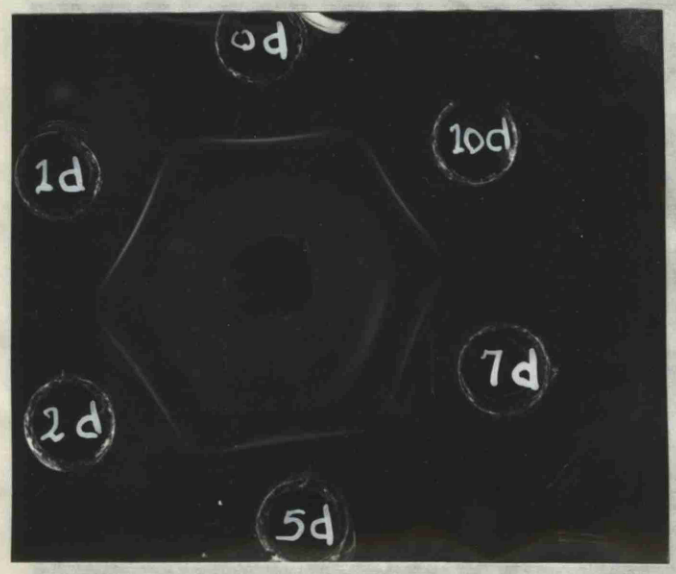
Photograph (14)

Diffusion patterns of suspending M.E. Broth at 5°C.



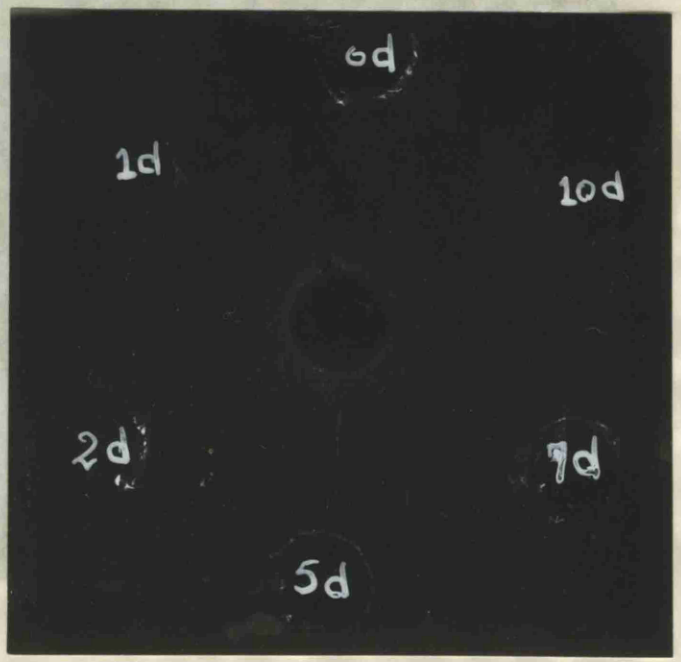
Photograph (15)

Diffusion patterns of cells in Saline at 22°C.



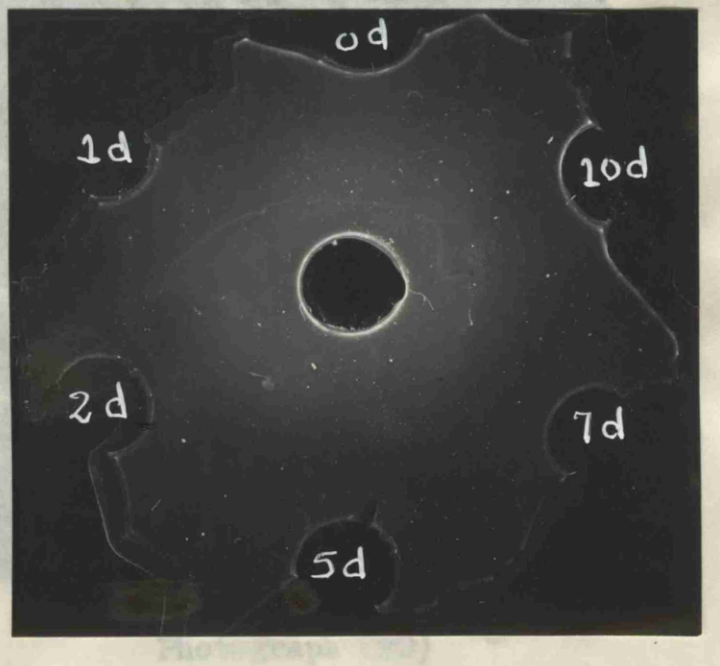
Photograph (16)

Diffusion patterns of cells in Saline at 5°C.



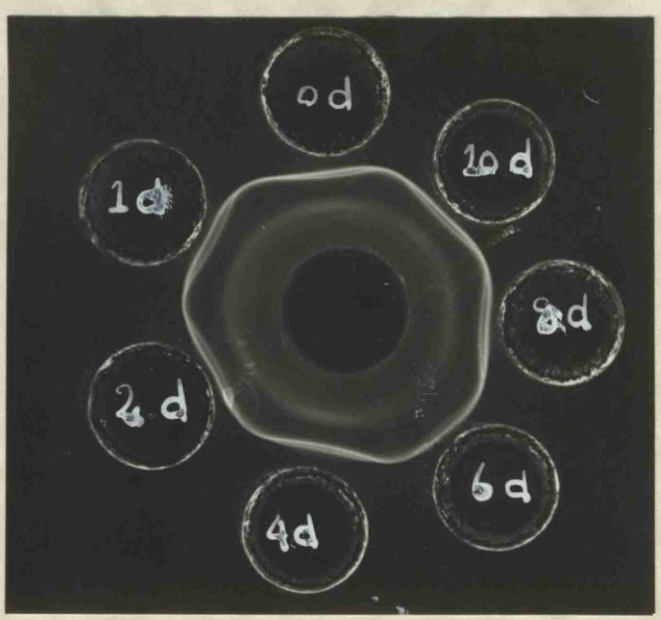
Photograph (17)

Diffusion patterns of supernatant Saline at 22 °C.



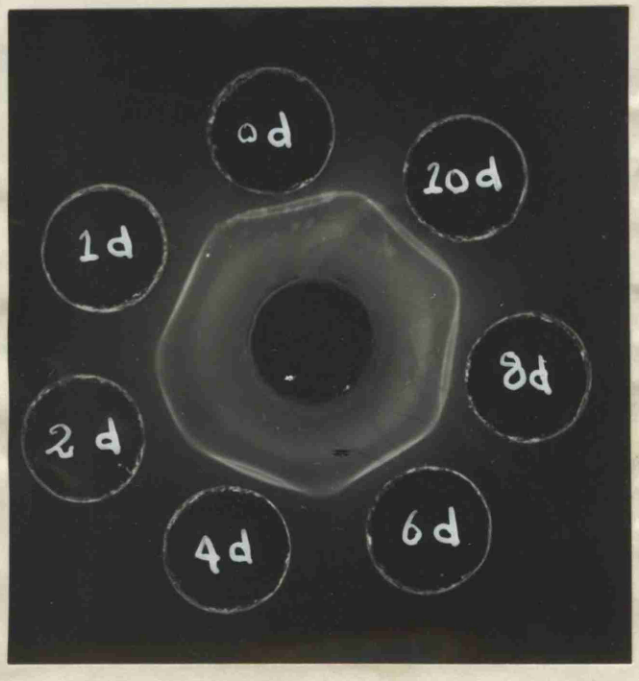
Photograph (18)

Diffusion patterns of supernatant Saline at 5 °C.



Photograph (19)

Diffusion patterns of cells
in Glucose-Phosphate at 22°C.



Photograph (20)

Diffusion patterns of cells
in Glucose-Phosphate at 5°C.