SOME PHYSICO-CHEMICAL ASPECTS OF BACTERIAL GROWTH.

The mechanism of nutritional training in coliform bacteria.

Abstract of a Dissertation submitted by Stanley Jackson for the Degree of Doctor of Philosophy.

October 1949. The Physical Chemistry Laboratory, Oxford.

MS. D. Phil. d. 717

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ABSTRACT OF DISSERTATION.

Ability to accept changes in nutritional supply, and to become resistant to the action of drugs, are striking characteristics of bacteria.

The mechanisms of these training processes have long been an issue of controversy. Alternative throries which have received some support have postulated, on the one hand, selection of mutant cells, and, on the other, direct adaptation of cells in response to their environment.

One type of 'training' which has received some attention is exemplified in the behaviour of Bact. coli mutabile in lactose medium. In this medium, the species does not, on the first transfer, commence growth until after a long lag phase, but thereafter grows readily in the medium.

Growth tests are usually made by inoculating a considerable number of cells into a medium containing the test substrate. The mutation theory suggests that a very small proportion of the cells in a test inoculum are, in virtue of chance mutation, able to grow on the new substrate, in this case lactose; the majority of the cells are not able to grow. Although the few mutant cells begin to grow very shortly after inoculation, the growth does not contribute to a detectable increase in total population until the number of mutant cells has become comparable with the total number of cells in the inoculum, so that there appears to be a long lag phase before growth begins. Once the culture has grown, the cells which are 'lactose-using mutants' are in a

majority, and are subsequently able to grow readily in lactose medium.

The alternative, adaptation theory postulates that all of the cells in the test inoculum are potemtially capable of reorganisation, in response to the presence of lactose, until they are able to grow on this new substrate. The reorganisation process takes time—hence the long lag phase. The changed cells are afterwards able to grow readily in lactose medium.

It is seen that the mutation theory requires that only a small proportion of the cells should be able to grow on lactose, fairly quickly, while the adaptation theory suggests that most of them are able to do so, but only after a long lag phase.

In PartII, experiments are reported in which the behaviour of small numbers of cells was observed. Small inocula were tested in both liquid and solid lactose medium; in each case, parallel tests were made with a medium which is readily used by all the cells of the strain.

With solid media, it was found that equal numbers of colonies appeared on lactose medium and on the control medium, in the former case after a long lag phase. Each colony springs from a single cell, or at most a very small number of cells. The result is in accord with the adaptation theory, but is incompatible with the mutation theory. With liquid media, it was found that inocula containing 100 cells or more grew in lactose medium after a long lag phase. Smaller inocula failed to grow, although inocula containing only one cell grew in the control medium. When a rich nutrient was added to the tests which failed

to grow in lactose medium, it was found that there was still no growth, and it was evident that the cells had died.

The estimate most frequently put forward for the proportion of mutant cells in strains of bacteria is about one mutant per 10^6 normal cells, and, certainly, only such a low proportion could account for the length of the lag phase in this case. The growth of inocula containing only 100 cells is incompatible with the mutation theory, and it seems clear that the smallest inocula failed to grow because they were overtaken by death before adaptation was complete.

Failure of small numbers of bacteria to grow in new substrates has frequently been reported, and has been interpreted as proof that only mutant cells were capable of growth in the new medium. Failure to grow is, however, inadequate evidence—only if it could be proved that cells which refused to grow after having had time to become adapted were viable would the mutant theory be vindicated.

Similar experiments were carried out to investigate the long lag of Bact. lactis aerogenes in D-arabinose medium, and of a coli-aerogenes intermediate in sodium citrate medium. In liquid media, all inocula, even those containing only one cell, grew in the new substrate after the appropriate long lag phase. On solid media, equal numbers of colonies of Bact. lactis aerogenes grew on D-arabinose medium (after the long lag phase) and on the glucose control medium; the behaviour of the coli-aerogenes intermediate on solid media was erratic. It seems probable that, in both these examples, the bacteria train to the new substrate by an

adaptation process.

Experiments with Bact. coli, tested in ammonium sulphate medium, led to the conclusion that the cells of this strain only trained to use ammonium sulphate, after a long lag phase, when traces of broth, carried over from the previous medium, were also present.

Part II is concerned with an attack on the problem from a different angle. The length of the lag phase of Bact. lactis aerogenes in D-arabinose medium was shown to be independent of the source of carbon previously used. If an attempt is made to account for this in terms of the mutant selection theory, it must be supposed that the strain contains an almost constant proportion of mutant cells which are capable of growth in D-arabinose medium.

This condition would be satisfied if the rate of mutation were very small, and the small proportion of mutants had been established over a considerable period of time. However, cultures grown directly from single colonies showed the normal behaviour, proving that this was not true.

If the phenomenon does depend on mutation, it is clear that a constant proportion of mutants can only be maintained by balance with back-mutation, in a state of dynamic equilibrium.

The implications of this theory were developed in mathematical form; it emerged that the minimum rate of back-mutation necessary to account for the observations would lead to steady 'de-training' if cells grown in D-arabinose medium were afterwards sub-cultured in glucose medium. The theory predicted that, as the 'trained' cells were grown in glucose medium, their lag, when tested in D-arabinose medium, should steadily increase.

Experiments were carried out to test this prediction. It was found that the actual behaviour was quite different. Initially, there was a fairly rapid, but not very great, ingrease in the lag in D-arabinose medium, but thereafter there was no further increase during the course of the experiments, which were continued until the predicted lag was very much greater.than the actual lag.

By this criterion also, the mutation theory was incompatible with experimental observation. Although the behaviour of Bact. coli mutabile with regard to lactose was not completely investigated in the same way, the characteristics appeared to be similar to those just described for Bact. lactis aerogenes and D-arabinose.

Adaptation may be supposed to occur by the synthesis of an enzyme pattern which plays an important part in metabolism of the new substrate. The pattern is probably present in a rudimentary form and in slight extent in the untrained cells. It appears that the adaptation process may result in a quite stable change in the structure of the cell, even if the process has not been completed.

If cells of Bact. lactis aerogenes are exposed to D-arabinose medium for a period less than the lag phase, and are then allowed to grow in glucose medium, they afterwards require a correspondingly shorter lag phase when tested in D-arabinose medium.

As already mentioned, cells of Bact. lactis aerogenes which have actually been grown in D-arabinose medium are subsequently able to grow in the medium after only a short lag phase.

Prolonged culture in glucose medium has little effect on this property.

In Part III, this stability is shown to be in sharp contrast with the changes observed in D-arabinose dehydrogenase activity. Glucose dehydrogenase activity, under the conditions used, was almost constant, at a high level, whatever the cultural history of the cells. Untrained cells possessed only very slight D-arabinose dehydrogenase activity. Cells which had grown in D-arabinose medium were equally active with either D-arabinose or glucose, but the D-arabinose activity was rapidly reduced by subsequent growth in glucose medium. The D-arabinose activity of very thoroughly trained cells was reduced less rapidly than that of cells less thoroughly trained.

'Training' by the criterion of the length of the lag phase in D-arabinose medium, is very stable during subsequent culture in glucose medium, but, on the other hand, the dehydrogenase activity in contact with D-arabinose is rapidly reduced by this treatment.

The length of the lag phase does not directly depend on the dehydrogenase activity—it appears that 'training' primes the cell for synthesis of D-arabinose dehydrogenase enzyme, when this is required.

These observations may be accounted for if it be supposed that, during the long lag phase, a fragment of protein in the untrained cells, an approximation to the ideal pattern for D-arabinose metabolism, is expanded, to form a suitable enzyme, and that the greater stability of D-arabinose activity in very thoroughly trained cells is due to perfecting and stabilising

of the enzyme configuration during prolonged training. Further, if it be supposed that 'trained' cells, cultured in glucose medium, retain a small fragment of enzyme, which is more suitable for reaction with D-arabinose than the imperfect fragment of untrained cells, and can be readily expanded and activated to permit D-arabinose metabolism, the stability of the short lag phase of 'trained' cells in D-arabinose medium may be accounted for.

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The author thanks the Department of Scientific and Industrial Research for the Maintenance Grant whuch has made it possible for him to carry out this work.

He is deeply grateful to Professor Sir C.N. Hinshelwood, F.R.S., for invaluable criticism and advice throughout the course of the work, to Dr. R.L. Vollum, for the supply of several strains of bacteria, to many colleagues, for valuable assistance, and to the staff of the Laboratory, for their unfailing help.

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INTRODUCTION.

Bacterial Growth.

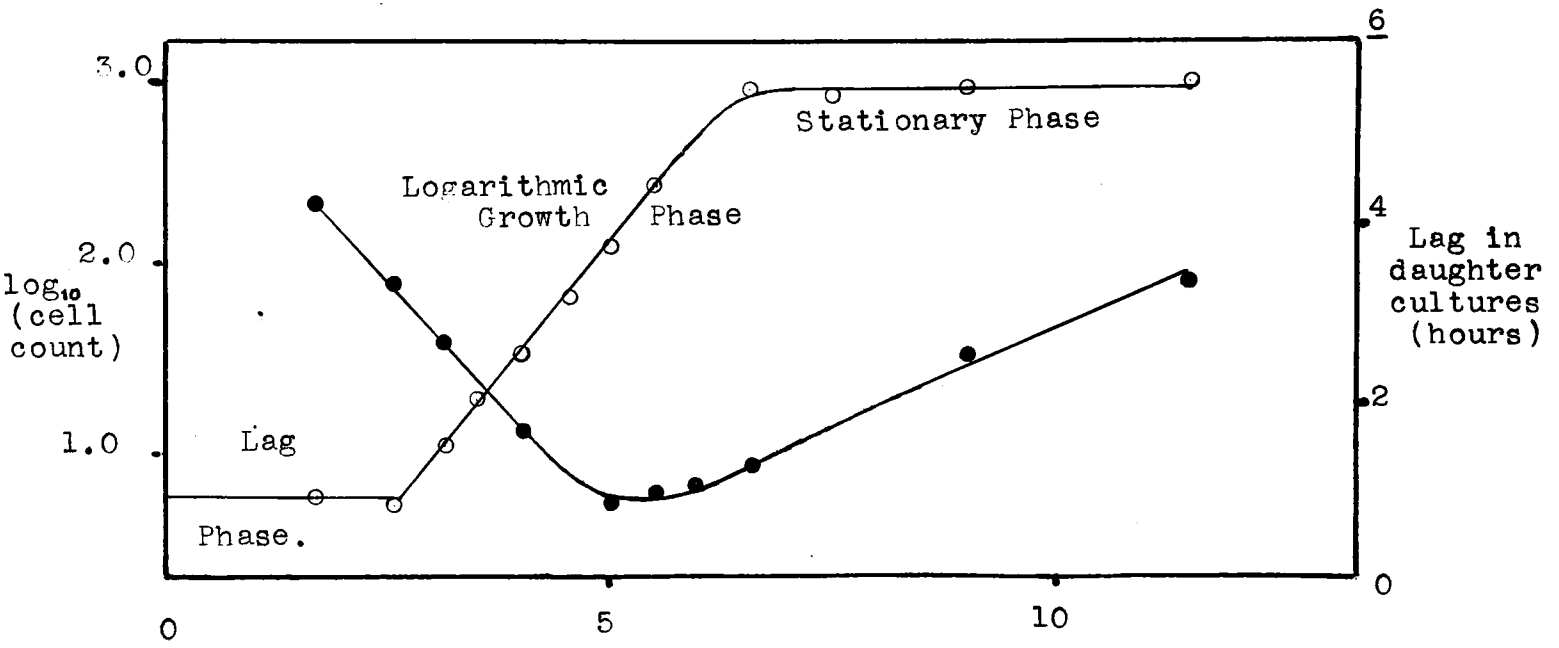
Bacteria are unicellular organisms. Their growth and reproduction, by cell-division, is rapid, and often requires only quite simple nutrient media.[1]

Coliform organisms, which were used in this investigation, may be cultivated in very simple media containing inorganic sources of nitrogen, phosphorus and sulphur, potassium, magnesium and trace metal ions, and an organic source of carbon, such as glucose, glycerol or acetate. The nutritional requirements of other bacteria are often more exacting. More complex sources of carbon or nitrogen, such as amino-acids, may be necessary, and some species require growth-factors, of which several are identical with certain vitamins.[2]

In a suitable synthetic medium, the growth of bacteria usually follows a fairly uniform pattern. A suitable example is the growth of Bact. lactis aerogenes in a medium containing glucose and ammonium sulphate as the only sources of carbon and nitrogen. (See Fig.1.) When a small number of cells is inoculated into a tube of the medium, there is an induction period known as the lag phase, before detectable growth and cell-division begins. Shortly after the beginning of cell-division (to which the term 'growth' is often applied — the growth in size of individual cells not being conveniently measurable) the rate of reproduction is usually found to be exponential. This reflects the fact that the time between the formation of each cell and its division is almost constant during this period, which is known as the logarithmic growth

FIGURE I.

The Growth of Bact. lactis aerogenes in Glucose-ammonium sulphate Medium.



Open circles: Growth curve. Black circles: Curve showing Lag in daughter cultures.

phase. Finally, growth in the culture is arrested, either by exhaustion of one of the nutrients, or by accumulation of toxic metabolic products, and the culture enters the stationary phase, when there is no further proliferation. As the stationary phase is prolonged, the culture is said to 'age'. During this period, cells may be damaged by the adverse conditions, or may decay and die.

The division of the growth cycle of bacteria into three phases is usually remarkably clear cut, and the change from one phase to another quite sharp, but the duration of each phase in a particular culture is very dependent on the composition of the medium and the state of the cells in the inoculum.

Obviously, if the concentration of one of the nutrients in the medium is reduced, the stationary population, (that is, the number of cells present when the stationary phase is reached) may be reduced, and the duration of the logarithmic growth phase may be shortened.The growth of a culture is affected in much more subtle ways by the state of the inoculum.

If a small sample is transferred from a culture to a new medium during the lag phase of the parent culture, the lag phase in the daughter culture is comparatively long. If the transfer is made during the logarithmic phase of the parent culture, the lag in the daughter culture is short. If the transfer is made during the stationary phase of the parent culture, the lag in the daughter culture is longer again. A plot of the lag in daughter cultures against the age of the parent culture when transfer is made, shows the form given in Fig.1. The initial

'early lag' decreases to a minimum, which may be zero, when the transfer from the parent culture is made near the end of its logarithmic growth phase. Thereafter, it begins to increase (late lag) as the stationary phase is prolonged.

This is explained as follows.[3] During the lag phase, the cells are engaged in synthesising certain intermediate metabolites (e.g. amino-acids) which are able to diffuse in the medium. When their accumulation reaches a certain threshold concentration, growth begins. The accumulation continues throughout logarithmic growth, and the concentration of diffusible metabolites reaches a maximum about the end of the logarithmic growth phase. If a small sample of the parent culture is inoculated into a new medium during the lag phase of the parent culture, any progress made in the accumulation of diffusible metabolites towards the critical concentration necessary for growth is nullified by the dilution caused in sub-culture to the new medium. The lag phase in the daughter culture is not short. However, during logarithmic growth of the parent culture, the concentration of diffusible metabolites becomes much higher, so that even after a sample is diluted in being transferred to a new medium, growth can occur almost at once. The lag in the daughter culture is short. If amino-acids are included in the medium,'early lag' is eliminated — organic nitrogen sources are immediately available.

The intermediate metabolites may be rather unstable substances, or may be destroyed by the conditions of the stationary phase (e.g. abnormal acidity). For this reason, the

lag in daughter cultures to which transfer is made during the stationary phase of the parent culture is again longer than in those inoculated from the parent culture during its logarithmic growth phase. Further, the damage, decay and death of the cells themselves during a long stationary phase increases the lag in daughter cultures inoculated at this stage. This is the main factor responsible for 'late lag' in amino-acid media.

Apart from, and in addition to, these 'lag-age' effects, changes in the inoculum size affect the lag in daughter cultures inoculated from a parent culture at the same time.[3] The concentration of diffusible intermediate metabolites necessary for growth in a daughter culture is more rapidly attained if a larger volume of parent culture is transferred — the initial concentration is higher and the lee-way is more rapidly made up by the larger number of cells. If the parent culture is diluted before an inoculum is transferred to a new medium, the lag may be prolonged somewhat because of disadvantage in these two respects.

The preceding discussion on the characteristics of bacterial growth, and, in particular, of the lag phase, has been concerned with the behaviour of bacteria in a medium in which they would grow reliably and readily. The effect of altering the composition of the medium has only been mentioned in its simplest case, the change in concentration of one of the established nutrients, which may affect the stationary population of the culture. More drastic changes are caused by the substitution of new nutrient substances in the medium.

The differences in nutritional requirements of different bacteria have been mentioned, but one of the most striking characteristics of bacteria is their ability to accept changes in nutritional supply. After suitable 'training', substances formerly indispensable for growth may be no longer required, or new, alternative substrates may be used more or less readily. These phenomena will be best described by reference to a few examples in the behaviour of the strains used in this investigation.

All the strains were maintained in meat-extract broth, sub-cultured monthly by transferring a little of one culture to a new medium by means of a sterile platinum loop. Such broth is very rich in organic nitrogen compounds etc. and supplies the nutritional requirements of many bacteria.

The strain of Bact. coli grew readily in a synthetic medium containing sodium glutamate and glucose as the sources of nitrogen and carbon. At 40°0° the stationary population of about 10^{10} cells was usually reached within 24 hours of inoculation from the stock broth culture, using a platinum loop which carried not more than about 10^{6} cells. Similar inoculation of a medium containing ammonium sulphate and glucose as sources of nitrogen and carbon never resulted in growth within 24 hours, and there was usually no growth at all. However, when the strain was cultured in a series of media containing glucose, and a mixture of sodium glutamate and ammonium sulphate, in which the proportion of sodium glutamate to ammonium sulphate was gradually decreased, it was found possible, in the end, to

eliminate the sodium glutamate completely, the strain being 'trained' to grow on ammonium sulphate as the source of nitrogen. This illustrates a procedure often used in 'training' a strain of bacteria to use a more simple substrate. An alternative method, for the particular example described, and its significance, will receive some attention below.

On the other hand, the strain of Bact. lactis aerogenes grew almost as soon in glucose-ammonium sulphate medium as in glucose-sodium glutamate medium, when inoculated from the stock broth culture. The growth rate and stationary population were erratic at first, however. When a small sample of the culture was transferred to a second tube of glucose-ammonium sulphate medium, and this was repeated in a series of sub-cultures, growth rate and stationary population became closely reproducible after about four sub-cultures.

These observations are characteristic of the changes in growth often associated with changes in the nutrition of bacteria. Once the strain of Bact. lactis aerogenes had passed through about four sub-cultures in glucose-ammonium sulphate medium, its growth followed the pattern already described as an example of the behaviour of bacteria in simple synthetic media. Its behaviour when transferred from glucose-ammonium sulphate to other synthetic media has been extensively studied in this laboratory.

When transferred to media containing glycerol in place of glucose or glycine in place of ammonium sulphate, the strain shows composite growth curves, in which there are two

distinct logarithmic phases of growth.[4] These are probably associated with different modes of metabolism. A second kind of change is observed when the strain is transferred from glucose-ammonium sulphate medium to media containing lactose,[5] maltose or sucrose in place of glucose. In these media, the initial growth rate is inferior to that in glucose-ammonium sulphate.

In neither of these types of behaviour is there any striking difference between the length of the lag in the new medium and that in a parallel test in glucose-ammonium sulphate medium. The phenomenon of a very long initial lag is observed when cells are transferred from glucose-ammonium sulphate to media containing D-arabinose[6] or sodium succinate in place of glucose. In all cases, the results may be erratic unless transfer is made at the optimum stage of growth of the parent culture, that is, at the point of maximum activity, just before the end of the logarithmic growth phase. If transfer is made at this point to tubes of glucose-ammonium sulphate and D-arabinose-ammonium sulphate medium in parallel, growth occurs almost immediately in glucose, but there is a lag phase of about 30 hours in D-arabinose. Growth then occurs at a rate inferior to that in glucose.

When growth has reached the optimum stage, transfer to a second tube of the new medium results in growth almost immediately _ the long lag phase of the first sub-culture is almost completely absent in the second.

This behaviour parallels that of Bact. coli mutabile in lactose medium,[7] in which the first lag phase occupies about 50

hours. Unless transfer to the new medium is made at optimum activity of the parent culture, growth may be even more delayed, or may fail completely, in a quite erratic way.

The lag phase is reduced to normal proportions in subsequent sub-cultures in lactose medium.

Usually, serial sub-culture in a new medium improves the growth rate until it is comparable with that in glucose-ammonium sulphate medium, although this does not appear to occur with Bact. lactis aerogenes in D-arabinose medium.

In addition to the ability to accept nutritional changes in these ways, bacteria have striking powers of becoming resistant to the action of inhibitory drugs. Drugs, added to the standard glucose - ammonium sulphate medium in low concentrations, often prolong the lag phase, affect the growth rate or reduce the stationary population of Bact. lactis aerogenes. Once cells have been cultured in a low concentration of drug, they are able to grow in a higher concentration, which would initially have been completely inhibitory. The resistance may be increased to a high level by sub-culture in a series of media containing progressively higher drug concentrations.[8]

The Mechanism of Training.

The interpretation of the phenomena of nutritional change and drug resistance of bacteria has been a matter of

contention for a considerable time. There have been two schools of thought, with, apparently, very little sympathy for each other.[7,8]

On the one hand, changes in bacterial properties are ascribed to mutations of genetic type. When the environment of a culture is changed, the bacteria most suited to the new conditions are selected, as the others are unable to grow, or are less vigorous. Thus, in a new nutrient medium, mutant cells able to grow on the new nutrient are selected; once growth has occurred in the new medium, the mutant strain thus selected is thereafter able to grow readily in the new medium. If the strain is exposed to drug action, only resistant cells survive and grow, so that, apparently, the whole strain becomes resistant.

On the other hand, it is suggested that changes in bacterial properties take place not simply by selection of abnormal cells, occurring by chance mutation, but by adaptation of cells to a new environment. According to the hypothesis, this occurs in at least the majority of the cells exposed to a new environment; in response to the new demands, the chemical apparatus of each cell is modified, quantitatively or qualitatively, to permit of corresponding change in the metabolic processes which constitute bacterial growth.

Investigations specifically directed towards experimental proof of the validity of either of these points of view have not been numerous. Most of the evidence adduced has been inferred from observations made under conditions which did not

provide a critical test of the alternatives. However, there have been some specific attempts to decide between the two theories.

The classical example of this behaviour is the growth of Bact. coli mutabile in lactose, already mentioned. The work of I.M.Lewis on this example is very important.[7] In one set of experiments, he compared the growth of one strain on solid media containing lactose as the sole source of carbon with the growth on solid media containing glucose, or peptone and beef extract.

An important feature of these experiments is the method of comparison used. The parent culture was grown on an agar solid medium, incubated for 24 hours. The agar was washed with 10 ml. of water, and the resulting suspension diluted in stages of 10. From each diluted suspension, a small volume was transferred to each of two solid media in parallel, one of them lactose agar, the other glucose, or peptone-beef extract agar. It was assumed that each cell able to grow yielded a colony after incubation on solid medium. Growth on glucose and peptone-beef extract media was reproducible at all dilutions; the number of colonies was consistent with the number of cells in the inoculum. On lactose agar, however, inocula containing less than 100,000 cells did not grow. Larger inocula yielded a number of colonies equal to about 10^{-6} of the number of viable cells in the inoculum (calculated from the countable number of colonies produced by smaller inocula on glucose or peptone-beef-extract). Although this is

not clearly stated in Lewis' paper, it may be presumed that there was a long delay before the appearance of colonies on lactose plates, since lactose in broth-indicator liquid or solid media was not fermented until after 3 days and 5 - 8 days respectively.

It was concluded from these observations that the strain contained a small proportion (about 10^{-6}) of mutants able to grow in lactose, and that these were selected in lactose medium.

This conclusion, however, does not seem to be fully justified by the evidence reported in the paper. Granted, only about 1 cell per 100,000 achieved growth on lactose, but the failure of the others to grow must be investigated further. With the strain of Bact. coli mutabile used in the experiments reported in this dissertation, growth in lactose liquid medium, of inocula containing about 10^8 cells, only occurred at all when the inoculum was transferred from the parent culture in its state of maximum activity. Inocula transferred at this stage grew after about 50 hours; they often failed completely if transferred at any other stage of growth of the parent culture, and especially after ageing in the stationary phase. This last was the case with the inocula used by Lewis. A test made in this way is not a fair test of growth in a new medium; the cells are, in any case, unlikely to be uniformly vigorous, or even uniformly moribund, after ageing in the stationary phase.

Such tests should, then, be made from a parent culture

in maximum activity. Failure of cells to grow may not be due to a fundamental dificiency of the bacteria, but to the fact that they are more or less moribund. This may be tested by making parallel tests in a number of tubes, and by adding a little of a readily utilised substrate to one or more of them at appropriate times. It may then be proved whether, at the time when growth is expected, there are any viable cells present, as all viable cells will grow on the readily utilised substrate.

(This criticism is also relevant to the work of Ryan,[9] of Luria and Delbrück,[10] and of Newcombe,[11] in which the ability of bacteria to make a nutritional change or to resist the action of phages is measured by analogous methods with similar limitations. The application of statistical analysis to the results is not justified by the methods of experimental control and observation).

Nor does the theory account for the delay in growth of mutant colonies.

The first object of the work to be described, was to investigate examples of the long lag phenomenon by methods similar to those of Lewis, with the refinement just suggested. It was found convenient and, indeed, necessary, to make similar experiments using liquid instead of solid media. A brief comparison of the methods will explain the procedure.

In tests with solid media, a parent culture is diluted in stages of 10. Small inocula from each diluted suspension, equal in volume, are inoculated to solid media, containing,

say, glucose or lactose, in parallel. Suppose the results are as follows (highly idealised):-

Dilution	Approximate Number of Cells in Inoculum.	Number of Colonies on Glucose	Number of Colonies on Lactose
1	10^8	Confluent	100
10	10^7	"	10
10^2	10^6	"	1
10^3	10^5	"	None
10^4	10^4	"	"
10^5	10^3	"	"
10^6	10^2	100	"
10^7	10	10	"
10^8	1	1	"
10^9	<1	None	"

Clearly 1 cell in 10^6 grows in lactose, assuming all viable cells grow on glucose. This is calculated from the ratio of the number of colonies which grow on lactose to the number of viable cells in the inoculum. The latter is calculated from the number of colonies which grow on glucose at much higher dilutions, when it is possible to count this number. But the ratio is also equal to the ratio of the highest dilution factors at which growth occurs in the respective media. The highest dilution from which growth in lactose occurs is 10^2 ; from which growth in glucose occurs

it is 10^8. The ratio is 10^6, as before.

The procedure with liquid media is precisely analogous. The actual number of cells which grow cannot be estimated, as can be done by counting colonies on solid media. But the highest dilutions at which growth occurs are readily observed, and the proportion of cells which will grow in lactose is easily calculated.

In Part I, the results of tests of this type, which may be called dilution tests, are reported, for four examples showing the phenomenon of a long lag before growth in a new medium. A note on the third example is appropriate at this point.

Earlier in this Introduction, the 'training' of a strain of bacteria to dispense with a complex nutrient substance, and to use a simpler one in its place, has been described. The example was the 'training' of Bact. coli, normally requiring sodium glutamate as the source of nitrogen, to use ammonium sulphate in place of sodium glutamate. This may be achieved, as described, by culture in a series of media in which the glutamate concentration is progressively reduced, and the ammonium sulphate concentration progressively increased. This is a typical 'training' procedure. But, in this case at least, the change in nutrition may alternatively be made in one step. If an inoculum of about 0·2 ml. of broth culture, containing about 10^8 cells, is introduced into 26 ml. of glucose-ammonium sulphate medium, growth occurs after a long lag phase of about 50 hours. Although the small supply of organic nitrogen compounds carried over in the inoculum seems

to be necessary for the achievement of growth, these facts suggest a much closer relationship than is obvious between the two ways in which growth in a new medium may be achieved.[12]

In addition to attempts to elucidate the phenomena of the long lag phase by means of experiments in which the dilution method is used, an extensive study has also been made of the effects of many different experimental conditions on the lag of a standard inoculum of 10^8 cells of Bact. lactis aerogenes in D-arabinose medium. The results, together with those of a less complete examination of the characteristics of Bact. coli mutabile in lactose, along the same lines, are reported in Part II.

These experiments provide considerable information about the reproducibility of the length of the lag phase under a variety of conditions, about the effects of culture of Bact. lactis aerogenes in media containing mixtures of D-arabinose with glucose or L-arabinose, and the effects of culture in glucose medium of cells previously grown in D-arabinose medium. It is possible to infer something of the nature of the change taking place in a strain of cells, during a long lag phase in a new medium, from observations of this type.

More direct evidence on this question is supplied by measurements of the changes in enzymes within the cell, which are associated with changes in cell nutrition. Bacterial metabolism consists of a series of linked enzyme reactions. Changes in metabolism made necessary by changes in nutrition therefore require changes, quantitative or qualitative, in

the enzyme equipment. The activity of certain enzymes, the dehydrogenases, may be conveniently measured by observing the time required for the consumption of a fixed amount of oxygen plus the reduction of a constant amount of methylene blue. A number of experiments made in this way are reported in Part III.

More detailed description of the scope and purpose of the methods used in Part II and Part III is deferred.

EXPERIMENTAL METHODS AND MATERIALS.

The strains of bacteria were stocked in broth cultures, sub-cultured at intervals of one month.

In the present work, bacteria were grown in both liquid and solid synthetic media. Strains were sub-cultured in a series of tubes of liquid medium, in which determinations of lag phase and growth rate were also carried out.

The synthetic liquid media were made up as follows:-

1 ml. magnesium sulphate solution (1 gm/l.)
10 ml. phosphate buffer (9 gm/l. potassium dihydrogen phosphate adjusted to pH 7·12 with sodium hydroxide)
10 ml. of a solution of the carbon source
5 ml. of a solution of the nitrogen source.

The last two solutions were, as far as possible, made approximately equivalent in carbon or nitrogen content; the actual concentrations were:-

glucose, 50 gm/l; lactose, 50 gm/l; sodium citrate dihydrate, 50 gm/l; D-arabinose, 5 gm/l; sodium glutamate, as carbon and nitrogen source, 50 gm/l; ammonium sulphate, 5 gm/l.

Tubes, usually 1 inch in diameter, containing cultures in liquid media, were suspended in water-baths at a controlled temperature of 40·0°. For serial sub-culture and for the measurement of growth rates, cultures were supplied with a stream of air bubbles from capillary tubes, which provided stirring and uniform aeration. The air was filtered through animal charcoal and sterile cotton wool. Such aeration was

not necessary in the measurement of long lag phases. It was proved that sufficient air was dissolved in the medium to permit growth to begin after the appropriate lag, and further growth to high cell populations was not required in these tests.

Bact. lactis aerogenes and D-arabinose.

The strain was Number 418 in the National Collection of Type Cultures. It was maintained in serial sub-culture in glucose-ammonium sulphate medium. The series was started by transferring an inoculum from the stock broth culture by means of a platinum loop. Thereafter, a small inoculum of the culture in glucose medium was transferred to a new tube of the medium once or twice daily, by means of a Pasteur pipette. Experiments with D-arabinose medium were not begun until the strain had passed through at least six sub-cultures in glucose medium. By this stage, growth in glucose medium was steady and reproducible, the lag being short when transfer was made at the appropriate time, and the mean generation time being about 33 minutes.

A general description of the growth of Bact. lactis aerogenes in synthetic media containing various sources of carbon has already been given in the Introduction. The importance of the stage of growth of the parent culture in the measurement of lag phases has been stressed.

Inocula transferred from a parent culture near the end of its logarithmic growth phase grow most reproducibly in daughter cultures; the lag phase in daughter cultures,

measured under these conditions, is minimal, and the lengths of lag in, for example, D-arabinose medium, are consistent within reasonable limits. Tests made at other stages of growth of the parent culture are less reliable.

All the tests reported later, were made from parent cultures at the optimum stage of growth. Tests of growth in D-arabinose medium were controlled by parallel tests of equal inocula in glucose medium. The standard procedure was to transfer an inoculum of 0·2 ml. of the parent culture, containing about 10^8 cells, to tubes of 26 ml. of the sterile test medium. If the lag in the control tube of glucose medium was greater than about 2 hours, the experiment was rejected, and the test repeated.

The standard glucose medium contained a supply of glucose excessive compared with the buffering capacity of the medium. In this medium, the stationary population of a culture was determined by the accumulation of acid toxic products and breakdown of the buffering capacity, some time after the optimum stage of growth for lag tests. At the latter stage, there was present a large amount of unused glucose. Transfer of inocula to daughter cultures at this stage resulted in sufficient glucose being carried over into the test medium for a certain amount of growth to take place, without use of the carbon source of the new medium. This vitiated control of the inoculum size.

The parent cultures for lag tests, therefore, were grown in glucose medium containing one-tenth of the

standard amount of glucose. In such a medium, the glucose was almost exhausted at the optimum stage of growth for lag tests, so that growth could only occur in the test medium by use of its constituents - the control of inoculum size was not invalidated by any nutrients carried over from the parent culture.

Bact. coli mutabile and lactose.

The strain was Number 2491 in the National Collection of Type Cultures. It was maintained in serial sub-culture in glucose - ammonium sulphate medium in a similar way to that described for Bact. lactis aerogenes. Parent cultures for lag tests were grown in dilute glucose medium in the same way. The length of the long lag phase in lactose medium was much less reliably reproducible than in the case of Bact. lactis aerogenes and D-arabinose, although careful control was exercised.

Bact. coli and ammonium sulphate.

The strain was a typical foecal strain, isolated by Dr.R.L.Vollum. When it was grown in serial sub-culture in a medium containing amino-acids as the source of nitrogen, it soon became able to grow in glucose - ammonium sulphate medium after only a short lag phase. Consequently, cells were tested directly from broth cultures, when, using the standard 0·2 ml. inoculum, they did not grow in glucose - ammonium sulphate medium until after a lag of about 50 hours. The length of the lag was independent of the age of the

broth culture.

Coli-aerogenes intermediate and sodium citrate.

One of the differences often regarded as distinctive between coli and aerogenes strains is their inability or readiness to use citrate as a source of carbon.

However, it has been recognised for some time that there exist intermediate strains which, although otherwise typical members of the coli group, are able to utilise citrate with more or less facility. Koser has described a number of such strains.[13]

In seeking another example of a coliform organism which grew in a new source of carbon only after a lag phase, an attempt was made to isolate such a strain from a few specimen strains of Bact. coli.

These were plated out on broth agar; individual colonies were selected, grown in amino-acid medium, then for several sub-cultures in glucose-ammonium sulphate medium, and finally tested in sodium citrate-ammonium sulphate medium.

After a lengthy and somewhat tedious search, a suitable strain was isolated. This grew in sodium citrate-ammonium sulphate medium after a lag of about 12 hours.

It was maintained in serial sub-culture in glucose-ammonium sulphate medium, and tested from dilute glucose-ammonium sulphate medium, in a similar way to Bact. lactis aerogenes and Bact. coli mutabile.

Measurement of Lag and of Growth Rate.

The growth rate of a culture was measured by sampling, at suitable time intervals, and counting the cell population. This was either done directly, using a Haemacytometer and a binocular microscope, or by measuring the light transmission of the sample, using a Spekker absorptiometer. Spekker instrument readings were calibrated against Haemacytometer counts in a special series of experiments. Samples were killed by heating to 100°, or by adding one small drop of formalin, immediately after removal from the culture.

Growth curves are most conveniently represented by plotting the logarithm of the cell count against time. The logarithmic growth phase is thus represented by a straight line. From this, the value of the mean generation time (the time required for the cell population, n, to double, i.e. for $\log_{10} n$ to increase by 0·30) is easily read off.

Once the growth rate in a given medium was proved to be reproducible, it was possible to determine the length of the lag phase by counting only two samples. The parent culture was sampled at the time of inoculation of the daughter culture. Knowing the volume of medium in the daughter culture and the inoculum size, the initial population of the daughter culture was calculated. The standard inoculum, and smaller inocula, did not immediately give rise to perceptible opalescence in a new, sterile tube of medium. The appearance of opalescence was a signal that growth had begun, when a sample was taken and counted. The time at which growth commenced was calculated by

extrapolating back to the initial count, using the known growth rate.

Inocula were measured either by pipette, used for 0·2 ml. inocula, or by carefully calibrated capillary tubes, used for 0·02 ml. inocula. The latter were used in most of the experiments with solid media.

Solid Media.

Solid media were prepared in two ways. In the first place, when a pure specimen of agar-agar was available, this was added to the liquid medium, lacking phosphate buffer, to a concentration of 2%. The resultant medium was a firm jelly at room temperature, but was easily melted by warming on a water-bath. Plates were prepared for inoculation by pouring the molten medium into sterile Petri dishes, allowing to set, and drying-off in the incubator for about 3 hours.

Later, silica gel media were used in place of agar media. They were made up by mixing the following solutions in sterile Petri dishes, allowing to set, and drying-off in the incubator for 12 to 15 hours. These media were less robust than the agar media, but the purity of the water-glass was superior to that of later supplies of agar-agar:-

10 ml. 10% water-glass in water.
1 ml. magnesium sulphate (1 gm/l).
1 ml. potassium dihydrogen phosphate (45 gm/l).
1 ml. of a solution of the carbon source (50 gm/l).
1 ml. of a solution of the nitrogen source (25 gm/l ammonium sulphate or 50 gm/l. sodium glutamate)

Normal hydrochloric acid to pH 7·1.

Cultures on solid media were incubated at 37·4°.

Before use, all glass-ware and solutions were carefully sterilized.

Solutions were initially boiled three times on successive days, and thereafter before use. Glass-ware was heated in an electric air oven at 180° for at least 30 minutes, and cotton-wool, used for plugging tubes, pipettes, etc. was sterilized in the autoclave, as were the agar media and broth media.

Stock broth cultures were sub-cultured using a platinum loop sterilized by heating to redness. All pipettes, previously sterilized in the oven, were carefully flamed immediately before use. Vessels were opened as briefly as possible, the necks being immediately flamed and cotton-wool plugs replaced.

The Characteristic reactions of the strains used were as follows:-

Test	Bact. lactis aerogenes	Bact. coli mutabile	Bact. coli	Coli-aerogenes intermediate
1. Glucose	+	+	+	+
2. Maltose	+	+	+	+
3. Mannite	+	+	+	+
4. Lactose	+	+(delayed)	+	+
5. Sucrose	+	−	−	+
6. Dulcite	−	+	−	−
7. Inosite	+	−	−	+
8. Citrate	+	−	−	+(delayed)
9. Methyl red	−	+	+	+
10. Vosges-Proskauer.	+	−	−	−
11. Indole production	−	+	−	−

For the tests 1-7,+ indicates the formation of acid and gas in a peptone water culture to which 1% of the test substance had been added. For test 8,+ indicates that the strain was able to grow in a medium containing citrate as the sole source of carbon The other three tests were performed according to the methods described in handbooks of bacteriology.

PART I.

DILUTION TESTS.

Bact. lactis aerogenes and D-arabinose.[6]

Theories of the long lag phase.

Inocula of about 10^8 cells of Bact. lactis aerogenes, taken from a parent culture just before the end of its logarithmic growth phase, begin to grow again, in glucose-ammonium sulphate medium, almost immediately. The mean generation time is about 33 minutes.

Parallel inocula in D-arabinose-ammonium sulphate medium, however, do not begin to grow until 20 to 30 hours after inoculation. Growth is then logarithmic, but the mean generation time is about 75 minutes. This behaviour has been consistently observed for over two years. (Previous behaviour is discussed later).

After one sub-culture in D-arabinose medium, cells are able to grow readily in the medium, and this property is very stable. These facts have been urged as support for the hypothesis that a strain of genetic mutants has been selected in the first sub-culture.

There is no detectable growth during the initial long lag phase in D-arabinose. According to the mutation and selection theory of change in nutritional habit, a few mutant cells , capable of growth in D-arabinose, are initially present; although they begin to grow when an inoculum is transferred to D-arabinose medium, the growth does not become detectable until the number of mutant cells is comparable with the number of

normal cells in the inoculum. The 'lag phase' is not a true lag phase at all, but is only the length of time during which the growth of mutant cells is masked by the presence of the normal cells in the inoculum. Some of the quantitative aspects of this theory will now be examined.

The experimental determination of the length of the 'lag phase' was carried out as follows. The initial cell count in the test culture, n_o, was calculated from the volume of the test culture, the inoculum size, and the cell count of the parent culture at the time of inoculation. The time at which growth began was calculated by extrapolating back to n_o from the cell count n_t of a sample taken at time t, soon after growth began. Under the actual experimental conditions, n_t was at least four times as great as n_o. In extrapolating back, the experimental value of the growth constant, k_A, was used. The expression relating these quantities and the 'lag', L, is:-

$$\ln\left(\frac{n_t}{n_o}\right) = k_A(t-L)$$

Now, if it be assumed that the actual process occurring was the multiplication of m_o mutant cells, present in the inoculum, to a number $(n_t - n_o)$,

$$\ln\left(\frac{n_t - n_o}{m_o}\right) = k_A t$$

Combining the two equations :-

$$\ln\left(\frac{n_t}{n_o}\right) = \ln\left(\frac{n_t - n_o}{m_o}\right) - k_A L$$

$$k_A L = \ln\left(\frac{n_t - n_o}{m_o}\right) - \ln\left(\frac{n_t}{n_o}\right)$$

$$k_A L = \ln\left[\frac{(n_t - n_o)\, n_o}{m_o n_t}\right] \text{-------} (1)$$

Now, under the experimental conditions, n_t was not less than four times n_o. If n_t is put = 4 n_o.

$$\ln\left[\frac{(n_t - n_o)\, n_o}{m_o\, n_t}\right] = \ln\left[\frac{3\, n_o^2}{4\, m_o n_o}\right]$$

$$= \ln\left[\frac{3\, n_o}{4\, m_o}\right] = \ln\left[\frac{n_o}{m_o}\right] + \ln\left[\frac{3}{4}\right]$$

$$= \ln\left[\frac{n_o}{m_o}\right] - 0{\cdot}3$$

If $n_t > 4\ n_o$, the numerical term becomes even less important.

The mean generation time in D-arabinose was 1·25 hours.

$$\therefore \ln 2 = k_A \times 1{\cdot}25$$

$$k_A = \frac{0{\cdot}3 \times 2{\cdot}3}{1{\cdot}25}$$

L ~ 30 hours.

$$\therefore k_A L \sim \frac{30 \times 0{\cdot}3 \times 2{\cdot}3}{1{\cdot}25} \sim 16{\cdot}6$$

Substituting in equation (1)

When $n_t = 4\ n_o$

$$16{\cdot}6 = \ln\left[\frac{n_o}{m_o}\right] - 0{\cdot}3$$

$$\ln\left[\frac{n_o}{m_o}\right] = 16{\cdot}9$$

If n_t is larger compared with n_o, $\ln\left[\frac{n_o}{m_o}\right]$ approaches the minimum value, 16·6

$$\therefore \log 10\left[\frac{n_o}{m_o}\right] \sim 7$$

$$\frac{n_o}{m_o} \sim 10^7$$

The proportion of mutant cells in the inoculum is about 10^{-7}. This is in fair agreement with estimates given in the literature for the proportion of mutants in bacterial cultures. I.M.Lewis suggested a proportion of 10^{-6} lactose - using mutants in the strain of Bact. coli mutabile used by him.

The hypothesis of mutation and natural selection, then, if applied to the standard lag test of Bact. lactis aerogenes in D-arabinose, suggests that only about 10^{-7} of the cells are capable of growth in D-arabinose medium.

On the other hand, the adaptation hypothesis suggests that the lag phase is a true lag phase, and that at least the majority of cells inoculated into D-arabinose medium are able to grow after that length of time in contact with the new substrate. The lag phase is necessary for reorganisation of the cell to metabolise D-arabinose, a process which occurs in response to the presence of this sugar.

In respect of the proportion of cells in a culture of Bact. lactis aerogenes expected to be able to grow in D-arabinose, the two theories are in very sharp contrast, and discrimination between the alternatives may be possible if this proportion can be measured.

The use of dilution tests, outlined in the Introduction, was a suitable method of attack on this problem. The separation of individual cells on solid medium makes possible a test of their individual ability to grow on the medium.

The growth of small numbers of cells in liquid media is also readily examined, by using small inocula from diluted

cultures.

Experiments with Solid Media.

In these experiments, the media were solidified by adding 2% agar-agar. The supply of this substance, which was available at that time, was pure, and did not support the growth of colonies when added to the basal medium lacking any other source of carbon. (The use of silica gel media in later experiments was necessary because later supplies of agar-agar were found to support bacterial growth under these conditions, even after prolonged washing of the agar-agar).

Complete medium containing 2% agar-agar became darkened on autoclaving, but this did not happen in the absence of phosphate buffer. It was found that the growth of colonies up to detectable size was independent of the addition of phosphate, so the buffer was omitted from the solid media used in these experiments.

Cultures of Bact. lactis aerogenes at the optimum stage of growth in dilute glucose medium were diluted in stages of 10; 1 ml. of culture was added to 9 ml. of phosphate buffer, and thoroughly mixed by shaking; 1 ml. of this diluted suspension (Dilution 10) was then transferred to a second tube containing 9 ml. of buffer, mixed (Dilution 10^2), and so on.

The activity of the cells was checked, as always, by testing the growth of standard inocula in liquid media at 40·0°.

0·1 ml. inocula of suitable high dilutions were transferred to the plates; the drops were dispersed over the surface of the solid media by tilting the plates slightly and by rotating them rapidly. The liquid was absorbed within half an hour, when the plates were transferred to an incubator at

37·4°. They were examined at suitable and convenient intervals.

All the solid media contained magnesium sulphate and ammonium sulphate in the same concentrations as in liquid media. Parallel inoculations were made to plates containing media with

(1) glucose in the same concentration as in liquid medium
(2) D-arabinose in the same concentration as in liquid medium
(3) no source of carbon.

No colonies had appeared on the control plates, containing no source of carbon, after one week's incubation. The numbers of colonies appearing on the other plates, and their time of appearance, are recorded in Table I, which contains the results of a number of experiments.

Each colony springs from a single cell, or, at most, a very small cluster of cells. In all experiments, similar numbers of colonies grew from parallel inocula on glucose and D-arabinose plates. There was a delay in the appearance of colonies on D-arabinose plates after those on glucose plates, which was comparable with the difference in lag in liquid media. In each experiment, all colonies, on the same medium, appeared at very nearly the same time, and no second crop of colonies was observed during a week's incubation.

It is clear that all cells which are capable of growth on glucose are equally capable of growth on D-arabinose, after the appropriate lag phase. The length of the lag phase is very similar for indivual cells.

There seems to be no question of selection of mutant cells - the behaviour is very nearly uniform for each

TABLE I.

The Growth of Bact. lactis aerogenes on Solid Media.

Dilution	Number of Colonies on Glucose Medium.	Time before last Colonies had appeared on Glucose Medium. (hours)	Number of Colonies on D-arabinose Medium.	Time before Colonies appeared on D-arabinose Medium. (hours)	Lag in D-arabinose Liquid Medium. (hours)
10^6	36	< 17	20	31-41	31
10^6	37	"	26	"	"
10^7	5	"	2	"	"
"	6	"	7	"	"
10^6	56	< 6	90	ca.33	22
"	44	"	70	"	"
10^7	1	"	6	"	"
"	1	"	2	"	"
10^7	11	< 14	3	ca. 33	23
"	8	"	3	"	"
	205	Total numbers	229		

individual cell. According to the mutation and selection theory as outlined above, colonies should not have appeared at all on the D-arabinose plates, as the smallest inoculum which might contain a mutant cell would contain a total of 10^7 cells.

However, it is well known that, on solid media, growth occurs in two stages. Micro-colonies may be formed very quickly, but their development, to form visible colonies, may be considerably delayed. Presumably, the first source of nutrition is some impurity in the solid medium (always possible with agar-agar), while the second stage of growth is that which makes use of the main nutrient supply.

It has been suggested that this phenomenon may permit an alternative interpretation to that of the adaptation hypothesis for observations of the type recorded in Table I. As has been shown, the mutation and selection hypothesis requires a proportion of 10^{-7} mutants to account for the lag of standard inocula of Bact. lactis aerogenes in D-arabinose liquid medium. Suppose that a cell on a D-arabinose plate grew, without use of D-arabinose, to form a micro-colony containing 10^7 cells. This might contain one mutant cell capable of growth on D-arabinose, which might then grow on the D-arabinose to form a visible colony

It seems very unlikely that a colony containing 10^7 cells would be invisible, and, in any case, the delay in appearance of colonies on D-arabinose is still unaccounted for, unless it be assumed that growth, either to or from the micro-colony stage, is abnormally slow.

Although a few early experiments yielded observations of

very slow growth rates of Bact. lactis aerogenes in D-arabinose, these were associated with very long lags of the order of 100 hours or more; nor have such abnormalities occurred at all during the experiments described in this dissertation, during a period of over two years.

However, this objection may be conclusively tested by dilution tests in liquid media.

Experiments with Liquid Media.

The dilution technique was identical with that used in the experiments with solid media. 0·2 ml. inocula were used. The tests in D-arabinose medium were made in duplicate at each dilution.

The results of a typical experiment are listed in Table II.

The smallest inocula capable of growth in glucose-ammonium sulphate medium, are equally capable of growth in D-arabinose-ammonium sulphate medium after the long lag phase has elapsed. Inocula which are statistically unlikely to contain any cells, fail to grow in either medium.

There is no agar-agar present in these media, so that there is no possible source of carbon apart from the test source. Any growth in D-arabinose medium must use D-arabinose.

The greater lag of tests from higher dilutions is in agreement with the account which has been given of 'early lag' and the effect of change in the inoculum size.[3]

These results confirm those obtained using solid media. There is no doubt that all cells of the strain of Bact. lactis aerogenes which was used in this investigation, are capable of

growth in D-arabinose - ammonium sulphate medium after a long lag phase.

TABLE II.

The Growth of Bact. lactis aerogenes in Liquid Media.

Dilution	Approximate Number of Cells in Inoculum.	Growth in Glucose Medium.	Approximate Lag in Glucose Medium. (hours)	Growth in D-arabinose Medium.	Approximate Lag in D-arabinose Medium. (hours)
1	10^8	+	<10	+	29
10	10^7	+	< 9	+	28
10^2	10^6	+	< 7	+	26
10^3	10^5	+	< 6	+	24
10^4	10^4	+	< 4	+	48
10^5	10^3	+	4	+	49
10^6	10^2	+	< 24	+	41
10^7	10	+	< 22	+	56
10^8	1	+	< 20	+	55
10^9	<1	-	> 8 days	-	> 8 days
Higher dilutions	<1	-	> 8 days	-	> 8 days

Bact. coli mutabile and Lactose. [7]

The strain of Bact. coli mutabile was much less easily cultivated in glucose - ammonium sulphate medium than was the strain of Bact. lactis aerogenes. The development of 'late lag' was much more rapid.

In making lag tests, very careful control of the state of growth of the parent culture at the time of inoculation was necessary. (See Part II).

With care, however, it was possible to maintain serial sub-cultures, and to make lag tests, by methods similar to those described for Bact. lactis aerogenes.

Standard, 0·2 ml. inocula, containing about 10^8 cells, if transferred at the correct stage of growth, began to grow again almost immediately in new tubes of glucose - ammonium sulphate medium, in which the mean generation time was about 30 minutes. Parallel inocula transferred to tubes of lactose-ammonium sulphate medium, however, did not begin to grow until after 50 or 60 hours. The growth rate was somewhat erratic, but usually corresponded to a mean generation time of about 90 minutes. This was considerably improved by serial sub-culture in lactose - ammonium sulphate medium. After the first sub-culture, the lag in lactose - ammonium sulphate medium was short.

It was shown, for the case of Bact. lactis aerogenes in D-arabinose, that, according to the mutation and selection theory, the length of the 'lag phase', L, for a standard inoculum, might be accounted for by masking of the growth of

m_o mutant cells in the inoculum by n_o normal cells which are incapable of growth.

The approximation relating these quantities, for the case of Bact. coli mutabile in lactose, is

$$k_L L \sim \ln\left[\frac{n_o}{m_o}\right]$$

where k_L is the growth constant.

For the growth of Bact. coli mutabile in lactose medium, the mean generation time is about 1·5 hours.

$$\therefore \ln 2 \sim k_L \times 1{\cdot}5$$

$$\therefore k_L \sim \frac{0{\cdot}3 \times 2{\cdot}3}{1{\cdot}5}$$

$L \sim 50$ hours,

$$\therefore k_L L \sim \frac{0{\cdot}3 \times 2{\cdot}3 \times 50}{1{\cdot}5} \sim 23$$

$$\ln\left[\frac{n_o}{m_o}\right] \sim 23$$

$$\log_{10}\left[\frac{n_o}{m_o}\right] \sim 10$$

$$\frac{n_o}{m_o} \sim 10^{10}$$

This estimate must be too low, as, in the standard inoculum, there are present only 10^8 cells. It is possible, in this case, that the growth rate, assumed similar to that later observed, which was somewhat erratic, might, in fact, be considerably slower during the earlier cell divisions.

But in any case, it is again seen that the mutation - selection theory requires that only a very small proportion of the cells should be capable of growth in the new medium, in sharp contrast to the adaptation postulate, that most of

the cells grow, after a long lag phase.

The problem was investigated by using the dilution technique, as before.

Experiments with Solid Media.

In these experiments, silica gel media, made as described in the Experimental Section, were used. Sodium glutamate was used in place of glucose - ammonium sulphate in the control media, since inocula of less than 10^6 cells of Bact. coli mutabile would not grow in glucose - ammonium sulphate medium. Fortunately, ammonium sulphate was a satisfactory source of nitrogen in association with lactose as the source of carbon.

Some little difficulty was at first experienced in the use of silica gel media. Control of the extent to which the plates were dried was very important in securing successful results. Unless the duration of the drying period was exactly right, syneresis occurred, and the medium was, to all intents and purposes, liquid, which vitiated the experiment.

The inoculation technique was slightly different from that previously described. Capillary tubes were cut square at the end, and were calibrated. Tubes which, when held vertically, delivered drops of 0·02 ml. each, were made by cutting the tubes at a suitable diameter, measured with a standard wire gauge.

The drops were placed on the surface of the plates, and were allowed to become absorbed without dispersing. The drops were absorbed within half an hour. Subsequently, growth occurred within a circular zone, around the site where the

drop was placed, over an area of about 1 sq. cm.

The suitability of sodium glutamate - silica gel medium as a control medium, was proved by checking against broth agar medium. The growth of a number of parallel inocula is recorded in Table III.

TABLE III.

Check of Sodium Glutamate - Silica Gel as a Control Medium.

Number of Colonies on Sodium Glutamate - Silica Gel Medium.	Number of Colonies on Broth - Agar Medium.
46	50
52	56
50	41
61	52
57	46
51	54
47	51
364	350

Table IV shows the results of an experiment comparing growth on sodium glutamate - silica gel and on lactose - ammonium sulphate - silica gel media. Blank plates, containing no source of carbon, did not yield any colonies within 5 days.

These results are in accord with those of Virtanen and Winkler.[14]

Comparable numbers of colonies appeared on lactose medium, and on the control medium. For the growth of Bact. coli mutabile on lactose medium, the lag before the appearance of different colonies was similar in each individual case. It seems clear that all the cells were able to grow on lactose medium, after the lapse of this lag phase.

TABLE IV.

The Growth of Bact. coli mutabile on Solid Media.

Dilution	Approximate Number of Cells in Inoculum.	Number of Colonies on Glutamate Medium.	Time before appearance of Colonies on Glutamate Medium.	Number of Colonies on Lactose Medium.	Time before appearance of Colonies on Lactose Medium.
10^4	4,000	Confluent	< 14 hours	Confluent	43-62 hours
"	"	Confluent	"	Confluent	"
10^5	400	110	< 14 hours	100	43-62 hours
"	"	106	"	204	"
10^6	40	13	< 14 hours	14	43-62 hours
"	"	10	"	30	"
10^7	4	0	> 5 days	0	> 5 days
"	"	0	"	7	43-62 hours

The standard 0·2 ml. inoculum grew immediately in glucose - sodium glutamate liquid medium, and after 60 hours in lactose - ammonium sulphate liquid medium.

Experiments with Liquid Media.

The method was similar to that described for the tests of Bact. lactis aerogenes in liquid media.

The results of a typical experiment are listed in Table V.

It will be noticed that, although inocula containing only one cell grew in sodium glutamate liquid medium, inocula containing less than 100 cells did not grow in lactose - ammonium sulphate liquid medium.

This might be held to support the theory that only special mutant cells were able to grow in lactose - ammonium sulphate liquid medium. Even so, it would be concluded that such mutants were present in a proportion of about one per 100 normal cells, a truly remarkable number compared with the figures generally accepted. But this interpretation makes the serious assumption that there is only one reason for the failure of very small inocula to grow, viz. the inability of the cells to use lactose in virtue of their genetic character.

The smallest inocula which did grow in lactose - ammonium sulphate liquid media, containing about 100 cells, only did so after a lag of about 94 hours. It seemed possible that, after this length of time, there was a considerable chance that many of the bacteria, in the rather unsuitable environment, had died. Perhaps this was the reason for the failure of smaller inocula to grow.

The tests in lactose - ammonium sulphate were made in triplicate at each dilution. While allowing the ordinary test

TABLE V.

The Growth of Bact. coli mutabile in Liquid Media.

Dilution	Approximate Number of Cells in Inoculum.	Growth in Glutamate Medium.	Lag in Glutamate Medium. (hours)	Growth in Lactose Medium.	Lag in Lactose Medium. (hours)	Growth in Lactose tests on adding Glutamate after 6 days.
1	10^8	+	<4	+	82	
10	10^7	+	<3	+	85	
10^2	10^6	+	1	+	103	
10^3	10^5	+	<14	+	116	
10^4	10^4	+	<12	+	113	
10^5	10^3	+	<10	+	110	
10^6	10^2	+	<8	+	94	
10^7	10	+	<6	−	>6 days	−
10^8	1	+	<6	−	"	−
10^9	<1	−	>6 days	−	"	−
Higher dilutions	<1	−	"	−	"	−

to proceed in one tube, two were available for secondary tests.

It had been shown that the cells grew readily and reliably in sodium glutamate medium. After 6 days, some sodium glutamate solution was added to two of the tubes containing tests of the 10^{7} and higher dilutions in lactose - ammonium sulphate medium, and incubation in the 40·0° water-bath was continued.

No growth occurred in these tubes, observed for several days. It was clear that, after 6 days, there were no viable cells left in the tests in lactose - ammonium sulphate liquid medium. Their failure to grow is scarcely attributable to the genetic character of the cells.

Apart from the possibility that death might overtake some cells before the completion of the long lag phase in lactose - ammonium sulphate medium, all cells of the strain of Bact. coli mutabile were capable of growth in lactose - ammonium sulphate medium. Before this could occur, the lapse of a long lag phase was necessary.

The numbers of colonies which appeared on solid media, and the maximum dilution at which growth occurred in sodium glutamate liquid medium, were in good agreement with the numbers of cells in the inocula, determined by counting under the microscope; as might be expected, cells in a culture near its most active stage of growth were almost all viable.

Bact. coli and Ammonium Sulphate.[12]

Some of the growth characteristics of the strain Bact. coli in synthetic media have already been described.

When a little of a stock broth culture was transferred to sodium glutamate medium by means of a platinum loop, growth occurred after only a short lag phase. Similar inoculation into glucose - ammonium sulphate medium, however, yielded very erratic results; sometimes there was no growth at all, and growth never occurred until after a long lag phase.

When the strain was sub-cultured in a series of tubes of sodium glutamate medium, and then transfer was made to glucose - ammonium sulphate medium, growth occurred after only a short lag. When standard 0·2 ml. inocula were transferred from a broth culture to tubes of sodium glutamate medium and glucose -ammonium sulphate medium in parallel, growth occurred almost immediately in the former. In the latter, there was a lag phase of about 50 hours before growth began, when the mean generation time was about 60 minutes. Transfer to a second tube of glucose - ammonium sulphate medium at the optimum stage of growth produced almost immediate growth in this second sub-culture. The growth rate was increased after serial sub-culture in glucose - ammonium sulphate medium.

The strain was plated out on broth agar medium. Individual colonies were selected, and grown in broth. The results of tests of these broth cultures, described below, were remarkably consistent. Cultures grown from different colonies yielded very similar results, and the length of the lag phase appeared to be

independent of the age of the parent broth culture.

If the postulate be applied that the 'long lag phase', is, in reality, the time during which the growth of mutant cells is masked by the presence of normal cells unable to grow in glucose - ammonium sulphate medium, it is found that a proportion of mutants equal to about 10^{-15} of the normal cells is required to account for the observations. This estimate is made by assuming that the growth rate during the masked period of growth is similar to that which is actually observed during the later stages of growth. It is possible that the concealed growth might occur at a much slower rate, which might bring the estimated proportion of mutants within the limit of 10^{-8}. This is the smallest possible proportion of mutants which could account for the growth of inocula containing 10^{8} cells.

The problem was again investigated by means of the dilution technique, using methods identical with those used for the experiments with Bact. coli mutabile.

Experiments with Solid Media.

The parent culture was grown in broth from a single colony, after plating out the stock culture.

Silica gel was used as the basis for glucose - ammonium sulphate solid medium, and also for the glutamate control medium.

Parallel inoculations were also made to broth agar plates.

0.02 ml. inocula, measured as drops from calibrated capillary tubes were used for the solid media. Standard 0·2 ml. inocula were tested in liquid media as controls.

The results of a typical experiment are recorded in Table VI (a), (b), and (c).

TABLE VI (a).

The Growth of Bact. coli on Sodium Glutamate - Silica Gel and Broth Agar Media.

Dilution	Approximate Number of Cells in Inoculum.	Number of Colonies on Sodium Glutamate Medium.	Time before appearance of Colonies on Glutamate Medium. (hours)	Number of Colonies on Broth Agar Medium.	Time before appearance of Colonies on Broth Agar Medium. (hours)
10^6	40	38	28	24	5 - 17
		28	"	23	"
		40	"	31	"
		35	"	26	"
		46	"	30	"
		42	"	30	"
		53	"	31	"
		37	"	30	"
10^3	4×10^4	∞	5 - 17	∞	< 5
		∞	"	∞	"

The symbol ∞ represents confluent growth within the circular zone of about 1 sq. cm. in which growth from 0·02 ml. inocula takes place.

TABLE VI (b)

Bact. coli and Ammonium Sulphate.

Growth of 10^6 Dilution on Glucose - Ammonium Sulphate - Silica Gel Plates.

(Lag of standard inoculum in glucose - ammonium sulphate liquid medium = 50 hours). About 40 cells in each inoculum.

Time after Inoculation (hours)	Number of Colonies.						
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
65	0	0	0	0	0	0	0
71	0	0	0	0	0	0	2
89	0	0	0	0	0	0	8
100	0	0	7	0	0	0	8
171	0	0	7	0	0	0	8

TABLE VI (c).

Bact. coli and Ammonium sulphate. Growth of 10^3 Dilution on Glucose-Ammonium sulphate-silica gel Plates. Each inoculum contained about 4×10^4 cells.

(Lag of standard inoculum in liquid medium=50 hours.)

Time after Inoculation (hours)	Plate 1 Sites 1,2,3,4	Plate 2 Sites 1,2,3,4	Plate 3 Sites 1,2,3,4	Plate 4 Sites 1,2,3,4	Plate 5 Sites 1,2,3,4	Plate 6 Sites 1,2,3,4	Plate 7 Sites 1,2,3,4
22							∞,∞,∞,∞
41	2		∞, 20,10	1	1	1	
53	10, 10, 10		100,100	4	8		
71	∞,50,3,20	0,0,0,0 Glutamate added	∞,2,100,100	0,0,0,40	0,50,0,0	5,0,0,0 Glutamate added	∞,∞,∞,∞
89	∞,13,100		30,∞,∞		2	∞,∞,∞,∞	
100	20			100	7,100,50,4		
114		1		∞ Glutamate added	50,200,100,5		
137	50			6,2	∞, ∞, ∞,200		
171	∞,∞,50,100	1,0,0,0	∞,30,∞,∞	6,2,1,∞	∞, ∞, ∞,200	∞,∞,∞,∞	∞,∞,∞,∞

For any inoculum, the first figure recorded in the appropriate vertical column is the number of colonies present when growth was first observed (at the time shown in the same level of the table). Blanks in the same column at earlier times indicate no growth; at later times, that there was no important change. The columns are filled in completely for 71 hours and 171 hours. The symbol ∞ indicates confluent growth.

Inoculations were made from two Dilutions of the parent culture. 0·02 ml. inocula contained respectively about 40 and about 4×10^{4} cells. The smaller inocula grew quite reliably on sodium glutamate - silica gel medium, (Table VI (a)), but usually failed completely on glucose - ammonium sulphate - silica gel medium (Table VI(b)).

The larger inocula rapidly yielded zones of confluent growth on broth agar and sodium glutamate - silica gel media, presumably due to simultaneous appearance of a very large number of colonies (Table VI (a)). On glucose - ammonium sulphate - silica gel medium their growth was very erratic. (Table VI(c))

Within 22 hours, 4 of the 28 inocula on this medium, had yielded zones of confluent growth. After 71 hours, six inocula had yielded zones of confluent growth, 4 of them on one plate (Plate 7). One plate had yielded no colonies at all (Plate 2) and another only 5, in one zone, and none in the other three (Plate 6). 0·02 ml. drops of 50 gm/l sodium glutamate solution were added to these plates in the neighbourhood of the inoculation sites. The Plate 2 subsequently yielded only one colony, appearing 114 hours after inoculation. Plate 6 yielded four zones of confluent growth, within 89 hours of inoculation and 18 hours of adding sodium glutamate, which was absorbed within half an hour.

These results suggest that, after 71 hours, the cells were at a very crucial stage of the test. Cells which had not achieved growth at this time were, apparently, very likely to

die before achieving growth. This was confirmed by the result of adding sodium glutamate, in a similar way, to Plate 4, after 114 hours, when only 9 colonies appeared in the 3 zones which had not previously shown growth.

Further, only half of the zones which had not achieved confluent growth after 71 hours, and to which no sodium glutamate was added at this stage, succeeded in achieving confluent growth later, the others finally yielding colonies from one to 200 in number.

The slow development of growth in many zones suggested that the growth of some of the later colonies might have been assisted by the diffusion of essential metabolites from the earlier ones.

This picture of erratic growth seems to suggest that there was a considerable difference in the ability of individual cells to use the new medium. The pattern of the results, however, does not justify the assertion that the parent culture contained a small proportion of mutants, capable of growth in glucose - ammonium sulphate, among a large number of 'normal' cells, which were incapable of growth.

It seems, rather, that the fate of individual cells was dependent on some environmental factor outside experimental control. If about 50 colonies were established in a zone within 71 hours, confluent growth was later achieved within the zone. It appears that such a number of colonies supplied a sufficient reservoir of essential metabolites, possibly amino-acids, to permit the growth of other colonies in the zone.

Perhaps the variable number of colonies appearing within the first 71 hours was determined by slight differences in the amounts of such metabolites carried over in the inoculum. It is possible that results might have been more reproducible if it had been possible to grow the parent culture in a synthetic medium instead of in broth.

Experiments with Liquid Media.

The methods used in this group of experiments were precisely parallel with those described for the dilution tests of Bact. coli mutabile in liquid media.

0·2 ml. inocula of each Dilution were tested in sodium glutamate liquid medium, and in 3 parallel tubes of glucose - ammonium sulphate liquid medium.

Table VII contains the results of a typical experiment. Although inocula from Dilutions up to 10^{8} grew in sodium glutamate medium, a result exactly consistent with the number of cells in the inocula, only the standard inoculum was able to grow in glucose - ammonium sulphate medium, after a lag of 62 hours. Smaller inocula entirely failed to grow in this medium.

This result, taken by itself and without further investigation, might be held to support the mutation and selection theory, with a proportion of ammonium sulphate - utilising mutants to normal cells of about 10^{-8}. But when glutamate was added to some of the parallel tests after appropriate times, it emerged that the cells died off fairly rapidly.

The tests containing inocula of 10^{5} cells or less, did

TABLE VII.

The Growth of Bact. coli in Liquid Media.

Dilution	Approximate Number of Cells in Inoculum.	Growth in Glutamate Medium.	Lag in Glutamate Medium. (hours)	Growth in Glucose-Ammonium sulphate Medium.	Lag in Glucose-Ammonium sulphate Medium. (hours)	Growth in Glucose-Ammonium sulphate Tests on adding Glutamate after : 5 days	7 days
1	10^8	+	5-12	+	62		
10	10^7	+	2-9	−	> 7 days	+	+
10^2	10^6	+	< 6	−	"	+	−
10^3	10^5	+	< 3	−	"	−	−
10^4	10^4	+	3	−	"	−	−
10^5	10^3	+	11	−	"	−	−
10^6	10^2	+	4	−	"	−	−
10^7	10	+	< 12	−	"	−	−
10^8	1	+	< 9	−	"	−	−
10^9	< 1	−	> 14 days	−	"	−	−
Higher dilutions	< 1	−	> 14 days	−	"	−	−

not grow when sodium glutamate was added after 5 days. Tests containing inocula of 10^6 cells were viable after 5 days, but not after 7 days. These results are in accord with those for solid media.

It would seem that the cells were unable to grow on glucose - ammonium sulphate medium without the assistance of small amounts of more complex nutrients carried over in the inoculum. Without this assistance, death occurred quite rapidly.

Apparently, the undiluted inoculum from the parent broth culture carried with it sufficient of these essential metabolites to permit growth to occur in liquid glucose - ammonium sulphate medium, but further dilution by only a factor of 10 made the concentration too low for growth to occur in the test.

On solid media, the localisation of all the cells of an inoculum near to each other apparently made possible the growth of some cells before the essential metabolites, perhaps initially within the cell, had become too dilute, by diffusion, and, in some cases, the supply was apparently enhanced by the growth of these cells.

This seems to be the most likely explanation of the results. In view of the proved high death-rate, it would be rash to attribute the success of some cells in growing to their genetic character rather than to their chance survival.

A Coli - aerogenes Intermediate and Sodium Citrate.

The isolation of the strain has been described in the Experimental section.

Cells taken from the stock broth culture grew readily in glucose - ammonium sulphate liquid medium after one passage through sodium glutamate liquid medium.

After a few passages through glucose - ammonium sulphate liquid medium, the mean generation time was about 30 minutes. Standard inocula, transferred at the optimum stage of growth, grew almost immediately in a new tube of glucose - ammonium sulphate liquid medium. In sodium citrate - ammonium sulphate liquid medium, they did not grow until after a lag phase of about 12 hours; the mean generation time in this medium was about 45 minutes. After the first sub-culture in sodium citrate - ammonium sulphate medium, the lag in this medium was short.

If the mutation - selection postulates are applied to this behaviour, as before, it is found that a proportion of citrate - using mutants of about 10^{-5} is required to account for the lag of the standard 0·2 ml. inoculum in sodium citrate medium. The problem was investigated by means of dilution tests, as before

Experiments with Solid Media.

Although the strain grew reproducibly on glucose - ammonium sulphate - silica gel medium, results on sodium citrate - ammonium sulphate - silica gel medium were not

consistent.

In one experiment, inocula of 10^4 and 10^5 cells produced zones of confluent growth on sodium citrate - ammonium sulphate - silica gel medium after about 50 hours. In another experiment, inocula of 10, 10^2, 10^4, and 10^5 cells failed to grow in this medium. Following the addition of sodium glutamate, after 114 hours, only a very few colonies appeared.

In each experiment, the viability of the cells was proved by the growth of consistent numbers of colonies on broth agar and glucose - ammonium sulphate - silica gel media, from inocula of 10 and 10^2 cells. These colonies appeared on broth agar after about 10 hours, and on glucose - ammonium sulphate - silica gel after about 50 hours. In each case, the lag of the standard 0.2 ml. inoculum in glucose - ammonium sulphate liquid medium was less than 2 hours, and in sodium citrate - ammonium sulphate liquid medium, about 12 hours.

However, the growth of the strain in liquid media was very reproducible. In view of this, experiments with solid media were not continued.

Experiments with Liquid Media.

The experimental methods were similar to those already described in the other three examples.

The results of a typical experiment are given in Table VIII.

Inocula from dilutions up to 10^8 grew in both glucose - ammonium sulphate liquid medium and sodium citrate - ammonium sulphate liquid medium. Smaller inocula did not grow in either.

TABLE VIII

The Growth of Coli - aerogenes Intermediate in Liquid Media.

Dilution	Approximate Number of Cells in Inoculum	Growth in Glucose Medium	Lag in Glucose Medium (hours)	Growth in Citrate Medium	Lag in Citrate Medium (hours)
1	10^8	+	1	+	13
10	10^7	+	1·5	+	11·5
10^2	10^6	+	4-7	+	8
10^3	10^5	+	14	+	23
10^4	10^4	+	7	+	30-48
10^5	10^3	+	11·5	+	55
10^6	10^2	+	10·5	+	45
10^7	10	+	12-23	+	53
10^8	1	+	10-21	+	67
10^9	<1	−	>5 days	−	>5 days
Higher dilutions	<1	−	"	−	"

(The 10^{8} dilution was the highest yielding on inoculum with a good statistical chance of containing one cell). It is clear that all the cells were equally capable of growth in glucose or sodium citrate media, in the latter after a longer lag.

As before, there was an increase in the length of the lag phase in both media as the number of cells, and the concentration of intermediate metabolites in the inoculum, was reduced. The exceptionally short lag from the 10^{2} dilution in this experiment was also occasionally observed in other experiments; it appears that the usual mean generation time in sodium citrate medium, about 45 minutes, was sometimes shorter, i.e. the growth rate was sometimes faster. Slightly erratic behaviour of this kind in the first sub-culture in a new medium is not unusual. In all the cases described, only an average figure can be ascribed to the mean generation time during the early growth in a new medium.

The non-reproducible behaviour of the strain on sodium citrate - ammonium sulphate - silica gel medium may have been a reflection or magnification of erratic growth rate, but was possibly due to some subtle environmental change. Two different samples of water-glass were used in making up the media for the two different experiments. Although growth on sodium glutamate - silica gel was equally reliable in each experiment, it is possible that the second sample of water-glass contained some impurity which interfered with citrate utilisation, while not affecting growth in glutamate.

Summary of Part I.

In introducing this section, it was pointed out that the alternative theories of the mechanism of change in bacterial nutrition were in sharp contrast in their postulates as to the proportion of the cells in a culture which are capable of growth in a new medium.

This was illustrated by use of observations on the length of the lag phase of Bact. lactis aerogenes when first transferred to D-arabinose - ammonium sulphate medium. To account for the lag, 30 hours, in terms of selection of mutant cells, it was necessary to assume that there was a proportion of about one D-arabinose - using mutant cell per 10^7 normal cells.

This contrasts with the adaptation postulate, that the ability to grow in the new substrate is not confined to a small proportion of mutant cells, but is developed by all of the cells during the long lag phase, in response to the presence of the new substrate.

Similar considerations apply to the other examples studied, in which there was a long lag phase before growth in a new medium.

All the examples were investigated by examining the growth of inocula containing small numbers of cells in the new medium, and in a suitable control medium. Experiments were carried out with both solid and liquid media.

For Bact. lactis aerogenes and D-arabinose, with both solid and liquid media, for Bact. coli mutabile and lactose,

with solid media, and for a coli - aerogenes intermediate and sodium citrate, with liquid media, it was found that all the cells of the strain were able to grow on the new substrate after the appropriate long lag phase.

By adding sodium glutamate solution, it was proved that cells of Bact. coli mutabile which had failed to grow in liquid lactose medium had died off quite rapidly, and that this mischance, rather than their genetic character, was probably responsible for their failure to grow.

In these three examples, it was clear that all cells of the strains were able to grow in the new substrates after a long lag phase, unless death overtook them before the lag phase was completed.

It is difficult to escape the inference that the long lag phase represented a necessary period of reorganisation of the cells, in response to the presence of the new substrate, before they were able to begin active growth in the new substrate. There was certainly no evidence in favour of the theory that the only cells able to grow in the new substrate were mutants, genetically different from the 'normal' cells of the strain.

The times at which individual colonies appeared on the new medium were very similar in the case of Bact. lactis aerogenes and D-arabinose. This was also true in the case of Bact. coli mutabile and lactose.

Nor did the pattern of behaviour of Bact. coli on media containing ammonium sulphate as the source of nitrogen

conform to a mechanism of selection of mutant cells. It appeared that traces of more complex nitrogen compounds were essential for growth. The failure of many cells to grow was probably due to chance deficiency in this respect; they also were proved to die off quite rapidly.

PART II.

TRAINING AND DE-TRAINING PHENOMENA AND THE LONG LAG PHASE.

The behaviour of Bact. lactis aerogenes when first transferred to various new media has been described in the Introduction.

This species grows very readily in synthetic media. In Part I, the results have been reported of dilution tests on a number of examples in which bacterial species grew in a new medium only after a long lag phase. Bact. coli mutabile, when first transferred to lactose medium, Bact. coli, when first transferred to ammonium sulphate medium, and a coli - aerogenes intermediate strain, when first transferred to citrate medium, did not grow until after a long lag. Bact. lactis aerogenes, however, grows in all of these media without any comparable delay. But there is a similar long delay before Bact. lactis aerogenes begins to grow when first transferred to D-arabinose or succinate medium.

In almost all these cases, the rate of growth is increased after serial sub-culture in the new medium, and, in the examples when the first lag phase is long, growth occurs in the second sub-culture after only a short delay, as in an accustomed medium.

It is clear that, when the nutrient medium is changed, changes in bacteria are required before growth can occur with maximum efficiency. In some cases, there is a delay before growth occurs at all. Presumably, in these cases, rather

serious changes are required before the new substrate can be metabolised.

The theory of mutation and natural selection suggests that such changes occur sharply, by chance, in a very small proportion of the cells, while the adaptation theory postulates gradual adaptive change in the whole cell population.

The dilution tests of Part I have supplied important evidence on the proportion of cells in some bacterial cultures which are able to grow on new nutrients. Study of the lag phase under various conditions, and the behaviour of cells which have grown in the new substrate, or been exposed to it, may be expected to throw more light on the way in which changes take place in the cells.

This section incorporates the results of a detailed study of the behaviour of Bact. lactis aerogenes with D-arabinose, and some observations on the behaviour of Bact. coli mutabile and lactose.

Bact. lactis aerogenes and D-arabinose.

The reproducibility of the standard lag test.

The careful control of lag tests throughout this work has been emphasised. Significant results can only be obtained by such careful methods.

All tests of the strain of Bact. lactis aerogenes were made from parent cultures in their most active state of growth. They were always controlled by making parallel tests in glucose-ammonium sulphate medium, and were not regarded as significant unless growth commenced in this medium within 2 or 3 hours.

The strain was maintained in serial sub-culture in glucose - ammonium sulphate medium each Term. Frequent tests were made of the lag in D-arabinose medium of cells grown in these series. As already described, the parent cultures for these tests were grown in dilute glucose medium, so that there was no significant transfer of glucose in the inoculum.

The standard concentration of D-arabinose in the D-arabinose medium was 1·9 gm/litre. The growth characteristics were unchanged in higher concentrations of D-arabinose.

In discussing the results in Table II (Part I) it was remarked that, as the number of cells in the inoculum was reduced, the lag in D-arabinose medium, as in glucose medium, increased. Presumably, in each case, growth occurs when certain intermediate metabolites have reached a sufficiently high concentration. A supply of such compounds is carried over in the standard inoculum from a culture at the optimum stage of growth for lag tests, but, during earlier stages of growth,

less is carried over, or, if the culture is diluted, very little is available in the daughter cultures inoculated from the diluted suspensions (see the discussion of 'early lag' in the Introduction)[3]. This factor might still be important in affecting the lag of larger inocula - inocula larger than the standard 0·2 ml. inoculum might grow earlier.

The growth of larger inocula in D-arabinose medium was tested to confirm the suitability of the 0·2 ml. inoculum as the standard. It will be seen from the results in Table IX that there is a sufficient supply of intermediate metabolites carried over in the 0·2 ml. inoculum to make this inoculum show the reproducible, minimum length of the lag phase in D-arabinose medium.

Using the standard inoculum, the lag of Bact. lactis aerogenes in D-arabinose medium has been consistently observed to be about 20 - 30 hours for over two years. The mean generation time , when growth occurs, is about 75 minutes.

The reproducibility of the length of the lag phase in D-arabinose medium is emphasised by the results listed in Table X. In the experiment, 0·2 ml. inocula were transferred from the parent culture, in dilute glucose medium, at various stages of growth. Tests were made in one tube of glucose medium and two tubes of D-arabinose medium, in parallel, at each stage. Mean values are given for the lag in D-arabinose medium - the difference between the lags in the two parallel tubes at each stage was never more than 4 hours.

Over a period of almost 2 days, the lag in D-arabinose

TABLE IX.

Inoculum Size and Lag in D-arabinose.

Inoculum Size. (ml)	Approximate Number of Cells in Inoculum.	Lag in Glucose Medium.	Lag in D-arabinose Medium.(hours)
0·04	2×10^7	0	28·5
0·1	5×10^7	0	26
0·2	10^8	0	23·5
0·5	$2{\cdot}5 \times 10^8$	0	24·5
1·0	5×10^8	0	26

TABLE X.

The Lag of Bact. lactis aerogenes in D-arabinose.

Tests at different Stages of Growth of the

Parent Culture, in Dilute Glucose Medium.

Age of Parent Culture at Time of Test. (hours)	Cells/ml in Parent Culture at Time of Test.	Lag in Glucose Medium. (hours)	Lag in D-arabinose Medium. (hours)
3	47×10^6	8	19
4	162×10^6	5	23
4·5	270×10^6	3	18
5	355×10^6	0	19
7	550×10^6	0	20
8·5	705×10^6	<10	23
11·5	790×10^6	< 6	23
22	830×10^6	< 3·5	24
34	840×10^6	< 7·5	24
45	850×10^6	3·5	24

of 0·2 ml. inocula transferred from this culture was very steady at about 20 hours. It will be noticed that the lag in glucose medium was only 3·5 hours when the age of the parent culture was 45 hours. This is not consistent with the account of lag-age relationships given in the Introduction. That account referred to observations made when using the standard glucose-ammonium sulphate medium, in which growth was arrested by accumulation of acid by-products and breakdown of the buffering capacity of the medium. Under such conditions, 'late lag' may increase rapidly. But, in the dilute glucose medium used for this experiment, as always, for the parent cultures tested in D-arabinose, growth is arrested by exhaustion of glucose , and 'late lag' develops very slowly.

In a few early experiments, much higher values for the lag phase and mean generation time were observed. Lag phases of 100 to 200 hours were followed by slow growth, corresponding to a mean generation time of as much as 400 minutes. Growth at the normal rate (mean generation time 75 minutes) followed a lag phase of about 60 hours, in other experiments.

This behaviour, never again observed during the course of over 2 years, during which the experiments described in this dissertation were carried out, must be regarded, in the light of the later, consistent behaviour, as abnormal.

Growth in D-arabinose after the first sub-culture.

After cells of Bact. lactis aerogenes have grown in D-arabinose medium in the first sub-culture, following the necessary long lag phase, they grow in later sub-cultures without any particularly long lag phase.

When the first sub-culture in D-arabinose medium has reached the optimum stage of growth, a standard inoculum, transferred to a second tube of D-arabinose medium, grows after a lag phase of not more than 3 hours. This behaviour is maintained in later sub-cultures. The growth rate, however, does not increase beyond that initially observed, corresponding to a mean generation time of about 75 minutes, even after 55 sub-cultures in D-arabinose medium; there is considerable fluctuation in the growth rate, but no regular increase.

Koser and Vaughan[6] showed that the ability of 'trained' cells of Bact. lactis aerogenes to ferment D-arabinose medium was a property of the cells themselves, not of diffusible substances which could be released into the medium. In their experiments, cultures were grown in broth to which D-arabinose was added. The D-arabinose was not fermented for several days. If the cells were separated from a culture, after fermentation had occurred, by centrifuging and washing, they would ferment the D-arabinose in a new sample of medium almost immediately. Further, the supernatant liquid did not accelerate the fermentation of D-arabinose by cells which had not been in contact with the sugar before.

It has been suggested that the inheritance of the ability

of 'trained' cells to grow in D-arabinose is evidence that the characteristic cannot be an acquired one, and that it must have occurred by mutation. But bacteria differ from higher forms of life in being unicellular - the whole organism is the germ cell. Adaptive changes in the neck of a giraffe would be unlikely to affect the remote and inaccessible germ cells, and would, therefore, probably not be inherited. On the other hand, adaptive changes in bacteria would be, ipso facto, changes in the germ cells. The fact that changes in bacteria are inherited is irrelevant to the question of whether they occur by mutation or adaptation.

The growth in D-arabinose of cells cultured in various sources of carbon.

The consistent length of lag in D-arabinose of Bact. lactis aerogenes cultured in a series of tubes of glucose - ammonium sulphate medium has been described.

Experiments were carried out to determine whether cells grown in similar series in media containing other carbon sources would show similar behaviour, or whether the lag of the strain in its first sub-culture in D-arabinose medium was dependent on previous nutrition.

In making up the tubes of these media, 10 ml. of a solution of the carbon source, 20 gm/l, was added to the usual quantities of magnesium sulphate, phosphate buffer (pH 7·12) and ammonium sulphate solutions.

Tests of the lag in D-arabinose medium were made at

suitable stages in the series of sub-cultures in the various media. The series were maintained by making sub-cultures once or twice daily. Tests were always made at the optimum stage of growth of the parent culture, the carbon content of the parent medium being almost exhausted when the most active state of the culture was reached.

The carbon sources used in these series were glycerol, lactose, maltose, cellobiose, sucrose, L-arabinose, mannose, xylose, galactose, glucose, acetate, fumarate and succinate. The strain of Bact. lactis aerogenes grew readily in all these sources of carbon after a few sub-cultures, and each test of the lag in D-arabinose was controlled by making a parallel inoculation into a tube of medium similar to that used for the parent culture. The series was thus continued in the control culture. If the lag in a control culture was greater than 3 hours, the result was rejected, and the test repeated.

One set of serial sub-cultures was maintained for six weeks, the results being recorded in Table XI Table XII contains the results of another set, which were maintained for eleven weeks. In each sub-culture, the all population multiplied by about 10^2 to 10^3, passing through about 7 generations.

These results make it clear that the lag of Bact. lactis aerogenes in D-arabinose medium is not dependent on its nutritional history. When tested at considerably separated stages in series of sub-cultures in very various media, the lag was very closely similar in all cases.

A lag phase of about 30 hours, in D-arabinose medium,

TABLE XI.

Lag in D-arabinose Medium and Culture History.

Number of sub-cultures, n	Lag in D-arabinose Medium (hours) after n sub-cultures in:								
	Glycerol	Lactose	Maltose	Cellobiose	Sucrose	L-arabinose	Mannose	Xylose	Galactose
4	31	28	36	30	29	30	29	30	29
6	38	34	35	33	34	38	30	38	30
8	38	36	36	36	38	36	36	36	36
10	35	35	35	35	38	35	35	35	35
20	33	33	33	38	33	33	33	33	33
29	35	31	29	34	32	31	32	33	29
35	38	30	35		38	38	33	32	33
41	30	30	30		30	30	30	35	35
52	38	31	22		33	24	25	24	24

TABLE XII.

Lag in D-arabinose and Culture History.

Number of Sub-cultures, n	Lag in D-arabinose (hours) after n Sub-cultures in :-				
	Glucose	Acetate	Fumarate	Glycerol	Succinate
4	21	28	22	24	22
9	19	33	33	36	24
16	31	28	28	38	30
24	22	34	30	37	27
31	24	36	33	38	29
45	34	24		27	24
51	27	24		24	25
58	34	28		24	28
69	25	35		25	28
74	29	35		22	

was characteristic of the strain of Bact. lactis aerogenes.

If it be supposed that this is due to masking of the growth of a few mutant cells by the initially preponderant 'normal' cells, the remarkable reproducibility of the length of the lag phase demands a correspondingly consistent proportion of mutant cells in the strain. There must, therefore, either be an extremely slow mutation rate, such that the proportion of mutants did not change significantly during the course of 74 sub-cultures (see Table XII), or the proportion of mutants must be maintained by dynamic equilibrium between mutation and back-mutation.

The former condition would demand an extremely slow mutation rate, as, during the course of 74 sub-cultures, there are about 10^{150} cell divisions. It is possible to decide whether this postulate is plausible by plating-out the strain, and testing the lag in D-arabinose of cultures grown by inoculating single colonies into dilute glucose medium.

Tests of cultures grown from single colonies.

Two possible mechanisms have just been suggested to account for the presence of a constant, minute proportion of about 10^{-7} D-arabinose - using mutants in the strain of Bact. lactis aerogenes.

If there were a very slow mutation rate, it is possible that the proportion of mutants would not change appreciably during 74 sub-cultures in synthetic medium containing various sources of carbon. In this case, only 1 in 10^7 cells of the strain, at any stage, would be able to grow in D-arabinose

medium. If the strain were plated out, and cultures grown from individual colonies, it would be expected that they would be incapable of growth in D-arabinose medium, even after a long lag phase. The chance of selecting a colony sprung from a mutant cell would be negligible.

On the other hand, if the proportion of mutants were maintained at a constant level by dynamic equilibrium between mutation and back-mutation, there is no theoretical way of predicting the respective rates. In plating out the strain, individual cells would be separated on the plate. Almost all of them would be 'normal' cells. During growth to form a visible colony, however, some mutations might occur; depending on the rates of mutation and back-mutation, more or less progress would be made towards restoring the equilibrium. If the colony were transferred to liquid medium, in order to grow a culture suitable for testing in D-arabinose medium, there would be the possibility of further progress towards the restoration of equilibrium. The lag in D-arabinose medium of inocula from such cultures would be expected to be equal to, or greater than, that of the unplated strain, depending on whether equilibrium had or had not been re-established.

In the first plating experiments made with these considerations in mind, no attempt was made to use conditions minimising the possibility of a return to equilibrium. The aim of the experiments was to determine whether cultures recently sprung from single colonies would grow in D-arabinose, or not. Failure to grow would be an indication that the mutation rate

was very low, and that the apparently constant proportion of mutants was, in fact, nearly constant because of the very slow change; growth would indicate that mutation was more rapid, and that the constant proportion was maintained in balance with back-mutation. Although the results have already been published[6], further comment is desirable.

In these experiments, the glucose - cultivated strain was plated out on glucose - ammonium sulphate agar. Colonies were taken from the plates, grown in broth, and then in dilute glucose liquid medium, from which the lag in D-arabinose was measured as usual.

Some of the tests showed lags greater than 50 hours. It was thought that this might be due to the culture history. Passage through broth only did not affect the lag in D-arabinose. The effect of growth on the agar media was further investigated.

Very large inocula were placed on the plates, so that growth was confluent. Samples of the film of growth were removed with a sterile platinum loop. They were then examined in the same way as the single colonies had been, by growing them in broth, sub-culturing into dilute glucose medium, and testing in D-arabinose medium.

Once again, some of the tests showed lags greater than 50 hours. This proved that the explanation of the longer lags was not that colonies sprung from abnormal cells had been selected; there was no question of growing sub-strains, with different lags in D-arabinose, from the different colonies. Growth on agar had resulted in a ~~general~~ random increase in the length

of the lag phase.

Abnormal lags of this type have occasionally been observed in ordinary tests. The plating experiments were continued in an attempt to elucidate the phenomenon.

It was thought possible that, if there were a reproducible effect on the lag in D-arabinose caused by plating out on glucose - agar, the most likely agents operating in this effect were either some compound or compounds present in the agar-agar, or the acid conditions of growth on plates which had no buffering capacity. (No phosphate buffer had been included in the agar medium used in these experiments).

The tests were therefore repeated, with a number of elaborations. The glucose - cultivated strain was inoculated, at maximum activity, to plates with and without buffer, to dilute glucose liquid medium with and without 0·2% agar (so that it was just liquid at 40·0°) and to dilute glucose liquid medium buffered to pH 5·5, instead of pH 7·12, with a different phosphate buffer solution.

After 37 hours, single colonies or small inocula were taken from these cultures and inoculated into dilute glucose medium, pH 7·12. From this, the lag in D-arabinose medium was tested.

The results are given in Table XIII.

It seems that the first observations, which showed a fluctuation in the length of the lag after culture on agar medium, were made when the strain was temporarily susceptible to some environmental factor which is normally without effect.

TABLE XIII.

Effects of Culture in Agar and Acid Media.

Culture medium for 37 hours.	Lag in D-arabinose (hours) (Tested from standard dilute glucose liquid medium) pH 7·12
Dilute glucose liquid medium. pH 7·12	27
Dilute glucose liquid medium. pH 5·5	29 *
Dilute glucose liquid medium and 0·2% agar pH 7·12	26
Glucose - ammonium sulphate solid medium (2% agar) pH 7·12	Colony 1 38 " 2 32 " 3 32
Glucose - ammonium sulphate solid medium (2% agar) Unbuffered	Colony 4 28 " 5 32 " 6 29

* Lag in D-arabinose 35 hours when test was made from a second tube of dilute glucose liquid medium, pH 5·5.

The later experiments showed that cells sprung from single colonies of Bact. lactis aerogenes, when tested in D-arabinose medium, grew after the normal lag phase of about 30 hours.

The origin of a single colony is a single cell or, at most, a very small number of cells. In the experiment just described, single colonies were inoculated into dilute glucose-ammonium sulphate liquid medium, and the lag in D-arabinose was tested when the most active stage of growth was reached.

At this stage, such a culture contains about 10^{10} cells. If the lag in D-arabinose is assumed to be due to masking of the early growth of a minute proportion of D-arabinose - using mutants, this proportion must be an equilibrium proportion, maintained by balance between mutation and back-mutation. The rates of these processes must be such that equilibrium is established during growth from one cell to 10^{10} cells. Since the equilibrium proportion of mutant cells must be about 1 per 10^{7} normal cells, it appears that the rate of back-mutation must be considerable.

According to the theory, only D-arabinose - using mutants are able to grow in D-arabinose medium; the normal cells which are produced by back-mutation are incapable of growth. (The rate of growth experimentally observed in D-arabinose medium is the net rate of growth, not the growth - rate of the D-arabinose mutants themselves, which is greater than this. In calculating the proportion of mutants in the equilibrium strain, in Part I, the observed growth rate was used, so that

the calculation is valid.)

On the other hand, in glucose medium, mutant and normal cells are able to grow equally well. As the back-mutation rate must be very much greater than the forward mutation rate, it is to be expected that a selected strain of mutants should undergo fairly rapid transition to the equilibrium strain during culture in glucose medium.

It is, therefore, of great interest to examine the actual behaviour of cells which have been grown in D-arabinose medium when they are subsequently sub-cultured in a series of tubes of glucose medium.

The effect of culture in glucose medium of cells which have been grown in D-arabinose medium.

In the Introduction, an account has been given of the behaviour of Bact. lactis aerogenes when transferred from broth to glucose - ammonium sulphate medium, and when transferred from the latter to other simple synthetic media.

The strain, after passing through a few sub-cultures in glucose - ammonium sulphate medium, grew reliably in the medium; in the course of serial sub-culture, transfers to new tubes of medium, made once or twice daily, led to growth without any very considerable lag phase, and the growth rate was quite stable, corresponding to a mean generation time of about 33 minutes.

On transfer to new media, various phenomena were observed. For example, in glycine medium there was no striking lag phase,

but in the early sub-cultures there were two distinct phases of logarithmic growth; in lactose medium, the initial growth rate corresponded to a mean generation time of about 100 minutes, although, again, there was no striking lag phase; and in D-arabinose medium, there was an initial lag phase of about 20 to 30 hours, followed by growth corresponding to a mean generation time of about 75 minutes.

Further sub-culture in a new medium usually resulted in an increase in the growth rate, i.e. a decrease in the mean generation time. Thus, in lactose medium, the mean generation time decreased progressively until, after about 5 sub-cultures, it was 33 minutes.[5] In D-arabinose medium, no consistent decrease in the mean generation time was observed, even after 55 sub-cultures in the medium. After the first sub-culture, however, the long lag phase was no longer observed in D-arabinose medium.

As was suggested in the concluding remarks of the last section, the behaviour in glucose medium of cells which have been grown in D-arabinose medium may be expected to throw some light on the mechanism by which growth in D-arabinose medium is achieved.

The usual pattern of events when cells which have been grown in a new medium are returned to glucose medium may be illustrated by a description of the behaviour of cells which have been grown in lactose.[5]

Cells which have passed through 5 sub-cultures in lactose medium grow with a mean generation time of 33 minutes. If they

are then sub-cultured in glucose medium, the 'lactose training' is rapidly destroyed, as shown by testing again in lactose medium. Thus, if returned to lactose medium after 5 sub-cultures in glucose medium, they grow at a rate corresponding to a mean generation time of about 100 minutes, which is the same behaviour as that of cells which have never been cultured in lactose at all.

On the other hand, if the cells are grown for 30 sub-cultures in lactose medium, and then sub-cultured in glucose medium, the ability to grow rapidly in lactose is retained during the first four or five passages through glucose medium. Thereafter, however, the 'training' is lost rather abruptly, the mean generation time in lactose increasing to nearly 80 minutes. But even more prolonged sub-culture in lactose medium makes the 'training' very stable. Thus, after 68 passages through lactose medium, the following observations were made. In the first place, the strain was sub-cultured 41 times in glucose medium, and then tested in lactose medium; the mean generation time was 30 minutes. In the second instance, the mean generation time in lactose medium was 27 minutes after 51 passages through glucose medium, and one through glucose medium containing proflavine. Finally, 41 passages through glucose medium and 5 through glycerol medium yielded cells which, when tested in lactose medium, grew with a mean generation time of 37 minutes.

Most of the observations made in this laboratory have been in accord with this general pattern - a few passages

through a new medium leading to unstable 'training' which was rapidly destroyed by growth in glucose medium; further sub-culture conferring 'training' of only temporary stability when the strain was transferred to glucose medium; and really prolonged sub-culture in a new medium conferring highly stable 'training' which was neither destroyed by culture in glucose medium, nor by more drastic treatment, such as exposure to drugs.

The media used in all these experiments were such that, in each sub-culture, there was a multiplication in cell population of about 10^2 to 10^3, i.e. the population passed through about 7 generations.

The behaviour of Bact. lactis aerogenes in D-arabinose was examined in a similar way. The series was maintained in serial sub-culture in D-arabinose medium, and, at suitable stages, transfers were made to glucose medium. Frequent tests of the length of the lag in D-arabinose medium were made from the series of sub-cultures in glucose medium.

Cells cultivated in D-arabinose medium, when transferred to glucose medium, grew at the same rate as cells which had never been grown in D-arabinose, with a mean generation time of about 33 minutes.

Lag tests were made in the usual way, taking a 0·2 ml. inoculum of a culture at the optimum stage of growth in dilute glucose medium.

The results of two series of experiments are recorded in Table XIV, and are graphically represented in Fig. II.

In both these series of experiments, the lag in D-arabinose

TABLE XIV.

The Lag in D-arabinose Medium of Cells cultivated in Glucose Medium after having been grown in D-arabinose Medium.

Cells tested after one passage through D-arabinose medium and N passages through glucose medium:-

N	Lag in D-arabinose Medium. (hours)	Lag in Glucose Medium. (hours)	Δ lag (D-arabinose lag - Glucose lag) (hours)
1	3	2·5	0·5
4	3	2·5	0·5
10	4	2	2
15	6	1·5	4·5
19	8	1	7
26	7	1	6
35	8	2·5	5·5
41	6	0	6
45	5·5	1	4·5
53	5·5	1	4·5

Cells tested after 50 passages through D-arabinose medium and M passages through glucose medium:-

M	Lag in D-arabinose Medium. (hours)	Lag in Glucose Medium. (hours)	Δ lag (D-arabinose lag - Glucose lag) (hours)
0	3	1	2
3	5·5	1	4·5
8	5	1	4
10	5	1·5	3·5
15	6	1	5
23	6	2	4
28	5	3	2
36	6	1	5
42	8	1·5	6·5
51	7·5	1	6·5

medium increased to about 5 hours after a few sub-cultures in glucose medium, and thereafter, apart from slight fluctuations, did not increase further, even after 50 sub-cultures.

It is of great interest to compare these results with that predicted if mutamt selection is presumed to be the mechanism of growth in D-arabinose medium.

The observations reported in the last section showed that, if mutant selection is the mechanism, then there is a constant proportion of mutants in the strain, and this proportion is established during growth from a single cell to about 10^{10} cells. It must therefore be an equilibrium proportion, maintained by balance between mutation and back-mutation.

If the chance of formation of a mutant cell from normal cells is μ per cell-division, and the chance of formation of a normal cell by back-mutation from mutant cells is μ' per cell-division, the following equations hold

Let the number of normal cells be n, and the number of mutant cells be m, then

$$\frac{dm}{dt} = \mu kn + km - \mu' km$$

$$\frac{dn}{dt} = kn + \mu' km - \mu kn$$

where k is the growth constant.

Let $R = \frac{m}{n}$

$$\frac{dR}{dt} = \frac{1}{n}\frac{dm}{dt} - \frac{m}{n^2}\frac{dn}{dt}$$

$$= \mu k + \frac{k\mu}{n} - \mu' \frac{k\mu}{n} - \frac{k\mu}{n} - \mu' \frac{k\mu^2}{n^2} + \mu \frac{k\mu}{n} = \mu k - \mu' kR - \mu' kR^2 + \mu kR$$

$$= \mu k \left[1 - \left(\frac{\mu'}{\mu} - 1 \right) R - \frac{\mu'}{\mu} R^2 \right] \qquad \text{(1)}$$

Put $\frac{\mu'}{\mu} = \beta$, when

$$\frac{dR}{dt} = \mu k \left[1 - (\beta - 1)R - \beta R^2 \right] \qquad \text{(1a)}$$

At equilibrium, $\frac{dR}{dt} = 0$

$$1 - (\beta - 1)R_{eq.} - \beta R_{eq.}^2 = 0 \qquad \text{(2)}$$

As $R_{eq.}$ is small, $1 - \beta R_{eq.} \sim 0$

$$R_{eq.} \sim \frac{1}{\beta}$$

It has been shown in Part I that, at equilibrium, i.e. the normal state of the strain in glucose medium,

$$\frac{n}{m} \sim 10^7$$

$$\therefore R_{eq.} = \frac{m}{n} \sim 10^{-7}$$

and $\beta \sim 10^7$

Now, when the strain was plated out on glucose - ammonium sulphate - agar medium, the single cells thus separated were, in such a majority, (10^7 to 1) normal cells. During their growth to form a colony, and then a culture in dilute glucose medium suitable for a lag test, they multiplied to about 10^{10} cells in each case. When these cultures were tested in D-arabinose medium, the lag was equal to that observed for the unplated strain. In these cultures, the value of R must be at least $\frac{R_{eq.}}{2}$.

For the establishment of equilibrium under these conditions, starting from a single normal cell, equation (1a) may be used in the form

$$\frac{dR}{dt} = \mu k (1 - \beta R) \quad \text{---------- (3)}$$

since R^2 is negligible compared with R, and 1 compared with β.

Integration of (3), with the condition that R=0 when t = 0, gives

$$R = \frac{1}{\beta}\left(1 - e^{-\beta\mu kt}\right)$$

when $R \sim \frac{R_{eq.}}{2} \sim \frac{0.5}{\beta}$

$$kt = \ln\left(\frac{N}{N_0}\right) \sim \ln (10^{10}) \sim 23$$

$$\therefore \frac{0.5}{\beta} = \frac{1}{\beta}\left(1 - e^{-23\beta\mu}\right)$$

whence $e^{-23\beta\mu} = 0.5$

and $\beta\mu = 0.03 = \mu'$

This is a minimum value, since equilibrium may have been attained earlier, i.e. k.t may have been less.

Now, it is postulated that cells which have grown in D-arabinose medium are the D-arabinose mutants, so that, after one sub-culture in D-arabinose medium, $R > 1$, and after many sub-cultures, R approaches ∞. It is possible to calculate the rate at which R decreases during subsequent sub-culture in glucose medium, as follows:-

When $t \to 0$, $R \to \infty$

When $t \to \infty$, $R \to \frac{1}{\beta}$

$$\frac{dR}{dt} = \mu k \left[1 + R (1 - \beta) - \beta R^2\right] \quad \text{---------- (1a)}$$

Under these conditions, we may not simplify the equation.

Re-writing:-

$$\frac{dR}{1 + R(1 - \beta) - \beta R^2} = \mu k dt$$

$$\frac{dR}{\beta R^2 + R(\beta - 1) - 1} = -\mu k dt$$

$$\frac{dR}{R^2 + R\left(1 - \frac{1}{\beta}\right) - \frac{1}{\beta}} = -\beta \mu k dt$$

Integrating, with the condition that $R \to \infty$ as $t \to 0$,

$$\frac{1}{\left[\left(1 - \frac{1}{\beta}\right)^2 + \frac{4}{\beta}\right]^{\frac{1}{2}}} \cdot \ln \left\{ \frac{2R + 1 - \frac{1}{\beta} - \left[\left(1 - \frac{1}{\beta}\right)^2 + \frac{4}{\beta}\right]^{\frac{1}{2}}}{2R + 1 - \frac{1}{\beta} + \left[\left(1 - \frac{1}{\beta}\right)^2 + \frac{4}{\beta}\right]^{\frac{1}{2}}} \right\} = -\beta \mu k t$$

$$= \frac{1}{\left[1 + \frac{2}{\beta} + \frac{1}{\beta^2}\right]^{\frac{1}{2}}} \cdot \ln \left\{ \frac{2R + 1 - \frac{1}{\beta} - \left[1 + \frac{2}{\beta} + \frac{1}{\beta^2}\right]^{\frac{1}{2}}}{2R + 1 - \frac{1}{\beta} + \left[1 + \frac{2}{\beta} + \frac{1}{\beta^2}\right]^{\frac{1}{2}}} \right\}$$

$$= \frac{1}{\left[1 + \frac{1}{\beta}\right]} \cdot \ln \left\{ \frac{2R + 1 - \frac{1}{\beta} - 1 - \frac{1}{\beta}}{2R + 1 - \frac{1}{\beta} + 1 + \frac{1}{\beta}} \right\}$$

$$= \frac{\beta}{\beta + 1} \cdot \ln \left\{ \frac{2R - \frac{2}{\beta}}{2R + 2} \right\} = \frac{\beta}{\beta + 1} \cdot \ln \left\{ \frac{R - \frac{1}{\beta}}{R + 1} \right\}$$

$$\left(\text{If } R_o \sim 100, \text{ Integration constant} = -\ln \left\{ \frac{100}{101} \right\} \right)$$

Now $\beta >> 1 \quad \therefore \frac{\beta}{\beta + 1} \sim 1$

$$\therefore -\beta \mu k t \sim \ln \left\{ \frac{R - \frac{1}{\beta}}{R + 1} \right\}$$

$$e^{-\beta \mu k t} \sim \frac{R - \frac{1}{\beta}}{R + 1}$$

$$R \sim \frac{e^{-\beta \mu k t} + \frac{1}{\beta}}{1 - e^{-\beta \mu k t}} \qquad \text{---------- (5)}$$

kt is conveniently expressed in terms of the number of sub-cultures in glucose

$$kt = \ln\left(\frac{N}{N_o}\right)$$

In each sub-culture, the multiplication factor is about 10^2. For S sub-cultures, it is 10^{2S}.

$$kt = \ln(10^{2S}) = 2S\ln 10 = 4{\cdot}6S$$

$\beta\mu$ has been shown $> 0{\cdot}03$, $\beta\mu kt > 0{\cdot}14S$

$$R \sim \frac{e^{-0.14S} + \frac{1}{\beta}}{1 - e^{-0.14S}} \qquad \text{---------- (5a)}$$

$$R \sim \frac{e^{-0.14S} + 10^{-7}}{1 - e^{-0.14S}}$$

If the strain is tested in D-arabinose, initially

$$R_o = \frac{m_o}{n_o} \qquad m_o = R_o n_o$$

at the end of the lag phase, $m \sim n_o$

(This is when $R_o < 1$; if $R_o > 1$, lag is zero)

$$\ln\left(\frac{m}{m_o}\right) = k_A L \quad \text{i.e.} \quad \ln\left(\frac{n_o}{R_o n_o}\right) = k_A L$$

$$\therefore \underline{-\ln R_o = k_A L}$$

$$\ln 2 = 1{\cdot}25 k_A \qquad k_A = \frac{0{\cdot}69}{1{\cdot}25} = 0{\cdot}552$$

$$\therefore L = -\frac{\ln R_o}{{\cdot}552} = -\frac{\ln\left(\frac{e^{-0.14S} + 10^{-7}}{1 - e^{-0.14S}}\right)}{{\cdot}552} = -\frac{\log_{10}\left(\frac{10^{-0.06S} + 10^{-7}}{1 - 10^{-0.06S}}\right)}{0{\cdot}24}$$

This relationship between L and S may be used to calculate the values listed in Table XV. They are plotted in Figure II for comparison with the experimental results.

TABLE XV.

De-training according to the Theory of Mutation and Back-mutation.

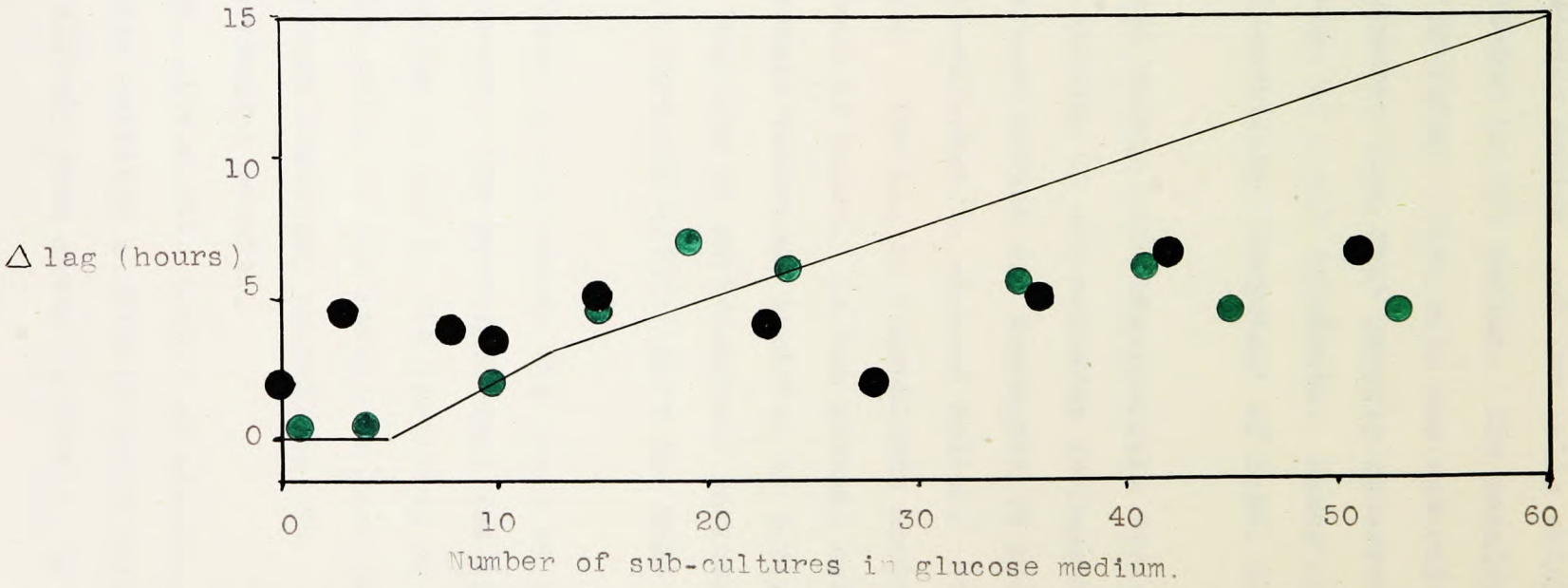
S	$10^{-0.06S}$	$\left(\frac{10^{-0.06S} + 10^{-7}}{1 - 10^{-0.06S}}\right)$	$\log_{10}$(ditto)	L (hours)
0	1	1	0	0
5	0·5012	1	0	0
10	0·2512	0·335	$\bar{1}$·525 = - 0·475	2·0
20	0·0631	0·067	$\bar{2}$·826 = - 1·174	4·9
30	0·0158	0·016	$\bar{2}$·204 = - 1·796	7·5
40	0·0040	0·004	$\bar{3}$·602 = - 2·398	10·0
50	10^{-3}	10^{-3}	$\bar{3}$·0 = - 3	12·5
60	0·00025	0·00025	$\bar{4}$·4 = - 3·6	15·0

It is seen that the theory is not in accord with the experimental facts. According to the theory, the lag in D-arabinose medium of 'trained' cells which are sub-cultured in glucose medium should increase gradually as the series of sub-cultures in glucose medium is continued. In fact, there was initially a more rapid increase than that predicted, until the lag reached a value of about 5 hours; thereafter, there was no further consistent increase in the lag throughout the experiments, which were carried on for about 50 sub-cultures in glucose medium.

FIGURE II.

The Effect of Sub-culture in Glucose Medium on Cells of Bact. lactis aerogenes which have been Grown in D-arabinose Medium.

The line shows the increase in lag in D-arabinose medium predicted from the mutation theory. The circles are plotted from experimental results. Comparable lags are obtained by adjusting to zero lag in glucose medium. (Δlag=lag in D-arabinose medium-lag in glucose medium).



Green circles: cells previously grown for one sub-culture in D-arabinose medium. Black circles: cells previously grown for 30 sub-cultures in D-arabinose medium.

This was true both for cells which had previously had 30 passages through D-arabinose medium, and for cells which had only once been sub-cultured in the medium. The considerable stability of D-arabinose 'training' after only one sub-culture in D-arabinose medium is greater than that usually observed after such a short experience of a new substrate. Koser and Vaughan[6] also report that D-arabinose 'training' of Bact. lactis aerogenes is very stable.

Some early experiments showed more considerable 'de-training' of cells which had passed through 17 sub-cultures in D-arabinose medium. The lag in D-arabinose medium was increased to about 25 hours after about 25 sub-cultures in glucose medium. However, before D-arabinose 'training', the lag in D-arabinose medium had been observed to be about 60 hours. As has already been mentioned, the somewhat erratic behaviour observed in early experiments seems to have been due to environmental influences which were either absent or were not effective in the main body of experiments.

These early observations do not weaken the force of those in the main body of experiments. The postulate that the long lag phase in D-arabinose medium is due to the inability of all but a minute fraction of the cells in the strain to grow in this medium, with the necessary auxiliary postulates, is incompatible with the experimental results.

On the other hand, the alternative theory of adaptive response to environment makes possible a unified account of all the observations. As has already been shown in Part I, in three

cases, growth in a new substrate is achieved by at least the vast majority of the cells in a strain of bacteria, a fact which is in accord with the adaptation postulate that the ability to grow in a new substrate is developed by each cell in response to the presence of the new substrate. The adaptation process presumably consists, like the process of bacterial growth, in a series of linked chemical reactions, in which enzymes play a very important part. Perhaps the critical factor is the synthesis of some new enzyme pattern.

The reproducibility of the length of the lag phase of Bact. lactis aerogenes in D-arabinose medium is a reflection of the reproducibility of the rates of the reactions involved in adaptation.

The phenomena of 'de-training' may be accounted for in similar terms. The stability of a new enzyme pattern during growth in glucose medium is not predictable. In some cases, it may be considerable, but in others, the new pattern may be eliminated during the course of only a few generations in glucose medium. It might be expected that the new structure would become more firmly established during further cultivation in the new medium. It appears that, in the 'training' of Bact. lactis aerogenes to use D-arabinose, the new structure may be very firmly established after only one passage through D-arabinose medium.

The scope of the adaptation theory is further seen in the account it can give of other phenomena in the growth of Bact. lactis aerogenes in D-arabinose.

Interruptions of the Long Lag Phase.

The reproducibility of the length of the lag phase of Bact. lactis aerogenes in D-arabinose medium is also observed if the lag phase is interrupted.[6]

This was proved as follows. A test in D-arabinose medium was begun in the usual way. After only part of the lag phase had elapsed, glucose or L-arabinose was added. Growth on the glucose or L-arabinose began almost immediately. When the optimum stage of growth was reached, a standard inoculum was transferred to a new tube of D-arabinose medium. The tests, were, of course, performed in duplicate, with the usual controls in glucose medium. It was found that growth occurred in the second test in D-arabinose medium after a shorter lag phase than that normally required; the lag was reduced by a time equal to the time spent in D-arabinose medium before glucose or L-arabinose was added. Whether the addition of glucose or L-arabinose was made early or late in the lag phase, this relationship was always found to hold.

This supplies further evidence that the change taking place was a change in the cell structure. If the process had consisted in the accumulation of a diffusible substance until a critical, threshold concentration was reached, the total time in the two parts of the lag phase would have been greater than the duration of the uninterrupted lag phase. The diffusible substance would have been considerably diluted in making the transfer to the second tube of D-arabinose medium, so that its accumulation would have been resumed from a lower concentration

than that attained when glucose or L-arabinose was added.

According to the adaptation hypothesis, the lag phase is spent in re-organisation of the cell structure in response to the presence of D-arabinose. The fact that the total time spent in the two parts of the lag phase was always equal to the duration of the normal lag phase, irrespective of whether the glucose or L-arabinose was added early or late, shows that, on transfer to the second tube of D-arabinose medium, re-organisation was resumed at the stage which had been attained in the first tube when glucose or L-arabinose was added. It seems that the rate of re-organisation is much the same throughout the lag phase, and that any progress made in re-organisation is retained during growth in glucose or L-arabinose.

Further evidence on this aspect of the problem was supplied by experiments on the effect on Bact. lactis aerogenes of sub-culture in DL-arabinose medium. These experiments were begun in an attempt to induce 'training' to D-arabinose. Tests of the lag in D-arabinose medium were made at different stages of the series of sub-cultures in DL-arabinose medium. It was found that the lag decreased as the series was continued, but examination of the results revealed that the 'training' was not continuous. The lag was reduced by a period equal to the total time spent in contact with D-arabinose when growth was not occurring. The stationary phases and lag phases were equally important in this respect. It appeared that, during growth in L-arabinose, there was no 'training' to D-arabinose,

but that any re-organisation achieved during stationary phases and lag phases was retained.

Similar results were obtained in experiments in which a mixture of glucose and D-arabinose was used in place of DL-arabinose.

Continuous Growth in a Mixture of D-arabinose and Glucose.

In order to confirm that there was no training of Bact. lactis aerogenes during growth in a medium containing a mixture of D-arabinose and glucose, an apparatus was designed to make possible the continuous growth of a culture for a period of days.[6]

By continuously syphoning out some of the culture, which was replaced by new medium running in through a capillary tube, it was possible to completely eliminate lag and stationary phases.

The culture was allowed to grow until it contained about 10^8 cells per ml. Then the flowing process was begun, and, after conditions had become stable, D-arabinose was added to the medium which was running into the culture. In this way, the culture was maintained in continuous growth in D-arabinose - glucose medium for 6 days.

At suitable intervals, samples were withdrawn from the culture and grown to the stage of optimum activity in dilute glucose medium, whence they were tested in D-arabinose medium. There was no change in the length of the lag phase in D-arabinose medium throughout the experiment.

It is clear that 'training' to D-arabinose medium only

occurs during contact with D-arabinose when there is no growth. Re-organisation of the cell structure occurs at much the same rate throughout this lag phase. Any progress made is retained during growth in glucose or L-arabinose, a fact which is in accord with the stability of D-arabinose 'training' which has already been described.

The Effect of Exposure to D-arabinose Medium lacking a Nitrogen Source.

The mutation hypothesis suggests that, before Bact. lactis aerogenes can grow detectably in D-arabinose medium, it is necessary for a very minute proportion of mutant cells to multiply until their number compares with the total number of cells in the inoculum.

An attempt was made to induce 'training' to D-arabinose by exposure of cells to a medium lacking a source of nitrogen, so that growth should not occur.

The medium contained the standard concentrations of magnesium sulphate, D-arabinose and phosphates for buffering, but ammonium sulphate was omitted.

A culture was grown in glucose medium until the optimum stage of growth was reached. The cells were then separated by centrifuging, and were re-suspended in phosphate buffer. They were again separated by centrifuging, and the procedure was repeated twice, in order to move the last traces of medium.

These carefully - washed cells were suspended in the incomplete D-arabinose medium so that there were about 10^8

cells per ml. The cell population was carefully counted, using the microscope.

After suitable intervals, samples were removed, and added to ammonium sulphate solution, so that the medium was completed. The lag was then observed. A careful count of the cell population in the suspension was again made at the time when the sample was transferred.

In some experiments, there was an increase in the cell population during exposure to the incomplete medium; these experiments were rejected. In other experiments, there was no detectable increase in the cell - population. The results of these are shown in Table XVI.

It is seen that, in some cases, there was a considerable reduction in the length of the lag in D-arabinose medium after exposure for a day or more to D-arabinose medium lacking a source of nitrogen. The lag was never reduced to less than 6 hours, and the rate at which D-arabinose 'training' developed was always inferior to the rate in complete D-arabinose medium.

The check of cell population by microscope count is not sufficiently sensitive to absolutely preclude the possibility that the reduction in lag was due to the multiplication of mutant cells, but the development of 'training' in the absence of a source of nitrogen suggests strongly that the process did not require the multiplication of cells.

P.C. Caldwell has shown, in this laboratory, that the organic phosphate content of cells of Bact. lactis aerogenes remains steady during the lag in D-arabinose medium until

TABLE XVI.

The Effect of Exposure to D-arabinose in absence of a Nitrogen Source.

Duration of Exposure to Incomplete D-arabinose Medium. (hours)	Subsequent Lag in Complete D-arabinose Medium. (hours)
0	19·5
24	6
0	28
43·5	6·25
0	27
20·5	22·5
43	27·5
0	20·5
28	12
56	14

shortly before detectable growth, when it begins to increase sharply, at a rate which requires the participation of most of the cells in the culture. These observations are similar to those on the lag phase in glucose medium. It seems clear that none of the cells are ready to grow until near the end of the lag phase. Just before the end of the lag phase, the sharp increase in the organic phosphate content shows that most of the cells are ready to divide.

The results described in the last three sections emphasise the adequacy of the adaptation theory to account for the phenomena of bacterial 'training', in this example. Bact. lactis aerogenes 'trains' to use D-arabinose during the course of a long lag phase in D-arabinose medium. No 'training' occurs during growth in L-arabinose or glucose, when D-arabinose is present. Any progress made in the re-organisation required in 'training' is retained during growth in glucose or L-arabinose. Considerable 'training' can occur during exposure to D-arabinose medium lacking a nitrogen source.

These facts are readily accounted for if the 'training' process is adaptive response to the presence of D-arabinose. Presumably, some new enzyme pattern is synthesised by the cells to permit metabolism of D-arabinose. Synthesis of this new pattern does not occur during growth in glucose or L-arabinose, but progress made in synthesis of the new enzyme pattern is not destroyed during subsequent growth in glucose or L-arabinose, which suggests that some substance, important as an intermediate in both processes, is preferentially consumed during growth.

Bact. coli mutabile and Lactose.

The length of the lag phase and the age of the parent culture.

In all respects, the behaviour of Bact. coli mutabile was less reproducible than that of Bact. lactis aerogenes.

It has already been mentioned that inocula of less than 10^6 cells would not grow reliably in glucose - ammonium sulphate medium, even when the strain had been cultured for some time in this medium.

The failure of some small inocula of Bact. coli mutabile to grow in lactose - ammonium sulphate medium has also been reported and elucidated.

The activity of cells used in tests of the lag in lactose-ammonium sulphate medium very seriously affected the result of the test.

This is illustrated by the results of an experiment in which the lag in lactose medium was tested at different ages of a typical parent culture (Reported in Table XVII).

The fluctuations in the lag of Bact. coli mutabile in lactose were much greater than those in the lag of Bact. lactis aerogenes in D-arabinose, but the pattern of events could be followed by using care in control of experiments and in repeating doubtful experiments. The results of tests made from a series of sub-cultures in glucose medium are shown in Table XVIII .

After the first sub-culture in lactose medium, Bact. coli mutabile grows in the medium without any remarkable lag phase. As sub-culture in the medium is continued, the growth rate increases.

TABLE XVII.

The Lag in Lactose Medium and Age of Parent Culture.

(Bact. coli mutabile)

Age of Parent Culture (from inoculation) (hours)	Approximate Cell Population of Parent Culture. (million cells/ml)	Lag in Glucose Medium. (hours)	Lag in Lactose Medium. (hours) (2 parallel tubes)
4	30	160	>10 days
5	70	3	>10 days
7	600	0	64 and 86
8	1,000	2·5	64 and 64
10	"	9·5	>10 days
11	"	8·5	60

TABLE XVIII.

The Lag in Lactose Medium of Bact. coli mutabile cultured in a Series of Tubes of Glucose Medium.

Number of Sub-cultures in Glucose Medium.	Length of Lag in Lactose Medium. (hours)
9	50
17	50
23	60
29	45
36	42
43	60
47	70
51	65
61	33
64	38

The Growth in Glucose Medium of Cells which have been grown in Lactose Medium.

A much less systematic study was made of the behaviour of 'trained' cells when grown in glucose medium than that already described for Bact. lactis aerogenes.[5]

However, it appears that, after 21 sub-cultures in lactose medium, the 'training' acquired by Bact. coli mutabile is very stable. Table XIX contains the results of lag tests made with cells sub-cultured in glucose medium after 21 passages through lactose medium.

Although 'trained' and 'untrained' cells of Bact. coli mutabile, cultured in glucose medium, behave less reproducibly in lactose medium than do 'trained' and 'untrained' cells of Bact. lactis aerogenes in D-arabinose medium, the general pattern of behaviour appears to be similar in both examples.

Exposure to Lactose Medium lacking a Nitrogen Source.

When Bact. coli mutabile was suspended in lactose medium lacking ammonium sulphate, for periods up to 140 hours, the normal length of lag was observed after the addition of ammonium sulphate. There was thus no 'training' to lactose in the absence of a nitrogen source.

TABLE XIX.

Lag Tests of Cells sub-cultured in Glucose Medium after 21 sub-cultures in Lactose Medium.

Number of Sub-cultures in Glucose Medium.	Lag in Lactose Medium. (hours)	Lag in Glucose Medium. (hours)
4	5·5	2·5
7	4	2
10	2·5	2
16	4	2
20	13	2
24	8	1·5
30	3	1
39	8	2·5
49	9	2
58	10	2·5

Summary of Part II.

It has been shown that the behaviour of Bact. lactis aerogenes in D-arabinose medium is very reproducible.

The mutation theory leads to predictions about the behaviour of cells which have been grown in D-arabinose medium, when sub-cultured in glucose medium, which are at variance with experimental observations. These, along with many other observations, may be readily accounted for in terms of the adaptation theory.

It appears that the 'training' process, which is a structural change within the cells, and probably consists of the synthesis or expansion of a new enzyme pattern, requires the participation of some substance which is also an intermediate in the mechanism of growth in glucose or L-arabinose, and is preferentially used in these processes. 'Training' only occurs when the cells are in contact with D-arabinose, and are not growing. Progress made in 'training' is not affected by subsequent growth in glucose or L-arabinose, even if the cells are not completely 'trained' to grow in D-arabinose.

Some 'training' is achieved in contact with D-arabinose medium lacking a source of nitrogen, but it occurs less completely and more slowly than in complete D-arabinose medium.

Bact. coli mutabile behaves less reproducibly in lactose than does Bact. lactis aerogenes in D-arabinose, but the experiments which have been made suggest that the general pattern of behaviour is similar, although no 'training' occurs in the absence of a source of nitrogen.

The results in Part II, especially taken in conjunction with those of Part I, make it clear that, in these examples, bacterial 'training' takes place by adaptation to the new substrate in response to its presence. The change in the cells required for growth is a structural change, and presumably consists in synthesis of a new enzyme pattern, or modification of existing cell material. The investigation of enzyme changes may be expected to throw more light on the phenomenon. The results of such an investigation supply the material of Part III of this dissertation.

PART III.

'Training' and Dehydrogenase Activity of Bacterial Cells.

Enzymes in Bacterial Cells.

It is well established that the process of bacterial growth consists of a series of linked chemical reactions, in which enzymes play an important part.

Biochemical reactions take place rapidly at comparatively low temperatures. This is attributed to the enzymes of biological tissue. Their action suggests comparison, on the one hand, with high temperature catalytic reactions, for instance, on metallic nickel, and, on the other hand, with the low temperature reactions of radicals.

Enzymes have been isolated from living tissue in varying degrees of purity. It has been shown that they consist essentially of protein, and that they are often associated with prosthetic groups, or co-enzymes, which, however, can be separated from them.

Bacterial cells contain quantities of protein material, which consists partly of enzymatic protein. They also contain considerable amounts of nucleo-protein, the material of which the chromosome nucleus of higher organisms is formed. The importance of chromosomes and their genes in controlling the development and functions of higher organisms is well known. The role of bacterial nucleo-protein is, as yet, obscure.

It has been claimed that the existence of nuclei in bacterial cells has been demonstrated by the use of special staining technique[15], and by other methods, but the evidence is by

no means decisive. Certainly, if nuclei exist, they are much less well defined than in higher organisms. It is probable that the distribution of nucleo-protein in bacterial cells is much more diffuse and much less rigid than in higher organisms, and it seems rash to suppose that there is a similarly strict genetic control in bacteria to that in higher organisms.

When a bacterial cell divides, two similar cells are produced, each with a similar amount of protein. It is clear that, during the course of bacterial growth, there is this much auto-synthesis of enzyme protein. In addition to this, enzyme protein acts catalytically.

The activity of enzymes may be measured in several ways. Enzyme material has been isolated by rather drastic methods, but the treatment may have a serious effect on enzyme activity. A better measure of the enzyme activity under normal cultural conditions may be made by using washed suspensions of cells in a saline or buffer solution. The cells may be separated from culture medium by centrifuging, and are washed by suspending in a suitable solution and centrifuging again, the process being repeated until the cells are sufficiently free from culture medium.

An enzyme reaction which may be conveniently measured is the transfer of hydrogen from a substrate, e.g. carbohydrate, to a suitable hydrogen acceptor, e.g. molecular oxygen. The rate of this reaction may be followed by measuring the rate of consumption of oxygen, using a Warberg micro-manometer.

Monod and Audureau[16] made some measurements, in this way, of the behaviour of Bact. coli mutabile. It was found that the strain consumed oxygen rapidly in the presence of glucose, but at only a very slow rate in the presence of lactose. The strain did not grow in lactose medium until after about two days. Cells which had been grown in lactose medium were found to consume oxygen as rapidly in contact with lactose as when in contact with glucose. However, after subsequent growth in glucose, the lactose activity was reduced to its original low level.

This is an example of the kind of changes observed in enzyme activity when bacteria are 'trained' to grow in new substrates. The consistent activity in contact with glucose, compared with the changing activity in contact with lactose, exemplifies Karström's classification of enzymes as 'indispensable' and 'dispensable' enzymes.[14] If the activity in contact with each carbohydrate is regarded as the functioning of a specific enzyme (glucose dehrogenase or lactose dehydrogenase) the glucose enzyme, being always present, is an indispensable enzyme, while the lactose enzyme, which is only synthesised during growth in lactose, is a dispensable enzyme. It is supposed that the protein available for use as adaptive enzyme acquires the configuration appropriate for metabolism of the substrate which is available, presumably by chemical reaction with it. When no longer useful, this configuration is replaced by another.

Monod and Audureau suggest that the enzyme protein can only cooperate with a new substrate to synthesise the appropriate adaptive enzyme if it has been made adaptable, by mutation,

to this substrate specifically, the mutation being a chance occurrence, independent of the presence of the substrate.

An alternative is to suppose that the enzyme protein is not precisely ordered in structure, but possesses regions of slightly different structure which are able to cooperate with different substrates. Suitable regions might be expanded at the expense of unsuitable ones, although the latter configurations would not necessarily be destroyed, and might replace the former under the influence of a different substrate.

The measurement of enzyme activity does not provide a crucial test of these alternatives, but there is one line of investigation which may be expected to throw some light on which is the more probable state of affairs,

The extent of 'training' of Bact. coli mutabile to lactose in the experiments of Monod and Audureau is not specified. If it could be shown that more prolonged 'training' induced more stubborn lactose activity, the formation of adapted enzyme would appear more significant than a mere complement to mutation. If the structural change can be made more emphatic by further culture in a new substrate, it seems likely that the new substrate plays at least the major role in originating the change.

Experiments to test this question were carried out with Bact. lactis aerogenes and D-arabinose and with Bact. coli mutabile and lactose.

Experimental Methods.

Dehydrogenase activity was measured by making use of the fact that methylene blue, a dye-stuff, is an alternative hydrogen acceptor to molecular oxygen, and that the reduced form is colorless, while the oxidised form has an intense blue colour. The time taken for enzyme material to reduce a saturated solution of air and then a constant amount of methylene blue, is an inverse measure of the dehydrogenase activity, and the end-point of the reaction is sharply defined by the disappearance of the blue colour. This method was originated by Thunberg[17]; such experiments are usually carried out in special tubes, which can be evacuated and filled with nitrogen. In this investigation, a simplified technique was found to give satisfactory results.

1 ml. of sugar solution, 0.2 ml. of methylene blue solution (400 mg./l.) and 5 ml. of bacterial suspension (about 10^9 cells per ml., in phosphate buffer, pH 7.12) were mixed, and immediately aerated, in 6"×½" tubes, suspended in the 40.0° water-bath.

Aeration was continued for one minute, in order to saturate the mixture with air. After the air supply had been cut off, the time before fading of the blue colour was measured. A stop-watch was used for the time measurements. Tests were made in two parallel tubes for each carbohydrate.

The time before disappearance of the blue colour is an inverse measure of the dehydrogenase activity. It was important that, in the different tests, the cells should be in a comparable state of activity. They were harvested from cultures in the

optimum stage of growth, and lag tests were made at the same time as when centrifuging was begun. These were a check on the activity of the cells, aswell as supplying useful data for comparison with the measurements of dehydrogenase activity.

The Scope of the Method.

With suspensions containing about 10^9 cells per ml., the usual reduction time in contact with glucose was 1 to 3 minutes.

In order to test the validity of appreciably longer reduction times, dilutions of active suspensions were prepared, and the reduction times of the diluted suspensions were measured. The results (Table XX) show that the reduction time is a good measure of dehydrogenase activity up to a value of about 20 minutes. Longer reduction times appear to give too low a value for the dehydrogenase activity, as the diffusion of air into the tube begins to significantly increase the amount of oxygen which must be consumed.

However, for the main purpose of the experiments, reliable reduction times up to 20 minutes were quite adequate, as will be seen in the account of the results.

Removal of the Last Traces of Culture Medium.

It was found that, if cells were only washed once after separation from their culture medium, sufficient of the latter was left in the suspension to permit it to achieve rapid reduction in the absence of carbohydrate (In all reduction tests, a control tube was used, which contained 1 ml. of magnesium sulphate solution instead of 1 ml. of carbohydrate solution).

TABLE XX.

Reduction Time and Dilution.

Bact. lactis aerogenes in contact with glucose:-

Dilution	Approximate Number of Cells per ml. in Reduction Test.	Reduction Time. (minutes)	Reduction Time calculated from Test of Dilution 1. (minutes)
1	5×10^8	2·25	(2·25)
2	$2{\cdot}5 \times 10^8$	4·0	4·5
4	$1{\cdot}2 \times 10^8$	9·5	9·0
8	$0{\cdot}6 \times 10^8$	20·0	18·0
12	$0{\cdot}4 \times 10^8$	> 3 hours	27·0
16	$0{\cdot}3 \times 10^8$	> 3 hours	36·0
20	$0{\cdot}2 \times 10^8$	> 3 hours	45·0
24	$0{\cdot}2 \times 10^8$	> 3 hours	54·0

Bact. coli mutabile in contact with glucose:-

Dilution	Approximate Number of Cells per ml. in Reduction Test.	Reduction Time. (minutes)	Reduction Time calculated from Test of Dilution 1. (minutes)
1	5×10^8	4·0	(4·0)
2	$2{\cdot}5 \times 10^8$	8·0	8·0
4	$1{\cdot}2 \times 10^8$	19·0	16·0
6	$0{\cdot}8 \times 10^8$	29·0	24·0
8	$0{\cdot}6 \times 10^8$	45·5 & > 3 hours	32·0
10	$0{\cdot}5 \times 10^8$	75 to 100	40·0
12	$0{\cdot}4 \times 10^8$	> 2½ hours	48·0
14	$0{\cdot}4 \times 10^8$	> 2½ hours	56·0
16	$0{\cdot}3 \times 10^8$	> 2½ hours	64·0

If the cells were washed several times, the reduction time was often increased, when in contact with glucose, to a level which made impossible any significant experimental result.

A method alternative to prolonged washing was therefore used in order to remove the last traces of culture medium from the cell suspension. After one washing, the suspensions were aerated for 3½ hours while maintained at 40.0° in the water-bath. This treatment was successful in increasing the reduction time in the control tube to a level where its effect could not be significant in the carbohydrate tests. Table XXI shows that this treatment did not impair the dehydrogenase activity of cells when in contact with carbohydrate.

TABLE XXI.

The Effect of Aeration on Reduction Times.

The experiment was carried out with cells of Bact. coli mutabile, washed once in phosphate buffer, and re-suspended in phosphate buffer (about 10^9 cells/ml.), after 43 sub-cultures in lactose medium and 4 generations in glucose medium.

Duration Of aeration at 40.0° (hours)	Reduction Time in contact with Glucose (minutes)	Reciprocal of Glucose Reduction Time (1/G)	Reduction Time in contact with Lactose (minutes)	Reciprocal of Lactose Reduction Time (1/L)	Reduction Time in Control Tube (minutes)	Reciprocal of Control Reduction Time (1/C)	$\frac{1}{G}-\frac{1}{C}$	$\frac{1}{L}-\frac{1}{C}$
0	1.75	0.57	3.0	0.33	17.25	0.06	0.51	0.27
0.47	2.25	0.45	3.25	0.31	ca.30	ca. 0.03	0.42	0.28
1.87	2.5	0.40	4.25	0.24	> 60	ca. 0.01	0.39	0.23
2.65	2.75	0.36	4.25	0.24	"	"	0.35	0.23
5.35	2.5	0.40	4.25	0.24	"	"	0.39	0.23
16.0	2.0	0.50	4.5	0.22	"	"	0.49	0.21

1/G-1/C and 1/L-1/C are measures of the dehydrogenase activity in contact with glucose and lactose respectively.

Bact. lactis aerogenes and D-arabinose.

The effects of growth in D-arabinose medium, and of subsequent growth in glucose medium, on the dehydrogenase activity of cells of Bact. lactis aerogenes, were measured by the method described. The results are recorded in Table XXII. The ratio of the reduction times in the two substrates is approximately equal to the inverse ratio of the respective dehydrogenase activities.

Normal cells of Bact. lactis aerogenes, cultivated in glucose medium, show very little dehydrogenase activity in contact with D-arabinose, although, in contact with glucose, reduction is rapid.

After only one passage through D-arabinose medium, however, the dehydrogenase activity when in contact with D-arabinose is comparable with that when in contact with glucose. After 2 sub-cultures in D-arabinose medium, the enzyme activity is equal for each substrate. It is clear that D-arabinose 'training' is associated with an increase in the dehydrogenase activity in contact with D-arabinose. After growth has been achieved in D-arabinose medium, the previously negligible activity becomes similar to the activity measured in contact with glucose.

The behaviour of cells which have been 'trained' to D-arabinose and subsequently grown in glucose medium is of great interest. Cells which have passed through up to 14 sub-cultures in D-arabinose medium lose a great deal of their D-arabinose dehydrogenase activity after only one passage through glucose medium, during which they pass through about 7 generations; the

Table XXII.

The Dehydrogenase Activity of Bact. lactis aerogenes.

Number of sub-cultures in D-arabinose medium.	Subsequent Number of Sub-cultures in Glucose Medium	Reduction Time in contact with Glucose (minutes)	Reduction Time in contact with D-arabinose (minutes)	D-arabinose Reduction Time / Glucose Reduction Time	Reduction Time in Control Tube (minutes)	Lag in D-arabinose Medium (hours)	Lag in Glucose Medium (hours)	Δlag(hrs) (Lag in D-arabinose Medium −Lag in Glucose Medium)
0	0	2.0	55.0	27.5	90.0	35	1	34
0	0	2.0	68.75	34.4	102.75	24	1	23
0	0	2.0	>300	>150	>300	30	0	30
1	0	2.0	3.0	1.5	>332	3	0	3
2	0	2.0	2.0	1.0	49.0	0	1.5	-1.5
13	0	1.0	2.0	2.0	60.0	4	1.5	2.5
32	0	3.5	3.5	1.0	142.0	2.5	1	1.5
1	1	3.0	55.0	18.3	>337	1	2.5	-1.5
1	1	1.25	25.0	20.0	>75	3	2.5	0.5
1	4	1.75	44.0	25.1	>251	3	2.5	0.5
1	21	2.0	>54.5	>27.4	>54.5	7	1	6
2	1	1.75	20.0	11.4	37.0	8	3	5
10	1	2.0	35.0	17.5	>60	5.5	2	3.5
14	1	1.5	20.0	13.3	>80	6	2	4
13	2	3.0	50.0	16.7	>150	9	1.5	7.5
13	6	1.5	20.0	13.3	46.0	7	1	6
13	27	1.5	30.0	20.0	>60	7	1	6
41	1	1.75	5.5	3.1	33.0	7	1	6
48	1	2.0	6.75	3.4	60.0	7	3	4
60	1	1.25	5.0	4.0	50.0	10	2	8
30	28	1.0	20.0	20.0	50.0	5	3	2

reduction time in contact with D-arabinose is increased 10- or 20-fold, or even more. The method is not sensitive enough to discriminate decisively between this reduced activity and the very slight activity of 'untrained' cells, but it appears that cells which have been grown in D-arabinose and then in glucose may retain a slightly higher activity than is shown by 'untrained' cells. The 'reversion' in the D-arabinose dehydrogenase activity contrasts with the comparative stability of 'training' by the criterion of the length of lag in D-arabinose medium, which was described in Part II.

The behaviour of cells which have had 41 or more sub-cultures in D-arabinose medium is different. After one passage through glucose medium, the D-arabinose dehydrogenase activity is only reduced to about one third or one quarter of the glucose dehydrogenase activity. In the time available, it was not found possible to investigate the effect of further sub-culture in glucose medium on these cells, but the D-arabinose dehydrogenase activity of cells which had been grown in D-arabinose medium for 30 sub-cultures was reduced to about 1/20th of the maximum activity after 28 passages through glucose medium. Again, as already described in Part II, the lag in D-arabinose medium was little affected by this treatment.

It is clear that the ability to grow in D-arabinose medium after only a short lag phase is not directly connected with high D-arabinose dehydrogenase activity; although the D-arabinose dehydrogenase activity of 'trained' cells may have been almost

eliminated by culture in glucose medium, the cells are still able to grow in D-arabinose medium after a lag which is very short compared with that of 'untrained' cells.

However, the way in which the D-arabinose dehydrogenase activity lapses durin-g growth in glucose medium is dependent on the extent of 'training' of the cells. The 'reversion' is very rapid for cells with only a small number of passages through D-arabinose medium, but not so rapid for cells which have been sub-cultured many times in D-arabinose medium.

This shows that the enzyme pattern for D-arabinose dehydrogenase becomes more firmly established in cells which have been thoroughly 'trained' to D-arabinose. There is a difference between the equally active enzymes of cells 'trained' very thoroughly and not so thoroughly. This may well be held to suggest that the synthesis of D-arabinose dehydrogenase is a process which need not wait upon mutation. If the process of 'training' consists in the expansion of a particular region of the cell protein, to permit a particular enzyme reaction to take place, the existence of different stages in the stability of this expansion suggests that the initial 'training' may simply consist of expansion from very small beginnings.

In view of the very strong evidence for adaptation as the mechanism of 'training' in the case of Bact. lactis aerogenes and D-arabinose, it seems that the interpretation of the observations on enzyme activity which has just been given is the most likely one.

Presumably, the long lag phase of 'untrained' cells of

Bact. lactis aerogenes in D-arabinose medium is spent in the expansion of a very minute region of the cell protein to form an enzyme capable of D-arabinose metabolism. Although the enzyme is not subsequently present in the same activity during growth in glucose medium, a sufficiency is retained for ready expansion or activation if D-arabinose metabolism is required.

Bact. coli mutabile and Lactose.

Experiments were carried out analogous to those described for Bact. lactis aerogenes and D-arabinose. (See Table XXIII).

It was found that 'untrained' cells had negligible lactose dehydrogenase activity compared with their considerable glucose dehydrogenase activity. Cells harvested after 2 sub-cultures in lactose medium possessed similar dehydrogenase activity in contact with either glucose or lactose. The lactose activity was, however, restored to a very low level after one passage through glucose medium. This was in accord with the results reported by Monod and Audureau. The lag phase in lactose medium was not seriously increased.

Cells which had previously passed through 18 sub-cultures in lactose medium had only suffered about a seven-fold decrease in their lactose activity after one sub-culture in glucose medium, while cells which had passed through 21 sub-cultures in lactose medium still retained some of their enhanced lactose activity after 30 sub-cultures in glucose medium. These are results which appear to suggest analogy to the greater stability of D-arabinose dehydrogenase activity in Bact. lactis aerogenes very thoroughly

TABLE XXIII.

The Dehydrogenase Activity of Bact. coli mutabile.

Number of sub-cultures in Lactose Medium	Subsequent Number of Sub-cultures in Glucose Medium	Reduction Time in contact with Glucose (minutes)	Reduction Time in contact with Lactose (minutes)	Lactose Reduction Time / Glucose Reduction Time	Reduction Time in Control Tube (minutes)	Lag in Lactose Medium (hours)	Lag in Glucose Medium (hours)	Δlag (hours) (Lag in Lactose Medium - Lag in Glucose Medium)
0	0	2.0	29.0	14.5	45.0	36	2	34
0	0	2.0	81.25	40.6	>135	43	1.5	41.5
2	0	3.5	3.5	1.0	>227	5	7	- 2
24	0	2.0	2.0	1.0	>309	2	4	- 2
1	1	2.5	35.0	14.0	>480	4	2	2
1	11	1.5	26.0	17.3	50.0	2.5	1	1.5
2	1	1.75	20.0	11.4	>95	5	1	4
18	1	1.0	7.5	7.5	57.0	3	2	1
21	4	4.0	60.0	15.0	>140	5.5	2.5	3
21	10	2.0	28.0	14.0	>247	2.5	1	1.5
21	30	2.5	17.5	7.0	>110	7	3	4

'trained' to D-arabinose medium. It appears that the 'lactose-trained' cells used by Monod and Audureau were not so thoroughly trained, although the two strains may not be comparable in this respect.

It seems that the lactose dehydrogenase activity of Bact. coli mutabile is made more persistent by prolonged sub-culture in lactose medium. This observation is not incompatible with the theory put forward by Monod and Audureau - it could be supposed that 'pre-enzyme' configuration was made more rigid under such conditions: but the effect may not be merely secondary to the disposition of the 'pre-enzyme' according to genetic control.

In view of the evidence against a mutation-selection theory of training, it is more likely that, in training, suitable regions of cell protein are expanded to form the enzyme required.

It was suggested, in discussing the changes in D-arabinose dehydrogenase activity of Bact. lactis aerogenes, that the lapse in activity during the growth of 'trained' cells in glucose medium represented a decrease in amount of the enzyme pattern concerned, but that there was a sufficient residue for quick expansion when the enzyme was again required. An analogous model may be postulated in the case of lactose dehydrogenase in Bact. coli mutabile.

Similar considerations apply to changes observed by Virtanen in the rates of fermentation of maltose, lactose and saccharose by Bact. coli.[14]

Summary of Part III.

The theory just enunciated may be summarised as follows. In untrained cells, there is present a minute and imperfect fragment of protein material which is able to synthesise enzyme in collaboration with the new substrate. During the long lag phase, this synthesis takes place, but if the cells are later transferred to glucose medium, the new enzyme pattern is superfluous, and is largely replaced by other patterns, but a fragment is retained. This fragment differs from that of untrained cells in having a more perfect configuration for collaboration with the new substrate, which fact is revealed in the short lag phase when the cells are returned to a medium containing the new substrate. Prolonged subculture in the new substrate results in the formation of a greater amount of more rigid pattern, of this type, which is less rapidly replaced by alternative patterns.

An alternative theory accounts for the 'permanent' stamp of training, which is exhibited in the short lag phase, by ascribing it to mutation. Perhaps the true state of affairs is, in a sense, intermediate between these two theories.

The presence of nucleo-protein in bacteria has been mentioned, and also its importance, as the chemical material of genes, in control of development and function in higher organisms. Although it seems unlikely that bacteria possess genetic structure, it is possible that their nucleo-protein may have a controlling effect on the behaviour of bacteria. Bacterial nucleo-protein is probably not concentrated in a nucleus, but sited in a more or

less diffuse array. Perhaps one effect of 'training' may be to induce changes in the nucleo-protein, so that trained cells possess 'trained' nucleo-protein, more stable than the 'trained' enzyme protein. This might be the reason for the stability of the short lag, which is so much superior to the stability of the enzyme activity.

Nucleo-proteins are easily degraded by the mildest chemical reagents. The tremendous stability of genetic structure in higher organisms must be due to the inaccessibility of the gene nucleo-protein to foreign substances. As this structural factor does not seem to be present in bacteria, it is possible that, in these organisms, the nucleo-protein may be affected by the substrate.

Astbury[18] has remarked on the similar spacings of complementary chemical groups in nucleo-protein and in unfolded protein molecules, and has suggested that the directing action of nucleo-protein may be via a kind of template effect during the synthesis of protein. If bacterial nucleo-protein is, in fact, able to react with the substrate, this mechanism may give a picture of the way in which bacteria can achieve adaptation. The possibility of mutation in nucleo-protein material is not excluded, but it is quite clear that mutation is not involved in the 'training' of Bact. lactis aerogenes to D-arabinose, or of Bact. coli mutabile to lactose, in view of the results of Part I and Part II.

A DISCUSSION OF SOME OBSERVATIONS REPORTED IN THE LITERATURE.

As mentioned in the Introduction, systems in which bacteria appear to grow in a new substrate after a long lag phase have received attention from various points of view. In particular, strains of organisms belonging to the coli-typhosum group, behaving in this way with regard to certain sugars, notably lactose, have been investigated by a number of workers.

The earlier work is excellently reviewed by Dobell.[19] The first reported observation of this type was made by Massini,[20] working in association with Neisser.[21] A coliform organism was cultivated on a solid medium, rich in other nutrients, but also containing lactose. The composition of the medium was so arranged that growth on the other nutrients did not affect the colour of an indicator which was also included in the medium; a change in the colour of the indicator could only occur as a result of lactose fermentation. Such media are often used to distinguish between coliform and typhoid bacteria - normal coliform strains produce colonies which rapidly ferment lactose, while typhoid strains do not ferment lactose at all. Massini and Neisser found that their strain did not ferment lactose if colonies were sub-cultured daily to new plates of the medium, but, if colonies were allowed to remain on one plate for two or three days, small nodules, of the acid colour of the indicator, appeared on the colonies. If these were picked out and sub-cultured they produced colonies which were all uniformly of this same colour. The white part of the original colonies, on sub-culture, repeated the original behaviour, forming, after three days, red papillae on the white colonies.

Müller[22] observed that Bact. typhosum behaved in a similar way with respect to rhamnose, and Bact. paratyphosum B with respect to raffinose.

Many other workers, e.g. Smith[23], Nungester & Anderson[24], Kriebel[25], Stewart[26], and Lewis[7], have since confirmed these observations, using similar strains of bacteria. Koser and Vaughan[6] found the behaviour of coliform strains towards D-arabinose to be analogous.

All these workers found that a parent strain, which remained homogeneous if sub-cultured frequently on media containing the test sugar, indicator, and other nutrients which did not affect the latter, produced some off-spring which were able to metabolise the sugar if they were allowed to remain on the medium for some time. The off-spring which were of the new type yielded progeny which were all of the new type - there was no reversion. Cells removed from the parts of the colonies which had not fermented the sugar, if sub-cultured frequently, did not ferment the sugar, but, like the original parent culture, produced some true-breeding sugar-metabolising off-spring if left on the medium for some time.

The complete stability of the new type was emphasised by the authors mentioned above. An observation of reversion to the parent type was made by Baerthlein.[27] A coliform organism which did not originally ferment lactose yielded some cells capable of growing on this sugar. These cells, when sub-cultured to medium containing lactose, after 6-8 days on a solid medium which did not contain lactose, were stated to yield a mixture of colonies; some of them were of the new type, and some of the original type. The literature

contains very few reports of reversion; Massini, even by the action of phenol, was only able to induce loss of the ability to use lactose in one instance, and was unable to reproduce the result.

It may be remarked here that, apart from these apparently abnormal exceptions, all the observations are in accord with the results reported in this Dissertation. The absence of training when cells were sub-cultured frequently on media containing both the new substrate and one which was more readily utilised is parallel to the observation, reported in Part II, that Bact. lactis aerogenes does not become trained to use D-arabinose if grown continuously in a medium containing a mixture of D-arabinose and glucose. Further, it has been emphasised in Part II that training seems to occur only while cells are exposed to the new substrate while not engaged on growth in another substrate. The authors mentioned above all reported that cells able to metabolise the new substrate only appeared after analogous stationary phases; however, it was usually reported that, even after the cells in some parts of the colonies were found to be capable of metabolising the new sugar, cells in other parts of the colonies were unable to do so. This contrasts with the results for Bact. lactis aerogenes reported in Part II, which led to the conclusion that all the cells became able to grow on D-arabinose, thus providing very strong support for an adaptive mechanism of training. It is possible that, under the conditions used by these other authors, there was local nutrient exhaustion or accumulation of toxic products, as a result of the growth of the primary colony, so that only some of the cells were able to achieve adaptation.

However, these workers usually interpreted their results, with some confidence, in terms of mutation of the parent strain. The latter was supposed to throw off mutant cells with ability to metabolise the new substrate, these mutants being produced by very rare, chance, abnormal cell divisions.

Observations comparable with those already described were made by Burri.[28] An organism of the coli-typhosum group was initially incapable of using sucrose, but yielded cells which were able to do so when it was cultured in media containing sucrose.

Lewis and Burri both carried out experiments to determine the proportion of the cells in their strains which were able to use the new substrate. Lewis found that small inocula of one of his strains would not grow in lactose medium which contained no other source of carbon. Larger inocula, tested, in parallel, on lactose and on glucose or beef extract-peptone plates, yielded only about 1 colony on lactose per 10^6 on glucose or beef extract-peptone. This prpportion was almost constant throughout a series of sub-cultures in a rich medium, and, further, when the parent cultures were grown from a number of different single colonies. Lewis concluded that the strain always contained about one lactose-using mutant per 10^6 normal cells. On the other hand, Burri found that, in a medium containing sucrose, his strain produced an almost constant number of colonies, independent of the inoculum size. The colonies appeared after 4-5 days, simultaneously with the commencement of sucrose fermentation. Many very small colonies were also present in cultures which had been heavily seeded. Burri interpreted his observations as implying that all the cells of his strain were

potentially capable of using sucrose, but that they took some time to achieve this. Only a limited number of large colonies could be supported by the quantity of nutrients present in the medium, so the larger inocula were not able to produce an proportionate number of large colonies.

Lewis criticised Burri's interpretation on the grounds that Burri had not established that the cells in his large colonies were of pure sucrose-using type.

An estimate of the significance of Lewis's results and his interpretation of them is made difficult by his inadequate account of the observations in respect of the times at which the various developments took place. The coli strains which he used apparently produced colonies fairly soon on lactose solid medium. Secondary colonies appeared after 5 to 8 days. If the original colonies were large, the secondaries were small, and vice versa. The earlier results were for a strain which differed from the others used by Lewis in that small inocula completely failed to grow on lactose medium. Apparently, larger inocula did grow, as he reported that secondary colonies appeared on lactose agar on the second day. However, Lewis gives figures for the numbers of viable cells of one of the other strains at various stages of culture on lactose and peptone media. Death began after 60-70 hours, and after 120 hours, only 10% of the cells were viable. This was due to the acid conditions - apparently, the stationary population was limited by this factor.

In view of this high death rate , the significance of Lewis's colony counts on lactose medium is not at all clear. His assumption

that failure to grow was an indication of unsuitable genetic character seems rash; the small proportion of cells which gave rise to colonies on lactose medium seems more likely to have been due to the small chance of survival for the necessary length of time - at least 36 hours, and possibly 8 days - Lewis does not make clear which.

In the case of his other coliform strains, Lewis claimed that the mixture of large and small colonies which they yielded on lactose medium permitted differentiation between those sprung from normal or from lactose-using mutant cells, and he estimated a mutant proportion in these other strains of about one per 10^6 normal cells. However, the photograph of such a mixture of colonies, included in his paper, scarcely suggests a clear-cut distinction between only two typical colony sizes.

Lewis induced an apparently comparable distribution in colony size in a strain of Bact. paratyphosum B on peptone-raffinose medium by the use of specific peptone and raffinose concentrations. With slightly different concentrations, however, the colonies were uniform in size; their total number was, in each case, similar to the number of colonies produced by equal inocula on peptone-beef extract agar. Similar adjustment of the conditions was required to vary the uniformity of colony size which was otherwise observed when Bact. typhosum strains were tested on peptone-rhamnose medium.

Lewis's work cannot be considered, as he would wish, as decisive proof that the appearance of new character in bacteria occurs by rare, chance mutation - in fact, much of his argument

appears to be special pleading.

Monod and Audureau[16] also carried out some experiments with Bact. coli mutabile and lactose. On a solid medium containing lactose as the sole source of carbon, with an inorganic source of nitrogen, they found that only large inocula yielded visible colonies in 24 hours, although even small inocula yielded micro-colonies. The number of visible colonies from large inocula was much enhanced after 48 hours, when the micro-colonies from small inocula also began to increase in size. After 3 days, all the micro-colonies from small inocula had developed into visible colonies. These authors postulated that micro-colonies were formed by growth on traces of impurities in the agar, which made possible the occurrence of a mutation, yielding a cell capable of growth on lactose. The small proportion of large colonies, from large inocula, after 24 hours, was regarded as having sprung directly from mutant cells in the inoculum. These considerstions might also be thought to apply to the experiments described in this Dissertation. The secondary assumptions involved are ingenious, but it is simpler to suppose that a long lag phase is normally required before growth can occur in lactose, although this may be somewhat shortened by the pooling of intermediate metabolites in the case of large inocula. It appears arbitrary to attribute colonies visible after 24 hours to direct growth from mutant cells, while those becoming visible after 48 hours are attributed to indirect growth from normal cells. Even more striking in this respect is Monod's report[29] of work on a strain of Bact. coli stated to require methionine. He made an estimate of the proportion of 'mutants' in the strain

by comparing the first possible count of visible cells on medium lacking methionine with the total viable count, although he reported that the number of visible colonies on Methionine-less medium was much augmented after 36 hours. It appears that methionine did narrow the distribution of colony size and/or lag phase, but the grouping of cells which differed so little into two sub-species supposed to possess different genetic character does not seem to be justified.

Some experiments on a phage-resistant strain of Bact. coli stated to be exacting for tryptophane, were carried out by Hollman.[30] Although the time-scale of events is not clear, it appears that she used a similarly arbitrary time limit to make the distinction between the supposed mutant and normal cells.

In these reports also, the stability of the so-called mutant cells is stressed - no reversion to the 'parent type' was reported for the cells which had exhibited the new nutritional capacity. The 'mutations' were assumed to occur by chance, independently of the new substrate. Lewis and Monod found that the 'proportion of mutants', as estimated by them, was almost constant, for tests made at different stages in a series of sub-cultures in a medium which did not contain the nutrient used for the tests. Lewis found very much the same 'mutant proportion' in cultures grown directly from single colonies. These two sets of observations do not appear to be compatible with the theory - if there are no back mutations, there is no means by which the proportion of mutants can be maintained at the approximately constant level which was observed. However, if the changes are not chance mutaions, but are adaptive

changes, occurring in response to the presence of the new substrate, the approximately constant proportion of cells which achieve the changes may be a reflection of the roughly equal statistical chances in different tests. The fact that the new character breeds true is not relevant to appraisal of the likely mechanism for its original appearance, in the case of bacteria. In these unicellular organisms, of indefinite structure, the possibility that adaptive changes may occur does not preclude the possibility that they may be stably inherited, especially if cultivation is continued in the new substrate. It appears that the distinction between 'mutants' and normal cells may be artificial.

This may also be the case in the work of Fildes and Whitaker[31] on a strain of Bact. typhosum which required tryptophane for optimum growth. In their experiments, cultures were grown in a medium containing tryptophane; the growth rate was measured by plating-out at suitable intervals on solid medium containing tryptophane. Each time the total viable count was determined in this way, a test was also made on solid medium lacking tryptophane. Colonies which appeared within 24 hours on this medium were assumed to have sprung directly from tryptophane-independent mutants; the number of colonies increased later, but those appearing after 24 hours were disregarded in computing the number of 'mutants'. Determinations made in this way led to the conclusion that mutant cells first began to appear in the cultures when the total population reached about 10^7 cells. Thereafter, they apparently increased in number at an approximately exponential rate. Cultures in which the total population was limited by tryptophane

exhaustion to about 10^5 cells did not yield any 'mutants'; inocula of this size, or less, did not grow in liquid medium lacking tryptophane although growth did take place when tryptophane was added to these tests. That the tryptophane was added to the small inoculum tests after the large inoculum tests had achieved growth is clear, but whether sufficient time had elapsed to make it quite certain that the cells in the small inocula were not going to achieve adaptation is not clear, which makes this result, at least as reported, indecisive. In the case of cultures whose stationary population was limited by tryptophane exhaustion, Fildes and Whitaker reported that the stationary population was proportional to [tryptophane] down to a population of about 10^7 cells, and in these cultures, the mean generation time was always 45 minutes. However, one-tenth of the tryptophane concentration which supported a population of 10^7 cells would only support 10^5 cells, and in this culture the mean generation time was increased to 170 minutes. It seems likely, after such obviously unfavourable conditions, that cells would be unable to grow in a medium lacking tryptophane altogether. On the other hand, cultures which have reached a population of 10^7 cells appear to be in a state of high activity.

Fildes and Whitaker suggest that the long lag phase of Bact. typhosum, observed earlier, might be accounted for by the very unfavourable constitution of the medium used. Perhaps the state of the parent cultures was also a contributory factor. The possibilities in the way of fluctuation in the character of a whole culture and of its individual cells is emphasised by the observations of Bunting and Ingraham,[32] working with Serratia marcescens. This species

always contains cells of different colours. The proportion in which each colour is represented fluctuates according to a consistent pattern during the growth of each culture - during the phase of most active growth, the population is predominantly dark red; shortly afterwards, paler colours are in the majority, but when the culture has aged for some time, dark red is again the colour exhibited by most of the cells.

Nevertheless, it is clear that, in the experiments of Fildes and Whitaker, cultures of Bact. typhosum which had attained a population of 10^7 cells contained some cells which, unlike the majority, were able to grow readily in the absence of tryptophane without any important lag phase. Of the cells forming the bulk of the population, it appears that some were able to grow without tryptophane, but only after an appreciable lag phase, while it is probable that some were unable to achieve growth, without the assistance of tryptophane, during the course of the experiment, and possibly would have been unable to do so before they were overtaken by death.

This is a different phenomenon from that observed with Bact. lactis aerogenes, training to use D-arabinose; in this case, individual cells apparently grow in the new medium after lag phases of similar length. It may be useful, at this point, to compare the phases of lag and of logarithmic growth. Although, during the latter phase, the mean generation time is remarkably constant, the individual intervals between formation and division for different cells are statistically distributed about the mean. A similar statistical distribution in the lengths of the lag phase for individual cells might account for the observations

32.5

of Fildes and Whitaker. The narrow distribution in the lengths of the lag phase of individual cells of Bact. lactis aerogenes in D-arabinose medium would hardly be expected to be typical of the behaviour of bacteria in new and unsuitable media. The cells of Bact. typhosum described by Fildes and Whitaker as mutants may have been cells with particularly short lags in tryptophane-less medium. Those which yielded colonies somewhat later may not have been genetically different from them, but may have represented a different region in a 'spectrum' of lags, while those which were not observed to grow may have represented the other end of the 'spectrum'. It is possible that the 'spectrum' only extended into the 'short-lag range' when the culture was flourishing, which would account for the absence of cells of this type in the cultures limited to a population of 10^5 cells by tryptophane exhaustion - an elaboration of the suggestion, already made, that such cultural conditions were not conducive to the appearance of really vigorous cells.

Some other recent information on the problem of nutritional training in bacteria has been supplied by the admirable work of Ryan and Schneider, in which experiments were made with an X-ray irradiated strain of Bact. coli which normally required histidine for optimum growth. Ryan[9] found that, from large inocula on a medium lacking histidine, about one colony appeared per 10^7 cells in the inoculum. On the other hand, Ryan and Schneider[33] found that, in another series of experiments, colonies were produced by inocula of less than 10^7 cells. This was attributed to the presence of a trace of histidine thought to be present in the sample of asparagine which was used in making up the medium - the anomaly

was not observed if asparagine was ommitted from the medium, or if the medium was passed through a column of Filtrol-Neutrol. In the ordinary asparagine medium, micro-colonies first appeared, and it was suggested that the growth of a micro-colony made possible the appearance of a mutant cell, which was then able to develop into a visible colony. Micro-colonies did not appear in the absence of asparagine, but this was not the case with other X-ray irradiated strains which were examined. Strains supposed to require lysine, methionine, proline, or leucine did form micro-colonies, and, later, visible colonies, on medium lacking asparagine, or any other amino-acid. Further, an irradiated strain stated to require arginine yielded visible colonies on the minimal medium without the previous appearance of micro-colonies. These observations, coupled with the fact that strains requiring threonine were unable to grow at all on the minimal medium, are not even fully accounted for if very specific assumptions are made about the presence of amino-acid impurities in the medium. The theory of adaptive response is able to account for the results more economically.

Ryan and Schneider further observed,[34] in the case of the strain which was supposed to be exacting for histidine, that proportionately fewer visible colonies, on minimal medium, were produced by the larger inocula, compared with those produced by the smaller inocula. In liquid media containing a range of histidine concentrations, the final stationary population changed in an unusual way. For histidine concentrations greater than one micro-gram per millilitre, the final stationary population was proportional to the histidine concentration, and there was no training. The final stationary

population attained in complete absence of histidine was equal to that attained in cultures containing ample supplies of histidine, and all the cells were trained to grow without histidine. In cultures containing histidine concentrations of less than one micro-gram per millilitre, the final stationary population passed through a minimum, while the proportion of trained cells progressively decreased. It appears that there was an arrest in the growth of these cultures, as Ryan and Schneider give figures for the population before growth was complete which are throughout proportional to the histidine concentration.

Ryan and Schneider suggested that the presence of numerous untrained cells inhibited the growth of trained cells, but did not devise a mechanism to account for this postulated effect. It appears to be quite likely that, initially, there was a phase of growth using histidine, if there was any present, followed by an arrest in which cells were engaged in adaptive processes to equip them for growth without histidine. During these processes, some important constituent in the medium, also used less rapidly in the processes of growth, was consumed at a considerable rate - perhaps even in a wasteful by-reaction; the extent to which such a substance was consumed would be dependent on the number of cells engaged in becoming adapted. If this substance were critical in limiting the final population of the culture, this mechanism would account for the observations - if there was no histidine present, the only cells engaged in adaptation would be those actually introduced in the inoculum; there would be very little waste of the critical substance, and the final stationary

population would be the maximum one: the introduction of histidine into the medium would permit the inoculum to multiply, in the first phase of growth, to a population proportional to the histidine concentration; there would then be a proportionate destruction of the critical substance, followed by the growth of those cells which had achieved adaptation, the final population being less than the maximum in proportion to the number of cells trying to achieve adaptation: when the histidine concentration had permitted the growth of a surficient number of cells to completely destroy the critical substance before any adaptations were achieved, the final population would be proportional to the histidine concentration, until the critical substance again became the limiting factor, by its exhaustion in the growth of cells on histidine, without any arrest; in such histidine concentrations, the final population would be the maximum one.

The observations of Ryan and Lederberg[35] on the loss of adaptation in neurospora do not seem to be comparable with the case just described. It appears that reversion occurred in the adapted mould, possibly by a mechanism involving the unadapted mould.

Ryan and Schneider comment on the importance of their results in assessing the significance of Lederberg's experiments,[36] in which strains of bacteria with different nutritional requirements were mixed, and tested on a simple medium lacking some nutrients for which each of the strains was exacting. It was claimed that the appearance of a small number of colonies was proof that gene recombination had occurred during sexual reproduction. Although the lactose reaction and phage resistance

also appeared to be appropriately segregated, the success of Ryan and Schneider in growing on a simple medium strains which were supposed to be exacting for more complex nutrients does demand a closer examination of Lederberg's experiments.

The potentially keen weapon of statistical analysis has not yielded a decisive answer to the problem of bacterial training in the cases where its use has been attempted. Luria & Delbrück[10] and Newcombe[11] have attempted to prove a mutational mechanism for the origin of phage resistance in Bact. coli. Different cultures were inoculated to plates on which they were subjected to the attack of phages. The number of colonies formed by cells which survived was then counted. Different statistical patterns for the distribution of the numbers of colonies on different plates are predicted by the alternative theories of adaptation and mutation. These workers found that the actual pattern resembled the mutation prediction more closely than the adaptation prediction.

Unfortunately, the different cultures were not tested under comparable conditions. They were grown from similar inocula for an equal length of time before being tested. Experience teaches that this method is not likely to give comparable results (cf. Bunting and Ingraham[32]). However carefully medium composition and inoculum size are controlled, when growing parallel cultures, their individual lag phases and stationary populations are likely to be different. Parallel cultures are most nearly comparable during the logarithmic growth phase, at the same population, not at some arbitrary time after inoculation - it is very unlikely that the same population should be attained in two cultures simultaneously. Even a comparison made at such a standard

population would be unlikely to yield precisely comparable results A test made at an arbitrary time after inoculation, probably during the stationary phase, cannot yield results to which the application of statistical analysis is admissible or significant.

Ryan[9], in attempting to secure comparable conditions for making analogous tests of nutritional change in his strain of Bact. coli, washed the cells grown in different cultures before testing them on histidine-free medium. Although this refinement is more likely to yield comparable results, the later work of Ryan and Schneider makes it clear that Ryan's first results and conclusions did not take into account all the factors affecting the system.

Recent developments suggest that the adaptation and mutation theories may have more points of contact than their more uncompromising advocates would admit. It is clear that the individual cells of bacterial cultures do not behave in exactly the same way at exactly the same rate as each other. This heterogeneity may be interpreted in terms of a continuous spectrum of potentiality and achievement, or in terms of a classification into a few groups, so different as to be comparable with allelic mutants in higher organisms. The theory to be preferred is that which is the more economical in its demands for secondary hypotheses.

The behaviour of Bact. lactis aerogenes appears to be far more economically accounted for by adaptive mechanisms - witness the possibilities of training to an almost unlimited range of nutrient substances, and to resist a number of drugs, the drug resistance being exactly graded to the drug concentration to

which the cells have been exposed.

It is possible that other examples may be better understood in terms of a mutation hypothesis, but close examination of many reports in the literature has convinced the author of this Dissertation that an adaptation mechanism is usually more economical in accounting for the phenomena of bacterial training. Mutation theories usually involve a certain arbitrariness in deciding the differentiation between mutant and normal cells, and also often require special, auxiliary assumptions. In most cases, it is very difficult to evolve a mechanism in which the environment has a merely selective influence on the development of the cells. Once the possibility is admitted that the cells may be affected in other ways by their environment, it is difficult to cling to the belief that adaptive processes must wait upon the chance occurrence of genetic mutation, especially in the absence of decisive independent evidence that bacteria possess a genetic structure comparable with that of higher organisms.

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