An investigation of factors determining growth and form of bacterial colonies.

A Thesis presented

by

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in part fulfilment of the requirements for the degree of Doctor of Philosophy University of London.

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September, 1968.

Abstract:

The literature pertaining to colony formation by microorganisms is reviewed and it is concluded, that while this property has been extensively utilised, it has been little studied for its own sake. Reasons are given why its further investigation is desirable.

The physical and chemical factors which are important in regulating the growth and form of a colony of Aerobacter aerogenes during its development from a single cell to a thin mass of cells up to 4 cm. in diameter, have been elucidated. The behaviour of large numbers of colonies of Aerobacter aerogenes and Saccharomyces cerevisiae, as populations, has also been given quantitative expression.

It was observed that colonies of Aerobacter aerogenes which were able to grow for prolonged periods of time, developed from the original circular disc shape into a complex 'floral' pattern. The nature of this phenomenon, which is closely related to the limitation of the rate of colony growth by the diffusion of a particular nutrient or toxic by-product, has been demonstrated. It seems that the development takes place by the self-reinforced deviation from a smooth line of the growing front, under the influence of a gradient of some growth regulating substance. The process has many similarities with the development of a dendritic crystal. This phenomenon and its pronounced modification by the addition of adsorbents to the gel, have been investigated and given semi-quantitative

description.

These studies have been paralleled with modified strains of Aerobacter aerogenes whose changed behaviour in forming colonies has been correlated, to some extent, with their altered metabolism.

It seems likely that the principles described apply to many non-motile organisms and possibly to other systems of expanding masses of cells.

I am indebted to the late Sir Cyril Hinshelwood, O.M., F.R.S. for the guidance and encouragement so freely given to me during the major part of this research.

I would also like to thank Dr. A.C.R. Dean for the assistance he gave me after Sir Cyril's untimely death. Professor E.B. Chain, F.R.S. kindly provided excellent laboratory facilities. Grateful thanks are also due to Professor R.M. Barrer, F.R.S., Dr. M. Carlile and Dr. J. Russell for many helpful discussions.

The Medical Research Council made the work possible by the provision of a research studentship.

Finally, I would like to thank Miss B.M. Heathcote and my wife Maggie for helping with the preparation of this thesis.

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CONTENTS.

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Chapter 1.

Introduction:

Bacterial colonies are closely packed aggregates of bacterial cells which are formed by the growth of isolated organisms within a solid medium or on the surface of it. For reasons which will become clear later, colonies which are formed inside a solid medium are of less interest than those which have grown on the surface and because of this, the latter will be discussed predominately. It is essential for the formation of a colony that the cells are non-motile on the surface in question, otherwise a thin film of growth is produced. Motile organisms, however, will produce colonies if steps are taken to inhibit their motility. In this discussion the colonies of other uni-cellular organisms such as yeasts, will also be included, since they are quite similar in many respects to bacterial colonies.

Most colonies of microorganisms are large enough to be seen with the naked eye, since they reach a size of the order of 1 mm, and although for the purposes of this study they have been produced by the deliberate inoculation of cells on to surfaces, they can be seen to occur in nature. Many decaying organic materials exhibit small specks, often white in colour, which are in fact colonies of microorganisms. It is well known that for a long time, man used microorganisms unknowingly in

such processes as the retting of flax and hemp, the leavening of bread and the production of beer, wine, vinegar and cheese. Man had suffered pain and even death at the hands of certain species and he had actually seen microorganisms in the form of naturally occurring colonies. In view of the above, his reluctance to accept the concept of 'microscopic creatures', although understandable, is still a little ironic.

As early as 1546, a view was advanced by Fracastorius of Verona, that a 'contagium vivum' was the cause of infective disease, (Wilson and Miles 1957). Such ideas were not tested experimentally and they remained as unproven hypotheses, much as the theories of the early Greeks concerning the ultimate nature of matter had done. Antony van Leeuwenhoek provided direct proof of the existence of microorganisms in 1683, with his invention of the microscope. He was able to make detailed drawings of large numbers of living organisms, including bacterial and protozoal forms. The existence of microorganisms had thus been clearly demonstrated, but their role in, for example, infectious disease and fermentation, was not yet clear.

At this time, (1859), there were two opposing schools of thought on the nature of microorganisms. One believed that such organisms could form spontaneously under suitable conditions, as, for example, in the presence of decomposing organic matter. The opposing idea was that microorganisms were produced in some way from similar pre-existing cells. The work of Pasteur demonstrated the correctness of the second

theory and also laid the foundations of modern practical technique. The methods employed by Pasteur and his colleagues were, however, unsuitable for the isolation of pure strains of organisms which was required for the further advance of the science.

In 1881, Robert Koch described a method for preparing cultures on solid media, which made possible the isolation of pure strains of bacteria from colonies derived from single cells. Koch used first gelatine and later agar, to solidify various liquid culture media. Many important discoveries followed the provision of this vital practical step and most of the experimental procedures then known have remained in common use, basically unchanged to the present day, although some new ones have been added.

On studying the literature it becomes clear that while colony formation has been used by many workers in a great variety of applications, there has been relatively little study of the phenomenon for its own sake. Nevertheless, the principal uses to which colony formation has been put will now be reviewed, since it illustrates the general properties of colonies and also serves to set the work in this thesis in its general context.

The main advantage of colony formation is that it can be used to provide large numbers of microscopic pure cultures, each derived from a single cell, easily and rapidly. For many organisms there is good evidence that when suspensions of cells

are spread on the surface of a suitable nutrient gel, the majority of colonies that form originate from single cells, (Stanier, Doudoroff and Adelberg, 1958). Thus this procedure provides a simple and direct method of counting the number of viable organisms in a cell suspension and is in common use. Under some conditions the number of viable organisms differs considerably from the total number, (Dean and Hinshelwood, 1966, p 48). Two limitations of this method should be mentioned. First, occasionally in liquid culture, organisms clump together in groups of two or more cells. This may be either a characteristic of the organism, (.e.g. Streptococci), or may be induced by the conditions in the liquid culture, (e.g. Aerobacter aerogenes in stale minimal medium). Obviously, if such suspensions are used in the above procedure, the viable counts will be lower than the true values, but it is possible to observe clumping of cells by viewing a sample of the liquid suspension under a microscope. A slight degree of clumping can be tolerated in view of a second limitation of this technique, which is that no absolute distinction as to whether a cell is viable or non-viable exists. Thus, when samples of a suspension of cells are plated on to full nutrient agar and minimal agar, (Chapter 2), a higher viability is usually obtained on the former. This finds a simple explanation in physico-chemical terms, (Dean and Hinshelwood, 1955). Each bacterial cell is being subjected to two types of physical process, the first is the spontaneous breakdown of the cell, the

loss of contents through diffusion, this corresponds to a thermodynamic increase in entropy. The opposing reactions are the synthetic reactions of the cell and various repair processes (Pirt 1965, 'maintenance energy'), these are entropy reducing reactions. When bacterial cells are placed on a gel surface, these two types of reaction compete; if the degrading process is sufficiently fast the cell never divides, but dies. However, if the synthetic reactions are fast enough the cell will divide and a colony subsequently forms. Nutrient media, such as meat broth, increase the rate of the synthetic reactions of the cells because the average lag times and mean generation times, (Chapter 2), are reduced while the degredative reactions, as we have defined them, are independent of the nutrient source and hence remain approximately constant. This explains the higher viability which is obtained on nutrient media compared with minimal media. The viability of organisms should, therefore, always be related to the conditions under which it is measured.

The formation of a colony by an organism generally classifies it as belonging to a non-motile species, although non-motile mutants of motile organisms have been reported, Armstrong et al.(1967). The colonies formed by a particular organism are usually characteristic in appearance for that species and the literature contains many descriptions of the colonies produced by various organisms under certain conditions, (Wilson and Miles, 1957 ; Burrows, 1955). Because

of this property, colony morphology often plays an important part in the identification of unknown organisms. This technique is not confined to pure cultures and although motile organisms can create difficulties on plates of mixed cultures, it is usually possible to use the method to give a first idea of the species present and also to isolate each organism for further tests. In screening samples taken from, say, infected wounds, the identification of the organisms present is often secondary to establishing which antibiotic they are most sensitive to. This is readily done by spreading some of the cell suspension on an agar plate which contains different antibiotics in separate sectors of the gel.

By mixing the cells with molten nutrient media containing agar, at $40^{o}C$, and then allowing it to set, it is usually possible to obtain small colonies inside the gel. The shape of such colonies, which is similar to a convex lens, is predominantly determined by the gel structure, (Stanier, Dourdorff and Adelberg, 1958), and hence they are less characteristic in appearance than surface colonies. This means that they are less useful for identification purposes and also less interesting in the present study.

The characteristic appearance of bacterial colonies may be markedly affected by alterations in the chemical composition of the gel and sometimes by the method of cultivation of the organism prior to plating, (for example see 'rough-smooth forms', below). Bacterial colonies will sometimes produce

quite specific colour changes in certain reagents which have been added to the gel before it sets and these colour changes may be used, in conjunction with colony morphology, in the identification of organisms. For example, colonies of Pseudomonas indoloxidans (and the gel close to them), grown on agar containing indole, (colourless), become deep blue as the indole is oxidised and condensed to indigo blue. Colonies of Corynebacterium produce characteristic colours when grown on gels containing tellurite and it is also possible, by this means, to distinguish between various types of this bacterium. Similarly, the colonies of Gonococcus and Meningococcus produce characteristic colour changes when tetramethyl-p-phenylenediamine solution is applied to them.

In screening the large numbers of microorganisms which is required when searching for new antibiotic producing strains, colony formation has been used extensively. Molten nutrient agar is inoculated with suitable numbers of test organisms, against which the antibiotic is required to be active, at 40°C, and allowed to set. A liquid suspension containing about 100 cells of the various organisms to be tested for antibiotic production, is spread on the surface of the gel, a suitable period of time after it has set. The surface colonies of any organism which produces a diffusible antibiotic substance are surrounded by a clear zone of agar where the growth of the test organisms within the gel has been inhibited. These inhibition zones may be produced by rather general factors, such

as adverse pH, but the method allows the detection and isolation of all possible strains for their further study in pure culture. A rather similar procedure, which is dependent on the theoretical solution of various diffusion equations, is employed as a bio-assay for antibiotics, (Cooper 1963).

In a similar way to screening mixed cultures, a pure strain of organism can be examined for the presence of mutant cells by using colony formation.

Occasionally the parent and the mutant cells will differ with respect to the production of a pigment. For example, it has been reported, (Gause 1966) that Staphylococcus aureus treated with the ultra violet light or various mutagenic chemicals, produced two types of colonies. Whitish colonies of the parent cells were formed and also orange coloured colonies from mutant cells. It was shown that the mutant cells were oxidatively deficient and produced an intracellular orange pigment. Gause reports that many mutant strains of Staphylococcus aureus and Staphylococcus afermentans which had oxidative deficiencies and produced orange pigments were also obtained. He suggested that respiratory defects in these strains might be accompanied by some disturbances in the metabolism of flavins. The production of a yellow pigment by an oxidatively deficient strain of Aerobacter aerogenes is reported in Chapter 7. Similarly, with organisms which normally produce pigments, mutant cells may often be detected through a deletion of this function. A bacterial colony represents a

very high concentration of cells and hence is ideally suited for the detection of the production of intra- or extra-cellular pigments.

A more general way of illustrating mutant formation through its modified metabolism is by the addition of some reagent to the gel which produces a differential colour reaction in or near the colonies. It has been shown, (Gause 1957a), that the colonies of some mutant cells of yeasts induced by trypaflavine, camphor and ultra-violet radiation could be readily distinguished from those of the parent cells by adding the colourless leucobase of methylene blue to the gel before setting. The normal colonies became deep blue in colour whereas those of the mutant cells which had respiratory deficiencies remained colourless because they were unable to oxidise the leucobase to methylene blue. Some mutants differ from the parent cells in their rate of production of some metabolite. Differences in the rate of acid production, for example, can sometimes be demonstrated by the addition of an indicator to the gel on which the colonies are growing. Such methods, which exploit quantitative differences in metabolic rates are in principle widely applicable to the detection of mutants.

One mutation which seems to be common to many species of bacteria, affects the appearance of their colonies and is known as the 'rough-smooth' mutation. It was observed that colonies formed by Streptococcus pneumoniae which had just

been isolated from an infected animal, formed circular colonies with a smooth glossy surface, on nutrient agar. On repeated sub-culture of such a strain in vitro, a second type of colony which had a rough dimpled surface, began to appear and was easily distinguished from the first type. It was later shown that the cells which produced the smooth colonies were surrounded by a capsule of polysaccharide, while those of the rough colonies did not have this capsule. The difference in the colony morphology of these two types seems to be closely connected with their respective cell surfaces, (Nutt 1927). The encapsulated cells were also found to be virulent in animals, but those of the rough colonies were not; the reason for this is not yet clear. It was found that 'rough' type cells could be transformed to 'smooth' type by contact with extracts from disrupted 'smooth' type cells. In 1944, Avery, McLeod and McCarty showed that the transforming substance involved was DNA. It seems likely, therefore, that the 'rough' type of colonies which appeared with the prolonged in-vitro culture of the 'smooth' type was a mutant strain which did not form a polysaccharide capsule. Although this capsule was in some way essential for the virulence of the organism in a host animal, it is superfluous in in-vitro culture. Many other genetic transformations were subsequently discovered, both in pneumococci and in other species of bacteria.

A very common difference between colonies of normal and mutant cells is that, while both are basically circular, they

differ in diameter, the mutants usually being smaller. The two types of colony are sometimes clearly distinguishable by inspection, (for example, Saccharomyces cerevisiae treated with sodium azide, Wild and Hinshelwood 1956), but when the difference in size is less marked, the presence of two types of colonies on a plate can often be demonstrated by constructing a histogram of colony diameters, (Saccharomyces cerivisiae treated with Crystal Violet, Wild and Hinshelwood 1956). Probably the most thoroughly studied example of these systems are the 'petite colonies' which are formed by Saccharomyces cerevisiae on exposure to acridines. The cells of these small colonies were found, (Ephrussi, Hottinguer and Chimenes 1949), to be genetically stable and to produce small colonies on sub-culture. It was shown that the mutant cells were oxidatively deficient and it was suggested, (Tavlitzki 1949) that the reduced size of the colonies that they formed was due to their enforced anaerobic metabolism of glucose which is less efficient than the almost completely aerobic mode of the normal cells. Gause (1957a) has reported the production of eleven oxidatively deficient strains of Saccharomyces cerevisiae by the use of trypaflavine, camphor and ultra-violet radiation. Oxidatively deficient strains of various genera of yeast including Saccharomyces, Candida, Schizosaccharomyces, Debaryomyces, Pichia and Torulopsis were described by De Deken (1961). Generally it seems that all oxidatively deficient mutant cells form colonies which are smaller than those of the parent.

The occurrence of various small colony mutants in bacteria has also been reported, (Gause 1956). It seems clear, however, that small colony forms of bacteria and yeasts can be produced by mutant cells which have normal respiration (Bianchi 1961a, 1961b : Gause 1966).

A brief survey of the types of work in which colony formation has been employed has been given. However, the amount of work which has been done in studying colonies for their own sake is quite small. Information concerning, for example, the overall kinetics of the growth of a colony, the mode of regulation of its growth, whether some cells are dividing more rapidly than others and if so, why this should be, have all been little studied. Also the factors which are instrumental in determining the morphology of a colony have not been elucidated. Clearly such information might be valuable in connection with many of the current applications of bacterial colonies and might also be useful in connection with related, but more complex phenomena such as the chemotaxis of motile organisms. In addition, bacterial colony formation is quite fundamental in nature and it is of interest in its own right. Views similar to the above were expressed by Hoffman (1964). In beginning a review article entitled 'Morphogenesis of Bacterial Aggregations' he said, 'The regular macroscopically visible microbial colonies on conventional laboratory media are not the characteristic aggregations which occur in nature. The wide variety of irregular

formations, however, which may be found in the natural habitat are, in all probability, expressions of the same mechanisms as those involved in the development of macroscopic colonies. Although these mechanisms are poorly understood in the case of most microorganisms, and particularly bacteria, it may very well be that increased attention to such simple models may allow their more effective isolation and definition. It is hoped that the present review will stimulate a greater interest in the problems of bacterial aggregation than is evidenced by the relatively meagre and uncollected literature. Increased understanding may not only clarify many aspects of microbial life, but possibly may also contribute significantly towards elucidation of the more complex processes of all aggregation in higher phyla'. Some studies of colony formation have been reported in the literature, however, notably by Pirt (1967); this and other work will not be reviewed here, but will be discussed later, at points where it is appropriate.

Bacterial colonies are also of interest because they bear a fundamental similarity to a tissue in that they are growing masses of closely packed cells which develop into morphologically characteristic forms. Hoffman, at the end of the above quotation, suggests that useful ideas might spring from this analogy. Likewise, Stanier, Dourdorff and Adelberg (1958) said,'Like the body of a multicellular

organism, a bacterial colony is a population of cells, and its appearance and organisation are determined by the specific properties of the individual cells and by the mutual relationship of the cells that are in close association'. Although not referring specifically to colonies, Sussman (1965), suggested that the study of the developmental phenomena of microorganisms would yield useful ideas about such processes in higher forms. 'Notwithstanding, one purpose of this essay is to convince the reader that there is, in fact, amply common ground between the developmental phenomena observed in microorganisms and those observed in higher plants and animals'. Pirt, (1967), supports the possible usefulness of regarding the bacterial colony as a model of a tissue, '..... one could regard the colony of a microorganism as a simple model of a differentiating tissue in which one can control the environmental factors and thus discuss their effects in quantitative terms'.

By studying the growth and development of a bacterial colony it might be possible to suggest mechanisms which could operate in the more complex formation of higher tissues. In addition, the techniques for the in-vitro culturing of mammalian cells to produce colonies are improving considerably and it is conceivable that a knowledge of the regulatory factors involved in bacterial colonies would be of use in this new field.

Chapter 2.

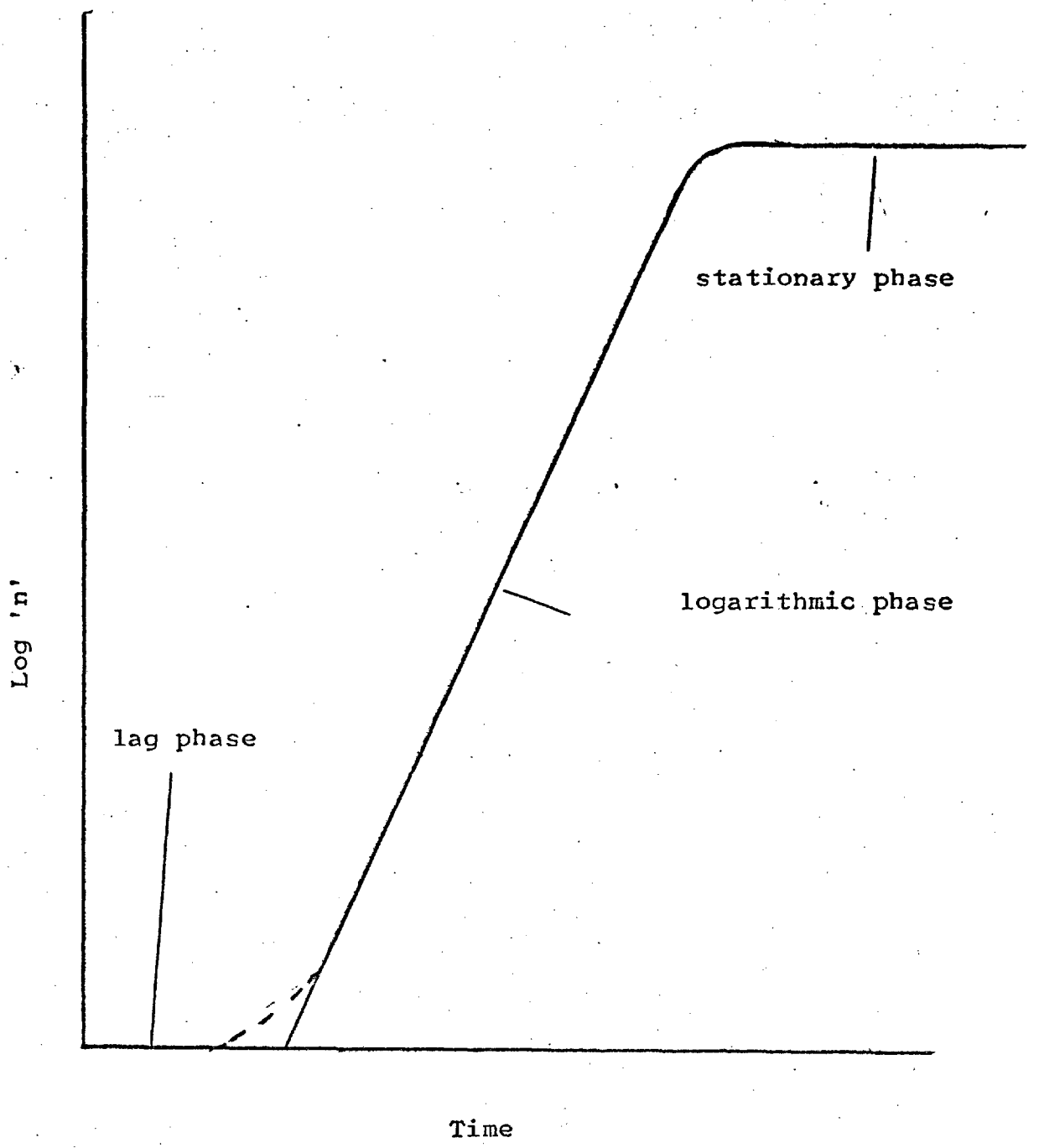
Bacterial Growth Cycle and Experimental Procedure.

When bacterial cells are inoculated into a fixed volume of a suitable liquid culture medium, their ensuing growth follows a characteristic pattern. This type of growth which is described below is also exhibited by the yeast strain employed in this work and by many other uni-cellular organisms.

A plot of 'log n' against time, (where 'n' is the number of bacterial cells per ml. at a given time) is given in Figure (2-1) and illustrates the basic bacterial growth cycle. The lag phase occurs immediately after inoculation into a fresh medium, is accompanied by little growth or division and is a period of adjustment to conditions for which the cells initially are not ready. The length of the lag depends on the nature of the medium, the age of the parent culture and the size of the inoculation. There is widespread evidence which suggests that the lag is in fact a period of intense chemical activity directed towards restoring the balance of cell metabolism and establishing growth and division.

During the logarithimic phase the cells grow and divide so that the rate of synthesis of new material is proportional to the amount of material already present. Although quite important variations in cell size occur during the course of a growth cycle, they do not affect the total cell mass. In logarithmic plots, moreover, moderate deviations do not show up to any marked extent even when actual numbers of cells are

Figure (2-1).



used. The growth rate is conveniently expressed as the mean generation time (M.G.T.) which is the average time interval between divisions and, therefore, the time required for the bacterial concentration to double. The mean generation time of Aerobacter aerogenes fully adapted to grow in a synthetic minimal glucose medium at 35°C is about 33 mins.

The fall in growth rate which heralds the onset of the stationary phase may be brought about by the accumulation of toxic products or by the exhaustion of some nutrient.

The accumulation of toxic products makes the medium unfavourable for growth. Some of these products may alter the pH to a value which is itself unfavourable for further growth. The growth rate then decreases until the stationary bacterial concentration is reached. As the stationary phase is prolonged, effects of the environment cause the cells to begin to die, often at an approximately logarithmic rate (Hinshelwood 1957).

When the exhaustion of a nutrient prevents further growth, the growth rate retains its optimum value almost to the very end and the lag following subculture to a fresh medium is small. When it is desired to ensure that the lag on subsequent subculture is as small as possible, the parent is usually grown in a 'limiting' medium in which the concentration of glucose is high enough to leave the growth rate unaffected and yet low enough to ensure its eventual exhaustion.

If, during aerobic growth, conditions allow the attainment

of a high bacterial concentration, the supply of oxygen may not be able to keep pace with its consumption. The cells then adjust themselves to this shortage of oxygen by partially utilising anaerobic growth mechanisms. These mechanisms involve the reoxidation of certain reduced carriers by the primary energy source rather than by molecular oxygen and in this process the yield of cells from a fixed amount of carbon source is lower than when completely aerobic conditions prevail.

Experimental Procedure:

Since most of the work to be described in this thesis has been performed with Aerobacter aerogenes the experimental procedures for its cultivation will be described first; the few modifications necessary for the cultivation of a yeast strain also employed, will be described afterwards.

Ions of heavy metals are toxic to bacteria and so growth media were made up with glass distilled water and 'Analar' grade chemicals. These solutions were prepared and used in Pyrex glass vessels.

Methods of Sterilisation:

The usual experimental precautions against infection by extraneous microorganisms were always observed. Glassware, after very thorough washing, ($Na_3 PO_4$ as detergent) and rinsing with distilled water was sterilised by placing in an air oven at 150°C for 40 mins. Liquid growth media, often as two or more constituent solutions in Pyrex glass flasks fitted

with non-adsorbent cotton wool plugs, were sterilised by autoclaving at 15 p.s.i. for 15 mins. The various solutions were mixed aseptically when cooler. Agar media, again in the form of two or more solutions, were autoclaved at 10 p.s.i. for 20 mins.

When it was necessary to sterilise solutions of less heat-stable substances the procedure of heating to 100°C on three successive days was adopted. This kills vegetative microorganisms and allows spores enough time to germinate and thus become vulnerable to subsequent boiling. Solutions of very heat-sensitive compounds can be sterilised by filtration through a Millipore filter, (Millipore Ltd., Heron House, Wembley, Middlesex).

Methods of Culture:

The organism was stored on nutrient agar 'slopes' in sealed McCartney bottles. With the aid of a sterile platium wire and observing the usual precautions, some cells were transferred to another bottle containing sterile meat broth and the new culture incubated until visible growth had appeared. This latter culture was used to inoculate minimal media. Such cultures of Aerobacter aerogenes in meat broth were maintained by subculturing at intervals of about one month.

Cells were grown in minimal media, (usually 20 ml), in 6" x 1" boiling tubes and subcultured daily. These tubes were placed in a thermostated water bath, (35.0 ± 0.1°C), and were fitted with cotton wool plugs through which passed Pasteur

pipettes for the purpose of aerating each tube. A small aquarium type pump was used to provide air for this purpose which was passed through a filter of activated charcoal to adsorb any oil vapour, followed by pads of glass wool and cotton wool to remove contaminating organisms before entry into the culture medium via the Pasteur pipette which itself contained a cotton wool filter. Occasionally anaerobic culture conditions were required and this was achieved by using 'oxygen-free' nitrogen from a cylinder instead of air, in the above system.

The composition of the principal minimal medium used and referred to later as 'normal minimal medium' was as follows:-

Solution A - Glucose, 200 ml. (50 g.p.l.)

Solution B - Ammonium sulphate, 100 ml. (5 g.p.l.)
Magnesium sulphate, 20 ml. (1 g.p.l. (+ Fe SO_4 5 mg.p.l.))
Potassium di-hydrogen ortho phosphate, 200 ml.
(9 g.p.l. brought to pH 7.1_2 with 4N Na OH).

Solutions A and B were autoclaved separately and mixed aseptically when cool.

The 'normal minimal agar' used in this work had the following composition:-

A

$MgSO_4 \cdot 7H_2O$	0.05 gm.
$(NH_4)_2SO_4$	0.6 gm.
Na_2HPO_4	3.16 gm.
KH_2PO_4	1.5 gm.
Agar Powder	10.0 gm.
Glass distilled water	600 ml.

B Glucose, 200 ml. (13.33 g.p.l.).

The two solutions were autoclaved separately at 10 p.s.i. for 20 mins. and mixed aseptically when the temperature had dropped to about 60°C. Difco Bacto agar powder was normally used, but in some of the experiments it was replaced by other brands, any changes being clearly indicated in the text. The alternatives were:-

Difco Purified agar	(Difco Ltd.)
Oxoid Ion agar	(Oxo Ltd.)
Oxoid Agar No. 3	
Oxoid Nutrient agar No.2	

To prepare agar plates, molten agar was pipetted into sterile Petri dishes, taking the usual precautions against infection and allowed to set. The plates were left overnight in a dry incubator at 35°C in order to remove excess moisture from the surface of the gel. At the beginning of this work, glass Petri dishes of 9 cm. diameter were employed. The bases of these dishes were somewhat irregular and 25 ml. of

agar was used to minimise any errors incurred by this. Later, disposable plastic Petri dishes were adopted, these proved much more satisfactory and were used in conjunction with 15 ml. of agar, (≏ 2.4 mm. deep).

Agar plates were either inoculated by spreading 0.5 ml. of a suitable dilution of cells in 1% sodium chloride solution over the surface of the gel with a bent glass rod or by a single inoculation using the tip of a platinum wire. The plates were then left for about 10 mins. to allow excess liquid to be adsorbed by the gel, before inverting and placing in an incubator.

Colony Measurements:

Colony sizes were measured under a low power microscope with a calibrated eyepiece scale or by calculation from measurements on enlarged photographs and drawings. The area of certain large single colonies was estimated by viewing against a ruled grid.

Measurement of Populations of Suspensions of Cells:

Measurements of the optical density of bacterial suspensions, as determined in a Unicam SP 500 Spectrophotometer, were used to estimate the number of cells per ml. in that suspension. The validity of this method has been demonstrated and fully investigated by Dean (1967). It was found quite suitable in the present study.

The spectrophotometer was calibrated in the following way. A fully grown culture of Aerobacter aerogenes in normal minimal medium was diluted, in known amounts, with phosphate buffer (pH 7.1). A calibration curve was constructed of optical density at 530mμ against the cell population as a fraction of the undiluted culture. The curve was then expressed in terms of cells per ml. of suspension by directly counting a sample using a haemocytometer chamber and a microscope. It was found that fully grown suspensions contained about 10^9 cells per ml.

Bacterial Strains:

The bacterium used, Aerobacter (Klebsiella) aerogenes, (N.C.I.B. 418 Bact. aerogenes no. 240), was kindly supplied by Dr. A.C.R. Dean, the Physical Chemistry Laboratory, Oxford. This strain of organism has been extensively studied in that laboratory for several years, (Dean and Hinshelwood 1966), and investigations are still being actively pursued there.

It was decided, for the purposes of the present investigation, to prepare modified strains of the above organism which were resistant to various toxic agents. It has been known for some time that by culturing an organism in the presence of a sub-lethal concentration of a toxic agent, ('training'), it is often possible to produce a strain which is much more resistant to the substance in question than the original cells were. Such resistant strains exhibit modified

biochemical properties, (in the absence of the toxic agent), and it is for this reason that they are of interest in the present investigation. Two opposing explanations for the phenomenon of 'training' have been advanced. One states that in the adverse environment, the selection of a small number of mutant cells which are resistant to the toxic substance occurs, while the other proposes that a modification to the majority of the population is taking place, (for example, Wolstenholme and O'Connor (1957); Dean and Hinshelwood (1966)). However, as it will appear later, for the purposes of the present investigation it is immaterial which of these mechanisms is operating in these particular examples.

Cells of Aerobacter aerogenes were cultured aerobically in normal minimal medium containing a low concentration of a particular drug or dye. The exact concentration initially employed depended on the toxicity of the agent, but in the absence of any published data, the percentage survival on various agar plates proved a very useful guide. By comparing the numbers of colonies which form on normal minimal agar and on agars containing various concentrations of a given toxic agent, when spread with the same volume of a given suspension of cells, it is possible to estimate a suitable initial concentration for training in liquid media.

Plating on agar media also provides a very good method for following the improvement in resistance of a particular

strain during training and this is illustrated in Figure (2-2) for a strain resistant to Brilliant Green.

A list of the resistant strains of Aerobacter aerogenes, (some of which were obtained from Dr. Dean), and the maximum concentrations of toxic agent to which they were resistant is given in Table (2-1).

Yeast:

Some experiments were performed with a yeast, Saccharomyces cerevisiae, Park Royal strain 4264, which was kindly applied by Messrs. Guinness Ltd. The procedure for the cultivation of this organism was the same as that just described for Aerobacter aerogenes except for the following points.

The organism was stored on wort agar slopes and transferred to strong hopped worts. It was grown in the following minimal medium at 20°C.

Solution A	500 ml.	(Glucose (100g.) (NaCl (0.1g.) (Ca $Cl_2.2H_2O$ (0.1g.)
Solution B	300 ml.	($(NH_4)_2 HPO_4$ (1.14g.) ($KH_2 PO_4$ (8.0 g.) ($CH_3 COO Na . 3H_2O$ (5.0g.)
Solution C	100 ml.	$Mg SO_4.7H_2O$ (0.5g)
Solution D	100 ml.	Hop extract (75 mg.)
Solution E	1 ml.	'Trace elements solution' (see below)
Solution F	1 ml.	'Vitamin solution' (see below).

Figure (2-2)

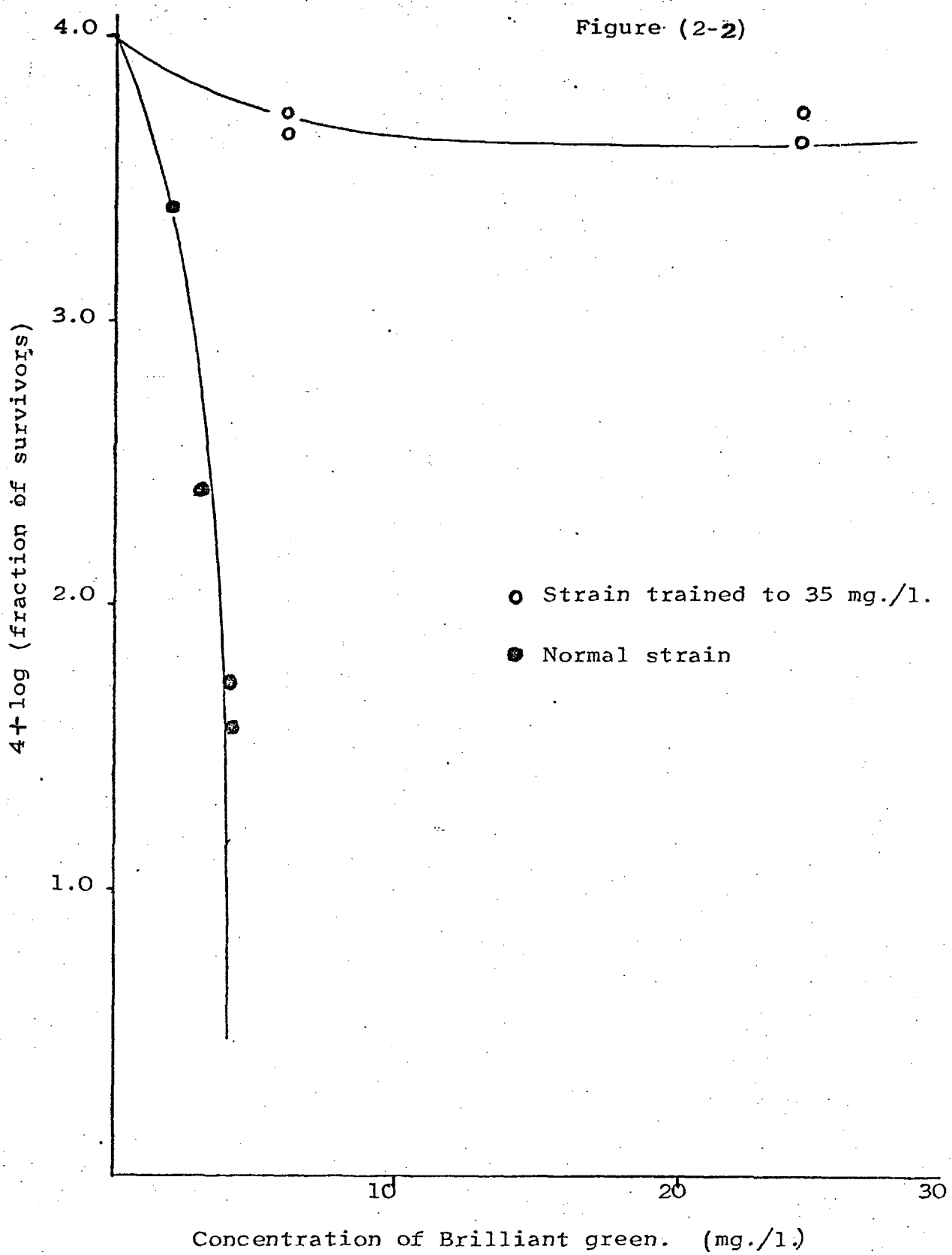

4.0
3.0
2.0
1.0
4 + log (fraction of survivors)
o Strain trained to 35 mg./l.
● Normal strain
10
20
30
Concentration of Brilliant green. (mg./l.)

TABLE (2-1) LIST OF DRUG-RESISTANT STRAINS.

Substance to which cells were made resistant by serial sub-culture.	Maximum concentration reached (mg/l)	Remarks.
Crystal violet	30	
Brilliant green	500	
Methylene blue	1400	
Janus black	690	
5- aminoacridine	91	
Streptomycin	2	
Barbitone	8500	
Chlorampenicol	59	
(Streptomycin	1)	Simultaneous
(Sulphanilamide	1000)	resistance induced
(Chloramphenicol	30)	(see Bolton et al 1967)
Anaerobic strain	200	sub-cultures.

The vitamin solution was sterilised by filtration through a 'Millipore' filter and the other solutions by autoclaving; They were mixed aseptically when cool. The compositions of the vitamin and trace elements solutions are given below.

Vitamin Solution:

Biotin (10 mg/l)

Calcium D-pantothenate (400 mg/l)

Trace Elements Solution:

Boric acid	(500 mg/l)
Cupric sulphate	(40 mg/l)
Potassium iodide	(100 mg/l)
Ferric chloride	(200 mg/l)
Manganese chloride	(400 mg/l)
Sodium molybdate	(200 mg/l)
Zinc sulphate	(400 mg/l)

To prepare agar plates the minimal liquid medium was solidified with 1.25% w/v of 'Difco Bacto' agar powder.

Chapter 3.

Factors affecting the Growth of Colonies on a Plate.

The Final Average Size of Colonies.

For these studies a suspension of cells was spread on the surface of agar, 15 ml. in a Petri dish, diameter 9 cm.

With the normal strain on the normal minimal agar at 35°C, isolated colonies became visible 10 to 24 hours after inoculation. They were whitish in colour and circular when viewed from above. They increased in size until all visible growth ceased from 2 to 10 days after inoculation. With more than 10^4 cells a confluent layer was produced: with smaller inocula the colonies remained as distinct entities when all growth had stopped.

Histograms of final colony diameters were constructed from measurements on plates having different numbers of colonies. The histograms have a single maximum and are approximately symmetrical, so that the most frequent diameter is nearly equal to the average. Gaussian curves have been verified to fit some of the histograms, most of which are of similar form, though some show a somewhat wider spread on the side of increasing diameter.

The spread of the distribution of diameters varies with the conditions on the plate. It is defined as the ratio of the width of the distribution curve between the points where the frequency is half the maximum to the most frequent diameter.

This is equal 2.3 times the coefficient of variation in a truly Gaussian distribution. The spread in the area is double that in the diameters. The diameter spread ranges from 0.1 to 0.6 according to conditions which will become clear later.

As shown in Figure (3-1) the final average diameter, D, decreases as the number, N, on the plate increases, all other factors being kept constant. Microscopic examination showed that the vertical thickness of the colonies was small compared with their diameter and for the time being we may accept the approximation of treating them as discs (cf. Pirt 1967). The value of ND^2 is proportional to the total volume of colony material on the plate. The horizontal portion of the graph in Figure (3-2) where ND^2 is plotted against N suggests that exhaustion of some medium constituent is limiting growth. The fall in ND^2 at lower values of N suggests, however, that with fewer growing centres this constant amount of total growth can no longer be attained, presumably because the limiting constituent is now being used less completely or in a less efficient manner. When N is small the colonies reach a larger size. They have a greater available area of agar from which to draw nutrients and into which to discharge products. The total time of growth for large colonies is greater than for smaller, both because the former present a smaller total interface with the medium and also because the greater amounts of nutrient which they eventually consume has had on the average further to diffuse.

Figure (3-1).

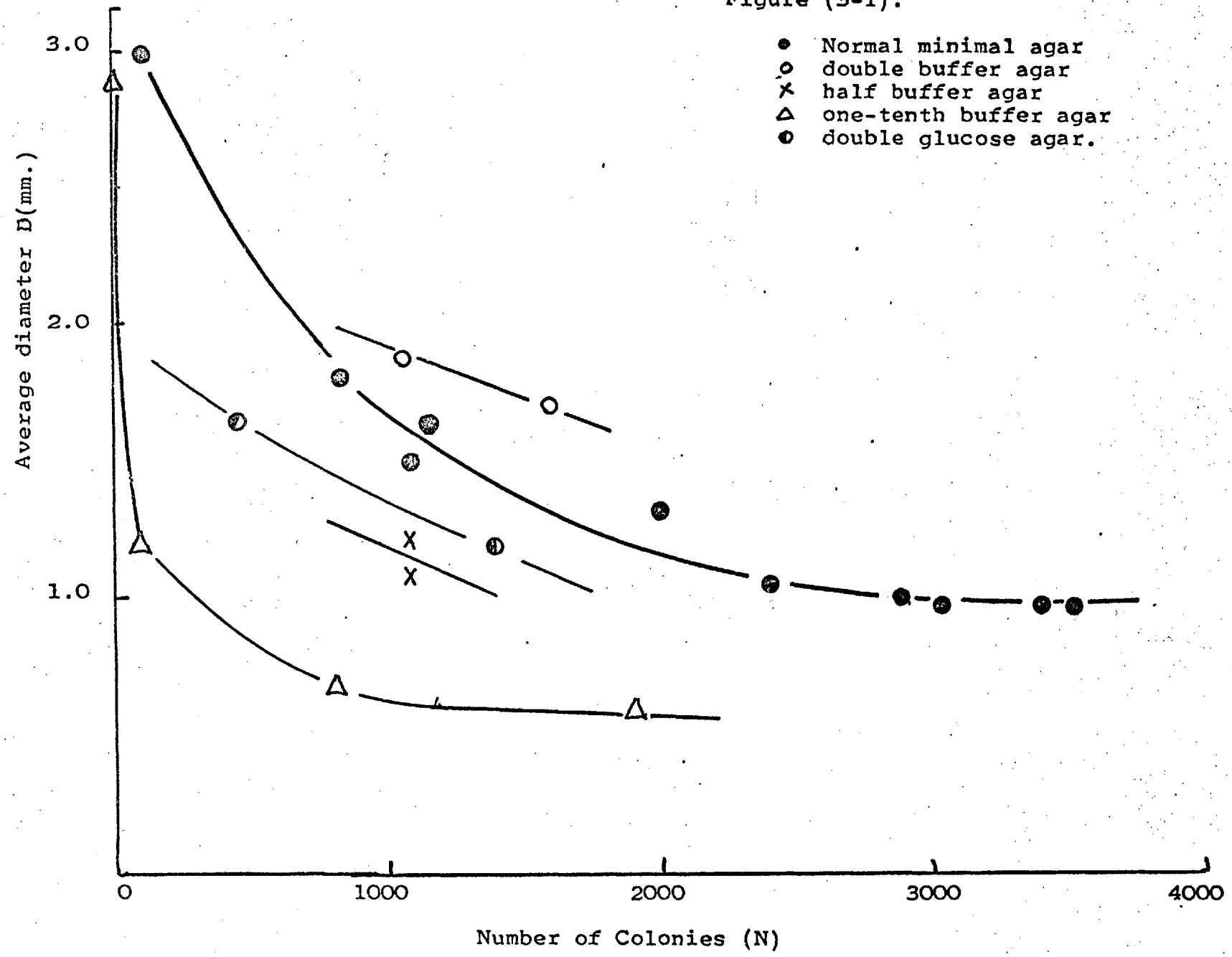

Normal minimal agar
double buffer agar
half buffer agar
one-tenth buffer agar
double glucose agar.
3.0
2.0
1.0
Average diameter D(mm.)
0
1000
2000
3000
4000
Number of Colonies (N)

Figure (3-2).

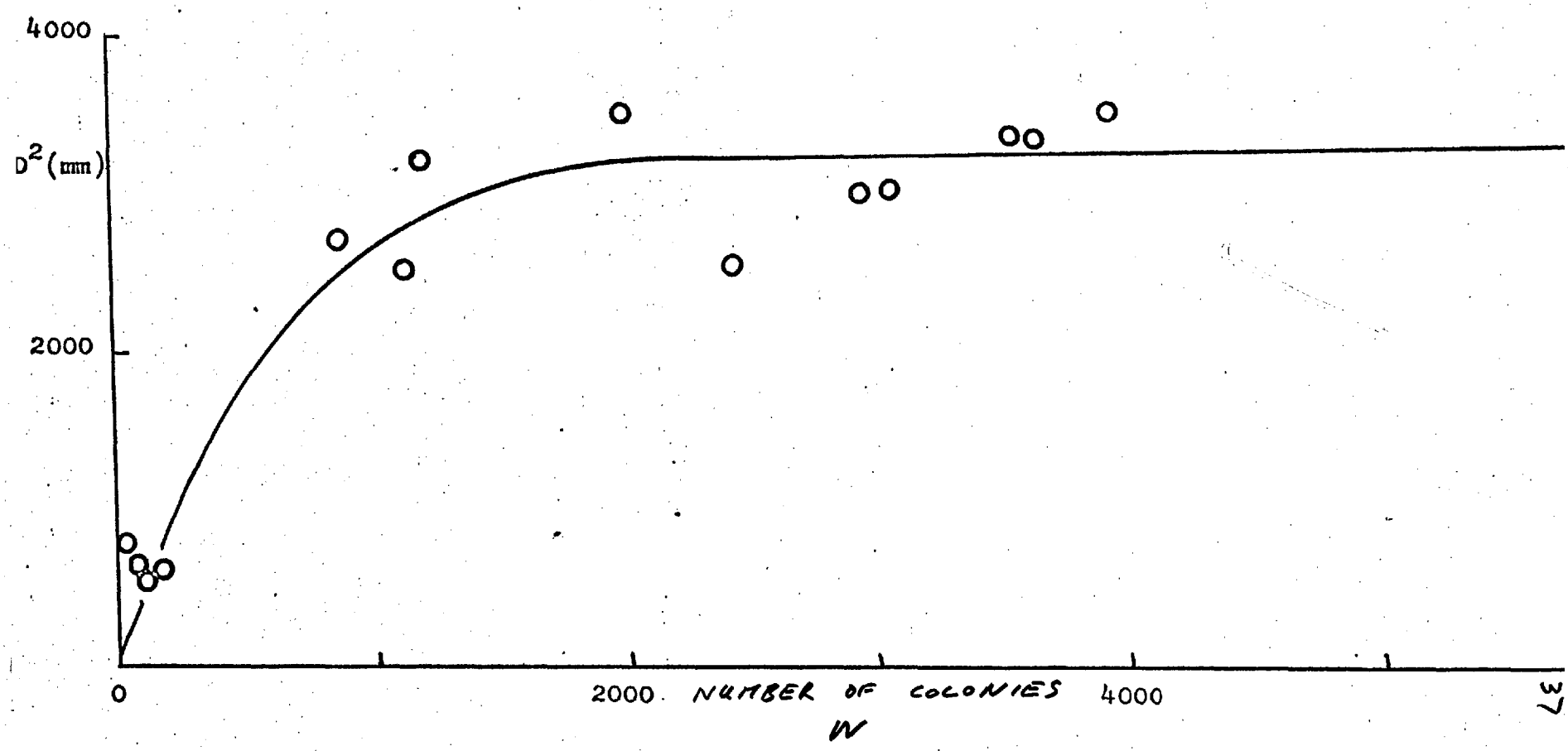


Figure (3-3) shows that for large values of N, ND^2 is linearly related to the volume of the agar, the diameter of the Petri dish being constant. This fact suggests that growth is limited by exhaustion of some constituent of the agar. The average colony diameter falls with reduction in the phosphate buffer concentration (Figure (3-1)) and an approximate linear relation is found between ND^2 and the amount of buffer in unit volume. Thus in these experiments the growth-limiting constituent seems to be the buffer.

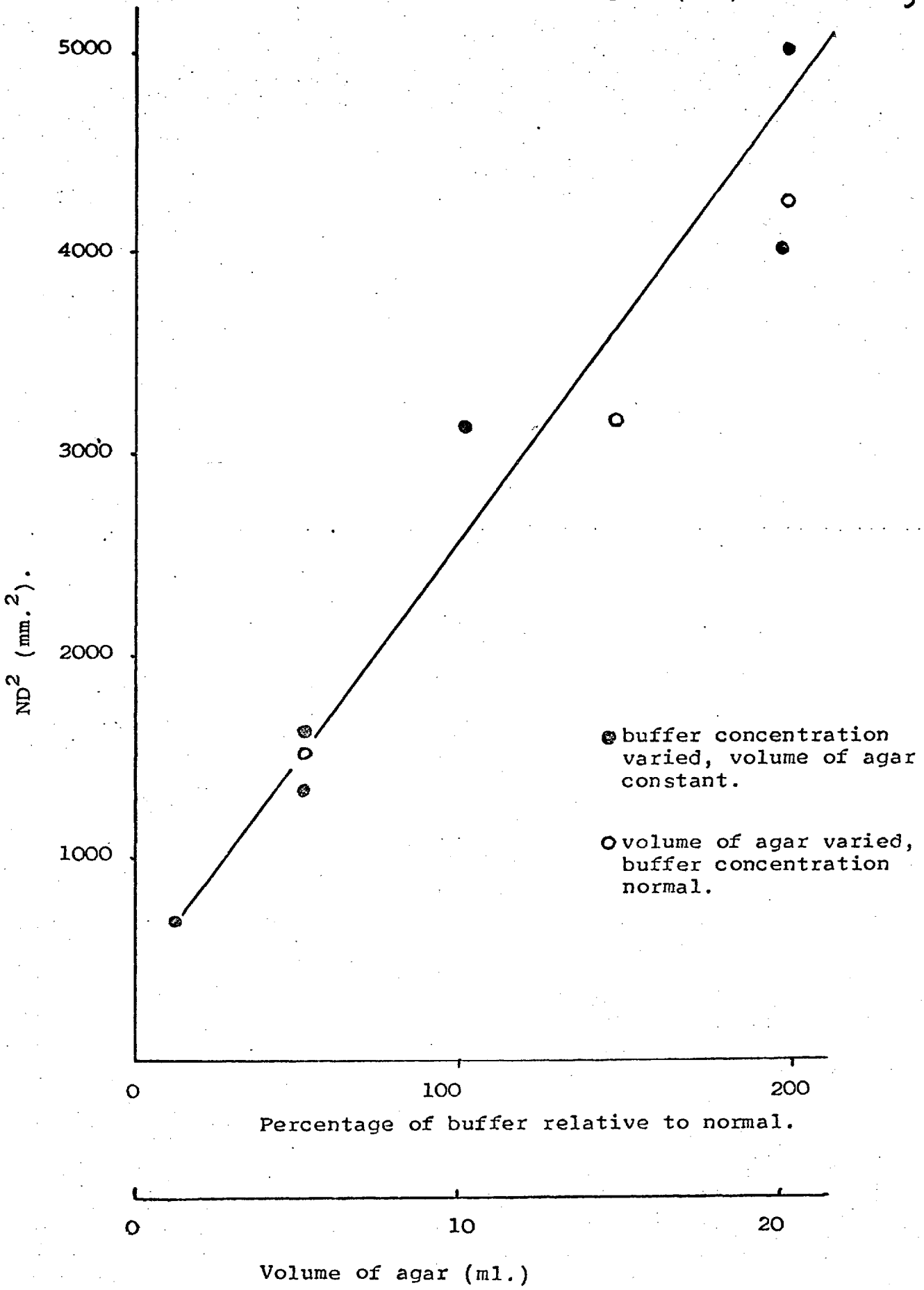
Doubling the standard glucose concentration gave smaller colonies (Figure (3-1)) rather resembling aged colonies on the standard agar. Possibly excess glucose reaching the inner parts of the growing colony is now wastefully fermented to yield more than normal amounts of products which can contribute to the stopping of growth. If this is so, it would imply that with the standard minimal agar the glucose concentration inside the grown colonies has fallen to a low value. At any stage during growth, and especially in the interior of a colony, increased acid formation may occur as a result of oxygen shortage (cf. Pirt 1967). Since, however, with Aerobacter the anaerobic growth rate is about two thirds of the aerobic rate, oxygen shortage itself will never be the sole limiting factor.

Factors affecting the Growth of an Individual Colony:

Lag Times:

As in liquid cultures, cells inoculated on to an agar plate show lags of varying lengths before they begin to divide.

Figure (3-3)


5000
4000
3000
2000
1000
ND^2 (mm.^{2}).
buffer concentration varied, volume of agar constant.
volume of agar varied, buffer concentration normal.
0
100
200
Percentage of buffer relative to normal.
0
10
20
Volume of agar (ml.)

Presently the colony radius increases linearly with time and because of the initial lag, plots of diameter against time cut the time axis at different points. The intercepts are not quite the same thing as the true delay before the first division, but they serve well as a measure of the relative delay which each colony suffers before a steady state of growth is established.

Histograms of colony lags were constructed by measuring under the microscope, the diameters of about 60 colonies on one spread plate at a series of times soon after visible growth appeared. Plots of these diameters against time were nearly parallel so that extrapolation to obtain the intercepts was reasonable. The type of histogram obtained is shown in Figure (3-4) and the spreads as defined earlier are shown in Table (3-1).

If the lag is L and the total time of growth T, the time of active growth T' is T - L. The absolute variations in T' will be due to those in L since T is a fixed period for all the colonies. If area were determined solely by T' then the spread in area would be greater or less than that in L according as T' were smaller or greater than L. In fact T' is often considerably greater than L. It will in fact appear that the spread of lags is insufficient to account for commonly observed spreads in colony diameter.

By the time a colony has reached about 0.3 mm in diameter it has entered the phase of growth where the diameter increases

Figure (3-4).

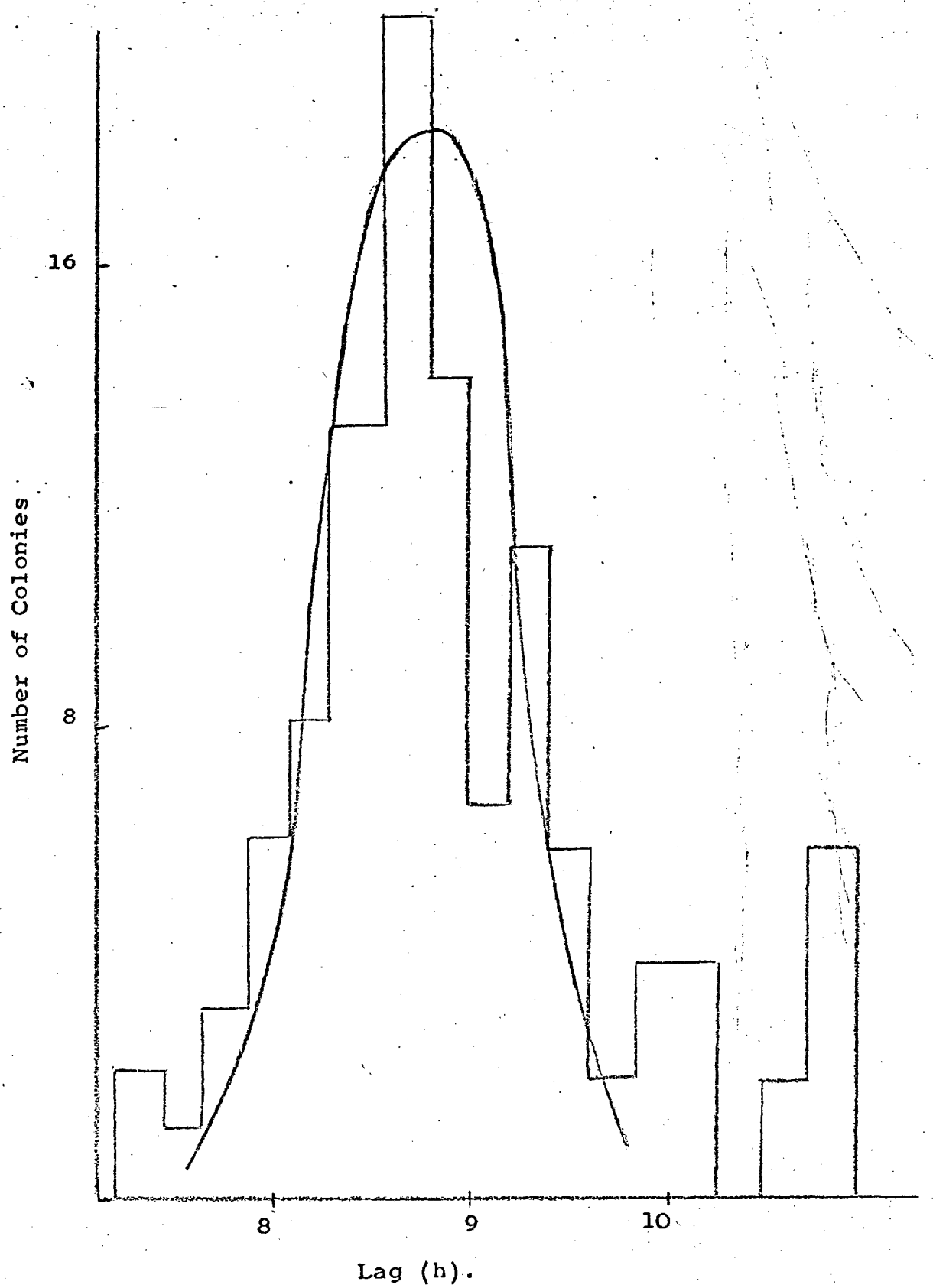


TABLE (3-1) DISTRIBUTION OF LAG TIMES OF INDIVIDUAL COLONIES.

Number of washed cells inoculated on plate.	Average lag time (h)	Spread
100	9.17	[illegible]
1900	9.25	0.15
3000	8.15	0.15

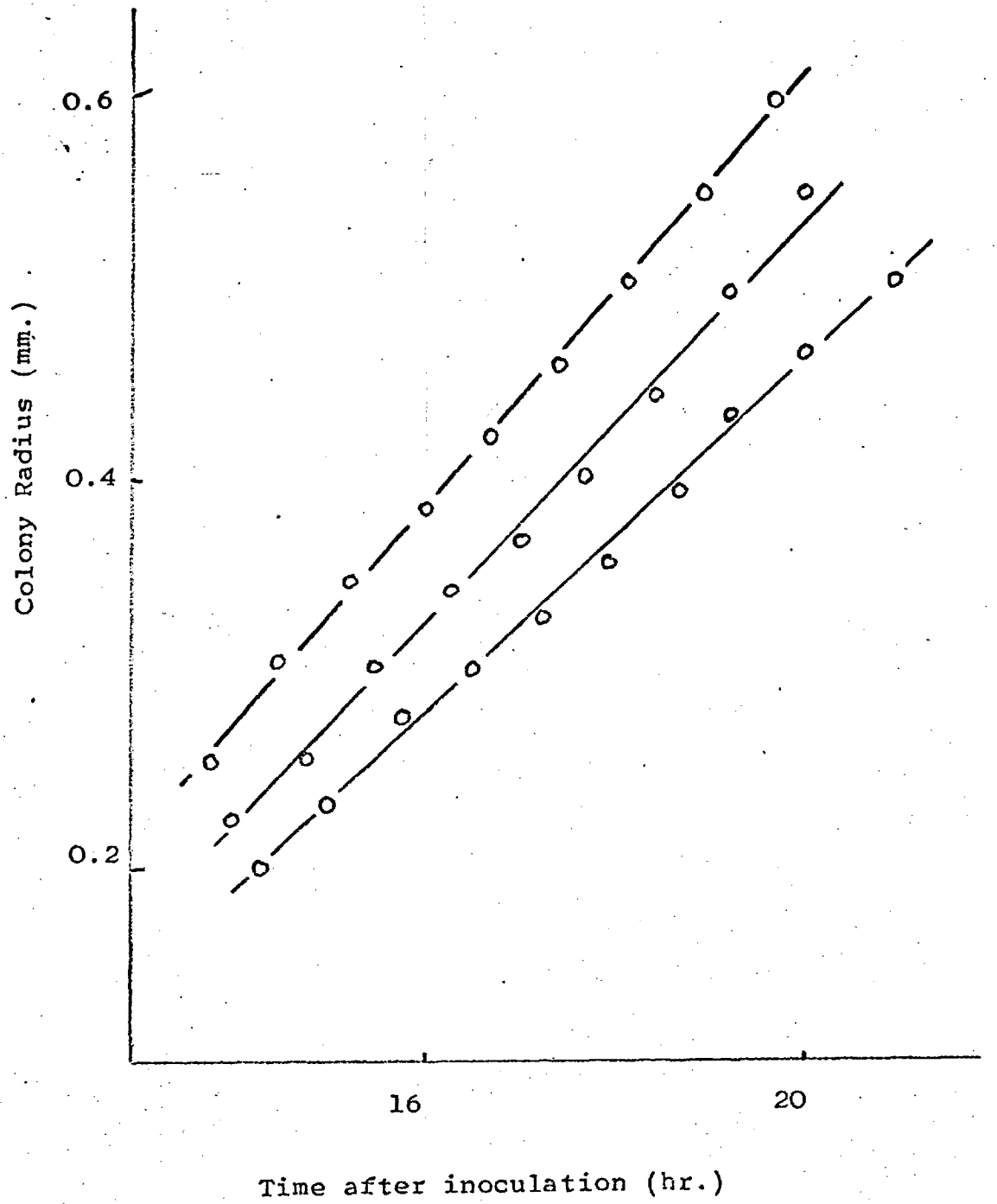
linearly with time. The kinetics of still earlier growth were not studied in these experiments but it is reasonable to assume that at the very outset it is exponential, as in a uniform liquid medium, and that then mutual interactions of the cells slow down the rate, (for general discussion cf Hoffman 1964).

Phase of Growth where Diameter increases linearly with time:

This linear law is often quite closely followed not only by Aerobacter but by other organisms (Dean & Hinshelwood 1956; Plomley 1959; Pirt 1967) and some typical growth curves are shown in Figure (3-5) which refers to spread plates containing about 100 colonies. On them, the linear phase continues until the colony reaches about 80 per cent of its final size. Later the law of growth changes. For similar inocula the upper limit of the linear region is affected by the buffer and glucose concentrations in much the same way as the final size. Table (3-2a) however, shows that the actual rate is practically independent of these concentrations. This implies that for the colonies growing on the normal minimal agar, the rate at which diameter increases in this region is not limited by the rate of diffusion of glucose or of phosphate buffer.

This phase of growth may be explained by a quite simple model in which active growth in the colony is regarded as being confined to an annulus of constant width and vertical thickness. At this stage, moreover, growth is limited by factors other than diffusion, except in so far as upward diffusion may have determined the vertical height (cf. Pirt 1967)

Figure (3-5).



EFFECT OF BUFFER OR GLUCOSE CONCENTRATION ON GROWTH RATE OF COLONIES.

TABLE (3-2a)

Concentration of buffer relative to standard.	Concentration of glucose relative to standard.	Mean rate.
'diameter growth rate'	mm/h.	
2.0	1	0.084
1.0	1	0.100
0.5	1	0.125
0.1	1	0.094
1.0	2	0.105

TABLE (3-2b)

'area growth rate'	sq. mm/h.	
1.0	1	0.98
0.5	1	1.30
0.4	1	1.90
0.3	1	2.30
0.2	1	0.58
0.1	1	0.09
1.0	2	0.06

The volume of the annulus is proportional to $2\pi r\Delta$ and by hypothesis the rate of increase of cell mass is proportional to this. The total mass is proportional to $\pi r^2\delta$ where δ is the vertical thickness.

Then $\frac{d(\pi r^2\delta)}{dt}$ = constant $2\pi r\Delta$ whence dr/dt is constant, so long as Δ is constant.

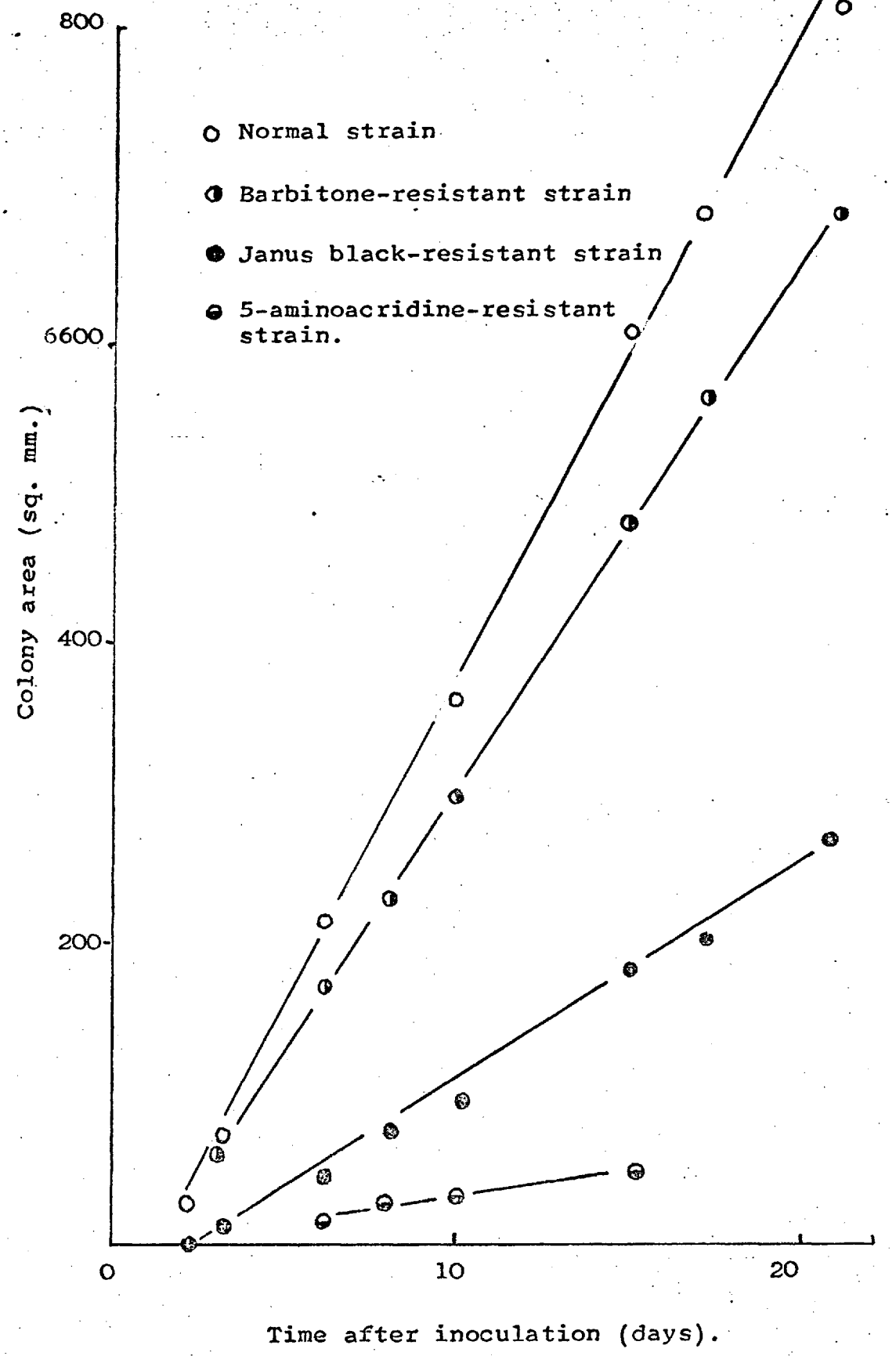
Attempts to verify this model were made by sprinkling fine particles of washed, sterile carborundum dust on to a colony growing in the linear phase and taking microphotographs at intervals. In a series of such photographs the particles showed no detectable movement relative to one another. Thus the colony is not expanding uniformly throughout its mass. If the annular model is correct the width of the growing zone must be quite small relative to the colony diameter (Pirt estimated about 0.09 mm. from the mean generation time and the colony growth rate of Escherichia coli). The experiments described do not rule out the possibility of growth in a very thin layer adjacent to the agar but the implied slip between the upper and lower parts which this would involve is questionable.

Later stages of Growth:

With small inocula, permitting the colonies to attain a considerable size, a slower phase ensues during which a different rate law holds. This phase has been studied in more detail with single colonies obtained by inoculating one point on an agar plate with tip of a platinum wire. The ensuing growth was followed for about 3 weeks and typical growth curves

are given in Figure (3-6) which shows that the area, rather than the diameter of the colony, now increases linearly with the time. The area law corresponds to slower growth than the radial law, and naturally in a gradual transition from growth according to the latter to complete arrest the area law must be followed transiently at some stage and so need not be of deep significance. The law, however, is sometimes followed over a quite considerable range, so that an approximate theoretical model to account for it has some interest. The radial law followed from the assumption that growth occurred in an annulus of constant width. The area law would require a steadily diminishing fraction of the annulus to be available for growth. This is what would happen if an outflowing tide of, for example, acid from the whole colony could no longer be neutralised throughout the whole annulus by an inflowing tide of buffer, or indeed if some other toxic product could escape progressively less easily. If we assume, as an example, that there is a critical acid concentration below which growth ceases, that the acid is formed throughout the colony at a rate proportional to its area while the rate of escape is proportional to the perimeter, then a simple calculation shows that the available width of the annulus is inversely proportional to $\underline{r}$, the radius of the colony. Thus the equation of the last section becomes $\frac{d}{dt}(\pi r^2) = \text{constant}$. $2\pi r \frac{\Delta}{r} = \text{constant}$, which is the area law. The model is only a rough one, but the law to which it applies is an approximate one. The main assumption, namely that diffusion is now beginning to play a more dominant role is,

Figure (3-6).


800
6600
400
200
0
10
20
Colony area (sq. mm.)
Time after inoculation (days).
Normal strain
Barbitone-resistant strain
Janus black-resistant strain
5-aminoacridine-resistant strain.

however, consistent with other evidence to be brought forward.

In the region of the radius law the rate is nearly independent of the buffer concentration, (Table (3-2a)), but in the region where growth approximates to the area law the relation of rate to buffer concentration becomes more complex. From 10 to 30 per cent of the standard concentration the rate rises steeply, passes through a maximum at about 40 per cent and then drops again. But at 100 per cent it is still greater than at 10 per cent, (Table (3-2b)). Toxic products such as alcohols formed by fermentation in the interior of the colony may well play a part and inhibit growth at the perimeter, a possibility strengthened by the observation that in this phase doubling the glucose concentration has a strongly inhibitory action.

The experiments of Pirt (1967) use conditions which are intermediate between the 'early' and 'late' phases of growth described above, but appear on the whole to be consistent with the present ones.

Measurements of Diffusion in Agar Gel:

Measurements of the diffusion of glucose into a cylinder of agar gel from an aqueous solution were made to verify that the standard diffusion laws could be used for the 1.25 per cent agar gel with which most of the experiments were made. The molten aqueous agar solution was sucked into a length of 6 mm. bore precision glass tubing and held in place by suction till set. Several such tubes were set up vertically with one end

immersed in a stirred glucose solution, and after suitable periods of time agar was removed and cut into portions of 1 to 2 cm. length which were analysed for glucose.

A typical graph of glucose concentration against distance from the face in contact with the solution is shown in Figure (3-7a). For simple one dimensional diffusion the following equation should apply

$$c = c_o \operatorname{erfc} \left(\frac{x}{2\sqrt{Kt}} \right)$$

where c is the concentration at time t and distance x and c_o the constant concentration in the reservoir. K is the diffusion coefficient.

Plots of ~~against~~ x against the square root of time should be linear for each value of c/c_o, and the approximate applicability of the formula is shown in Figure (3-7b).

Comparison of experimental and theoretical curves shows that glucose diffuses more slowly at its higher concentrations. To obtain accurate agreement with the classical diffusion formula rather elaborate corrections would be needed. For the present, however, it will appear that very accurate values for diffusion coefficients will not be required and it can be concluded that the behaviour of glucose in 1.25 per cent agar gel is not very different from its behaviour in water, (cf. Stiles and Adair 1921; Hill 1928, 1965; Friedman and Kraemer 1930).

Figure (3-7a)

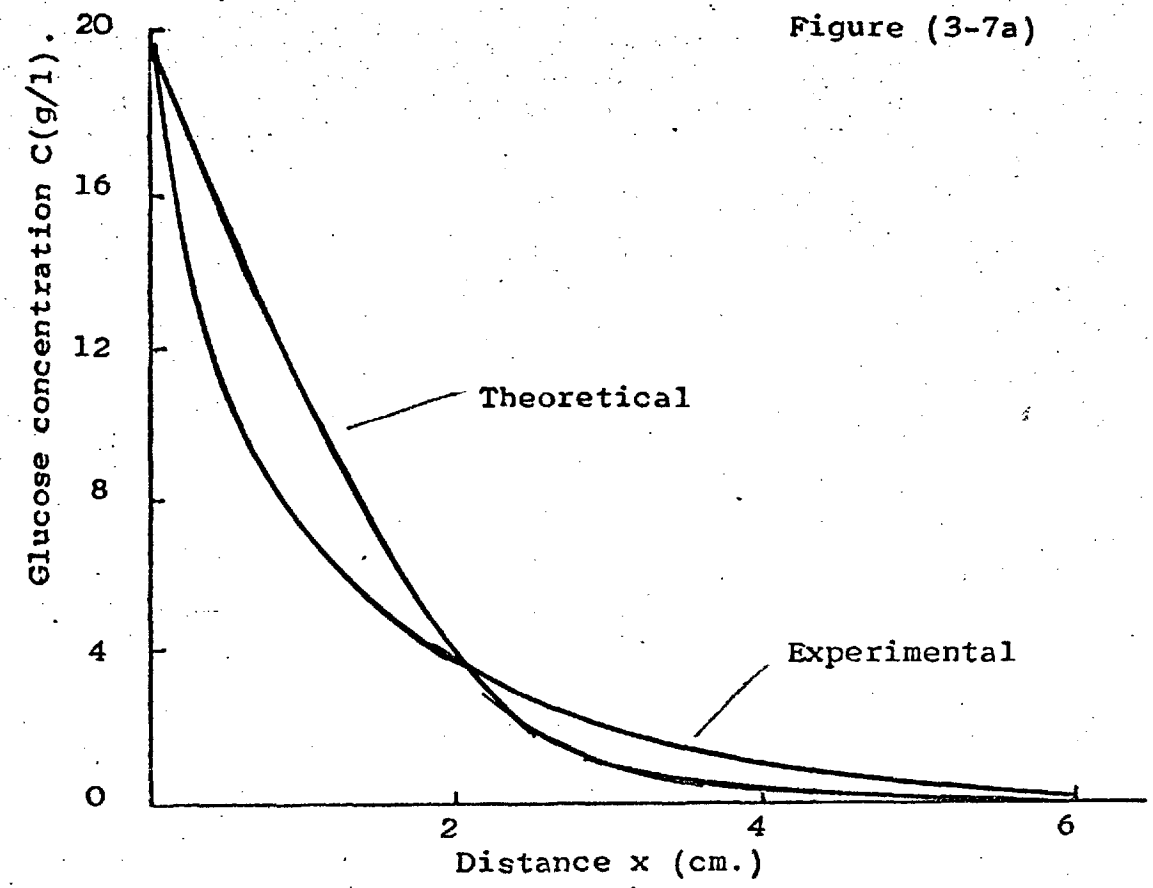
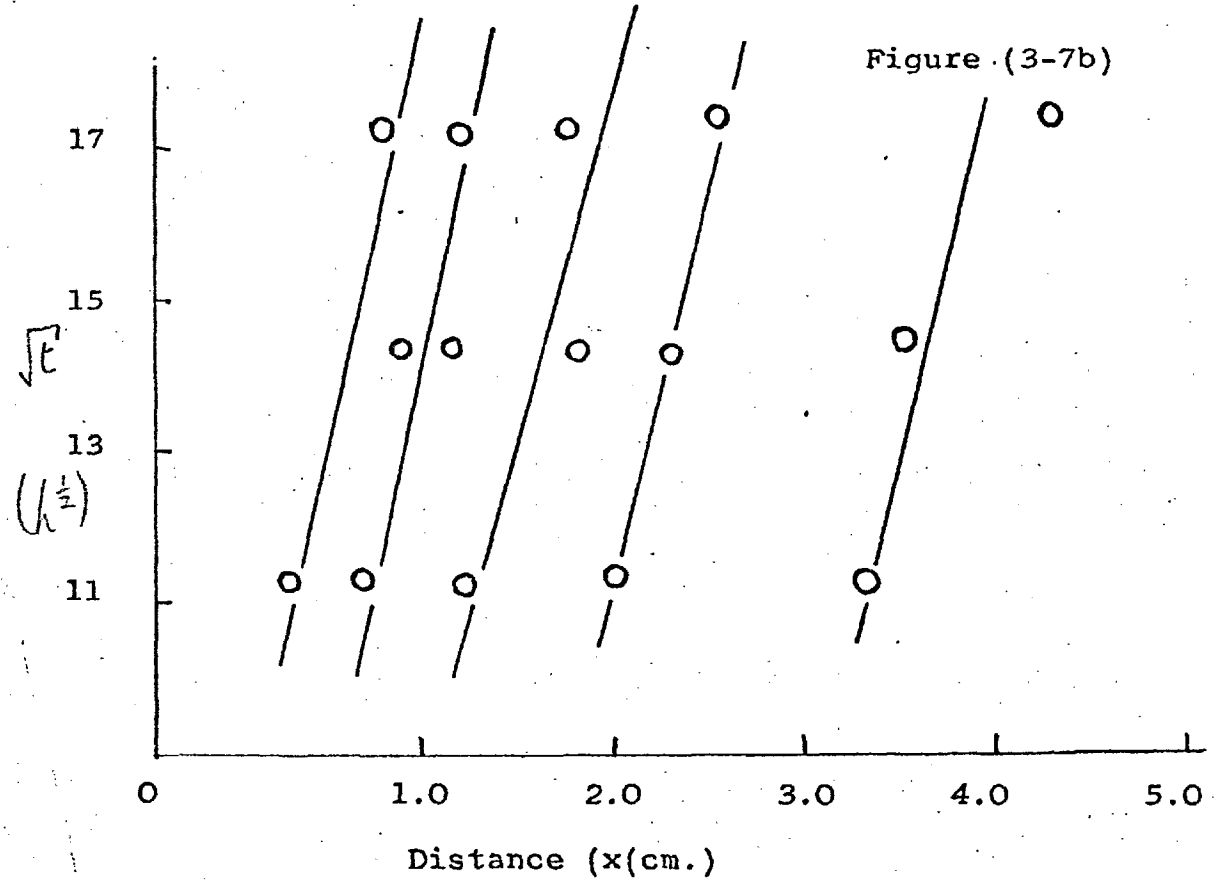


Figure (3-7b)



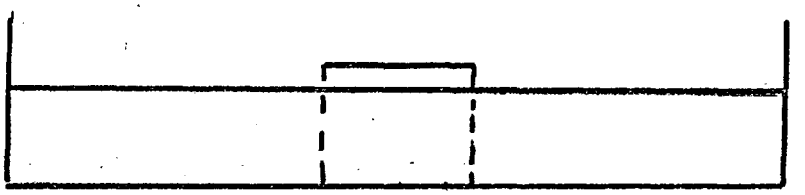
Likely Role of Diffusion in Colony Growth on Plates:

The standard diffusion equations apply well enough to justify the following approximate calculation which indicates, with some margin of safety, that rate of diffusion, whether of glucose or buffer, up to the colony is unlikely to determine the rate of increase of diameter on plates containing a considerable number of colonies, where the final diameter is in the neighbourhood of 1 mm, and where most of the growth is over within a day.

On the other hand, with the protracted growth of a few colonies on a sparsely covered plate, the final rate is likely to be governed by diffusion.

Let Figure (3-8) represent a colony on a plate. The complicated geometry of the situation is idealised as follows. The volume indicated by the dotted lines is allocated as private to the colony vertically above it, medium constituents which have once diffused into it being assumed unavailable for any of the competitors. Let the area of the clyindrical walls be A. Diffusion from remoter parts of the plate through this area will not be less than from a square prism of cross section A (since in fact with the actual circular section of the plate material will converge from a larger volume than with the square prism. We shall be on the safe side if we use the simple formula of [Page 50]. In that formula $c = c_o$ at $x = 0$ and at $t = 0$, $c = 0$ everywhere else, but for the present situation these conditions must be changed to $c = 0$ at $x = 0$ and at $t = 0$, $c = c_o$

Figure (3-8).



everywhere else. Thus the c of the former equation becomes $c_o - c$ and c/c_o becomes $(1 - c/c_o)$ and the solution is now $c/c_o = \text{erf } x/2(Kt)^{\frac{1}{2}}$. We will take K to be the 10^{-4} cm^2 min^{-1} and plot curves in Figure (3-9).

At time t areas such as OAB in this Figure represent the concentration per unit cross section lost to the system, the amount having diffused into the dotted region of Figure (3-8) (at $x = 0$) being given by c_o x area OAB. At $t = 10$ the mass lost is found by measurement of area to be approximately 0.04 c_o g/cm^3. If c_o is taken as the initial concentration of phosphate in the agar, 10^{-2} g/cm^3, the flux into the sink is 4×10^{-4} g/cm^3 in 10 min.

Now a total area of colonies of 25 cm^2 consumes about 0.2 g phosphate so that a colony 1 mm. in diameter and 0.31 cm. in circumference would have consumed 6.3×10^{-5} g. But in 10 min. diffusion could provide up to 4×10^{-4} g per unit area of the cylindrical dotted surface in Figure (3-8). The area of this surface is approximately 0.31×0.5 cm^2, the depth of the agar on the plate being at most 0.5 cm. Thus in 10 min the supply is $4 \times 10^{-4} \times 0.15$ which is 6×10^{-5} g. This is nearly the same as the estimated requirement for the actual growth which in fact occurred not over 10 but over more like 1000 min.

Thus we may conclude with some margin of safety that in the early stages of reasonably densely spread plates diffusion will have played very little part in limiting growth.

Figure (3-9).

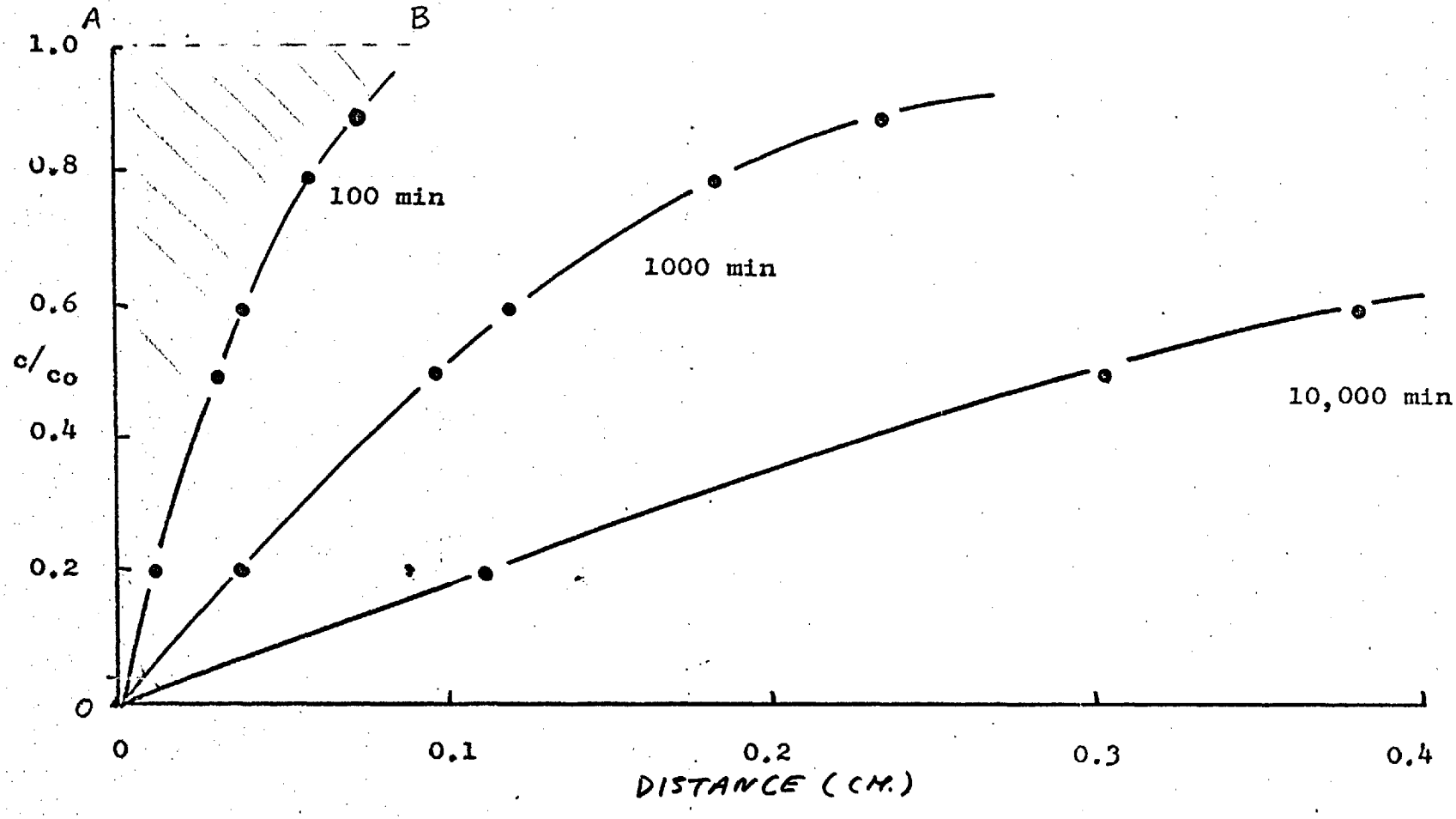

A
B
1.0
0.8
0.6
c/co
0.4
0.2
0
100 min
1000 min
10,000 min
0
0.1
0.2
0.3
0.4
DISTANCE (CM.)

Figure (3-9) however, shows that in 1000 min only about three times as much will be supplied by diffusion as in 10 min, and by the time a colony has reached a diameter of a centimeter, which may take a week or more, diffusion will almost certainly have assumed the major role.

Later, an estimate of the time at which diffusion becomes limiting with a single colony will be made. The final size of colonies on spread plates is, however, a separate matter since limitation of actual growth rate, and limitation of final size are different problems. On a spread plate the final sizes will be partly determined by competition between the colonies themselves. If the colonies on a standard plate are surrounded by circles just touching one another, the average radius would be about 0.1 cm. with 2000 colonies and about 0.45cm. with 100. With the more populous plates the diffusion times will remain small until the plate is nearly exhausted, so that we have the equivalent of a stirred medium. Thus competition will remain relatively unimportant until the end of growth. as numbers fall and diffusion times lengthen the system more and more assumes the character of competing sinks, and as shown later, the final size of individual colonies is more and more determined by the free area which they happen to have around them.

Populations of Colonies on Plates:

In the light of the preceding sections it appears that different colonies have varying starts one on the other in virtue of varying lags, and that thereafter for some time they

grow according to the law of linear increase in diameter with time, diffusion at this stage not seriously limiting their actual rate of increase : that before growth ceases the law changes, and in a way which can be explained in terms of increasing dependence on diffusion. This change will explain several other interesting phenomena. With spread plates the diffusion stage involves competition for nutrient or for buffering capacity with neighbouring colonies. These facts prove to be very relevant to the statistical distribution of colony sizes at various times.

Probability Considerations relating to Colony Size Distribution:

Colonies randomly distributed over a plate differ from one another in the extent of the competition from neighbours and in the relative areas of plate available for supplying them or for receiving their waste products. Since the rigorous solution of this diffusion problem is somewhat formidable, geometric measures might be chosen to represent the 'free area' around a colony, or conversely the degree of exposure to competition or interference by neighbours.

A test was first made by scattering grains of sand on a circular area of squared paper and marking their positions by dots. A given dot was joined by pencil lines to its next neighbours in every direction, and the radial lines were then bisected so that the mid-points could be joined to give an irregular multi-pointed star. The areas of these stars were

measured by square counting and histograms were constructed for the frequency distribution of the areas. Smoothed curves from the histograms were roughly Gaussian, with some distortion probably attributable to the finite size of the circle. The modal values of the areas, which are not far from the average values, were recorded, and also the width of the distribution, which was again measured by the ratio of the distance between the points where the frequency was half the maximum, to the average value. Table (3-3) shows that if the final area of a colony was determined by the free space measured in this way, the width or spread of the distribution would be in the range 1 to 1.4. In terms of actual colony diameters the corresponding spreads would be 0.5 to 0.7.

For fully grown and exhausted plates at long times the spread is indeed in this sort of range, suggesting that free space may well be a largely determining factor.

Another way of assessing the degree of potential mutual interference of colonies is to count the number of competitor colonies included within a circle of assigned radius about the centre of the given colony. The radius must be chosen neither too great nor too small. If too great, the numbers of intruders into the domains of the individual colonies will be determined only by the average density : if too small, the number will tend always to zero. For randomly distributed colonies there will be for each a most probable number of intruders within a given circumscribing circle, and whether or not the actual number is above or below this value can be

TABLE (3-3) STATISTICS OF RANDOM GRAIN DISTRIBUTION.

Total grains in circular area.	Modal value of 'free area' (sq. mm.).	$NA \times 10^{-4}$	Spread.
N	A		
44	320	1.25	1.22
100	125	1.40	1.05
284	50	1.40	1.40

correlated with observations on the size attained by that colony.

Consider a small area a on a plate of area A containing N randomly distributed colonies. The chance that there are r colonies in the area a is given by

$$P = \frac{N!}{r!\,(N-r)!}\left(\frac{a}{A}\right)^{r}\left(1-\frac{a}{A}\right)^{N-r}$$

The maximum value of P can be found by the standard method and the use of Stirling's approximation to occur when $r/N = a/A$. The value of P/P_{max} can also be calculated for various values or r and a frequency distribution curve plotted. In two examples where r_{max} was 6 and 10 respectively the spread of the distribution was about 0.7. This simple criterion of the number of intruders into the assigned domain of a given colony can sometimes be informative. A rather slow-growing strain of Saccharomyces cerevisiae which according to the consideration advanced above would be unlikely for a long time to have its growth limited by diffusion, was plated on an agar medium and the colonies were photographed as soon as they had ceased to increase appreciably in size. On an enlarged print of this the diameters were measured, and for each colony the number of intruders within circles of 1.0 and 1.75 cm. radius respectively was counted. For 11 colonies above the median diamater the total numbers of these intruders were 14 and 33 respectively and for 11 colonies below the median they were 15 and 36. Thus the influence of competition is here seen to be small.

On the other hand, when a similar test was carried out with the faster growing Aerobacter aerogenes, although competition had little effect on colony diameter in the early stages of growth, as time increased and accordingly diffusion became more and more important, paucity of intruders increasingly favoured above average diameter. This is shown in Table (3-4) where the results refer to a plate containing about 200 colonies, which was photographed at various times after spreading.

These photographs were also used in a more elaborate test of mutual interference. In them each colony was joined by a straight line to any other colony not eclipsed by an intervening one. If the distances are x_1, x_2, x_3 --- the expression $\sum \frac{1}{x_1^2}$ now gives a qualitatively reasonable measure of interference or competition and $1/\sum \frac{1}{x_1^2}$ a corresponding measure of 'free space', f . This can be elaborated by a rough allowance for the differing sizes of the neighbours and f can be replaced, by f' which is $1/\sum \frac{D_1^2}{x_1^2}$ where D_1, D_2, D_3 ---- are the diameters. In Figure (3-10) the colony diameters at 22 and 135 hrs. are plotted against f'. There is little or no dependence on the free area at the earlier time, but a marked dependence at the later time. To eliminate subjective bias lines through the observational points were calculated by the method of least squares with the following results:

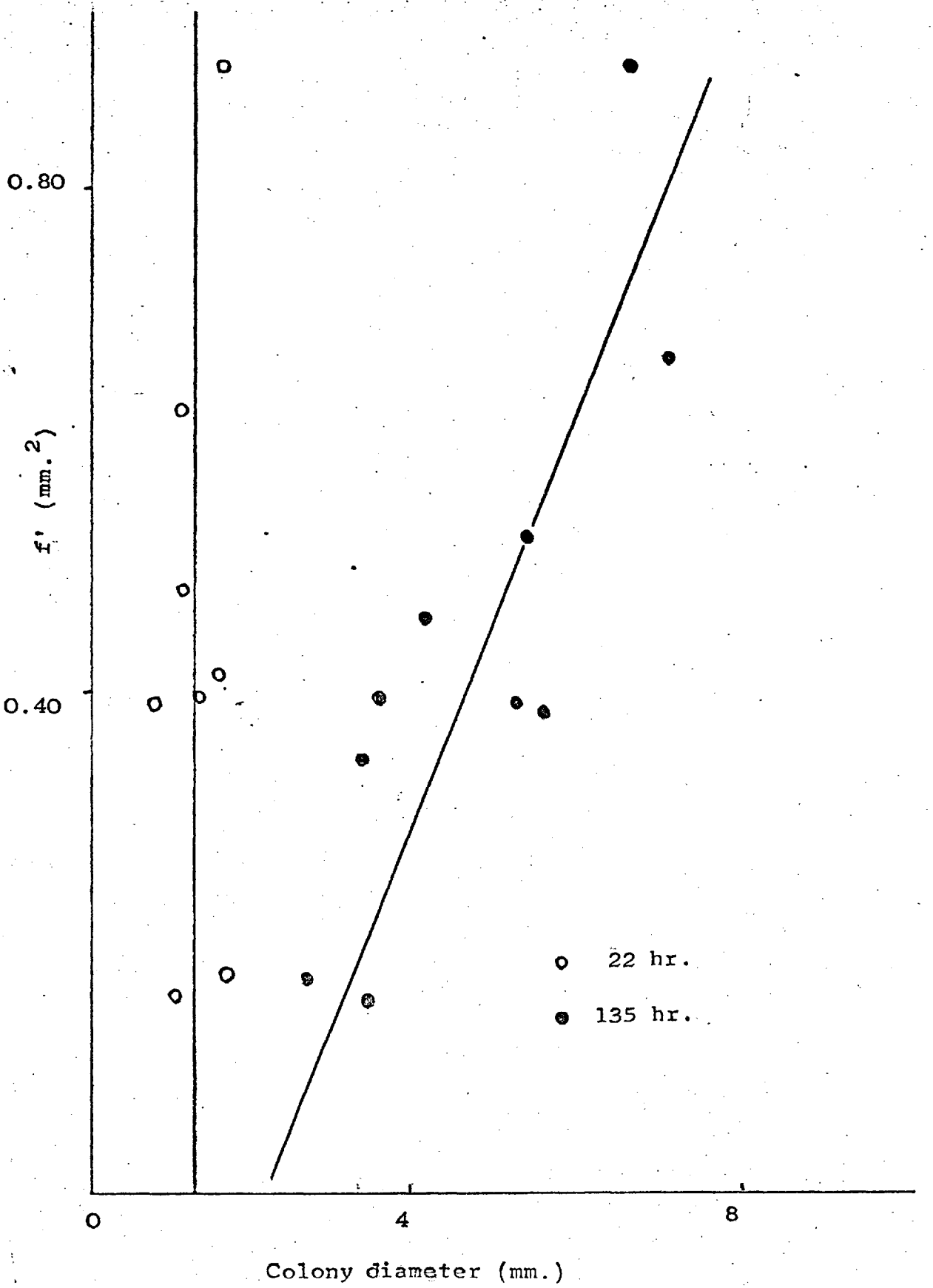
at 22 hrs. $D = 1.15 + 0.02\ f'$

at 135 hrs. $D = 2.34 + 5.32\ f'$

TABLE (3-4) EFFECT OF FREE AREA AROUND COLONIES ON COLONY DIAMETER.

Time (h)	Average number of competitors within chosen reference circle.		Ratio.
	For colonies above average diameter.	For colonies below average diameter.	
17.3	4.4	4.04	1.09
22.8	5.0	4.80	1.05
40.5	4.14	5.18	0.80
83.0	3.23	5.83	0.55
135.0	3.33	5.90	0.57

Figure (3-10).



The dependence of diameter on f' is shown by the ratio of the term involving f' to the intercept. This is 0.017 at 22 hrs., but 2.31 at 135 hrs. and thus the dependence increases 135 times between the earlier and later measurements. When the less elaborate function, f, was used the corresponding increase was 30-fold.

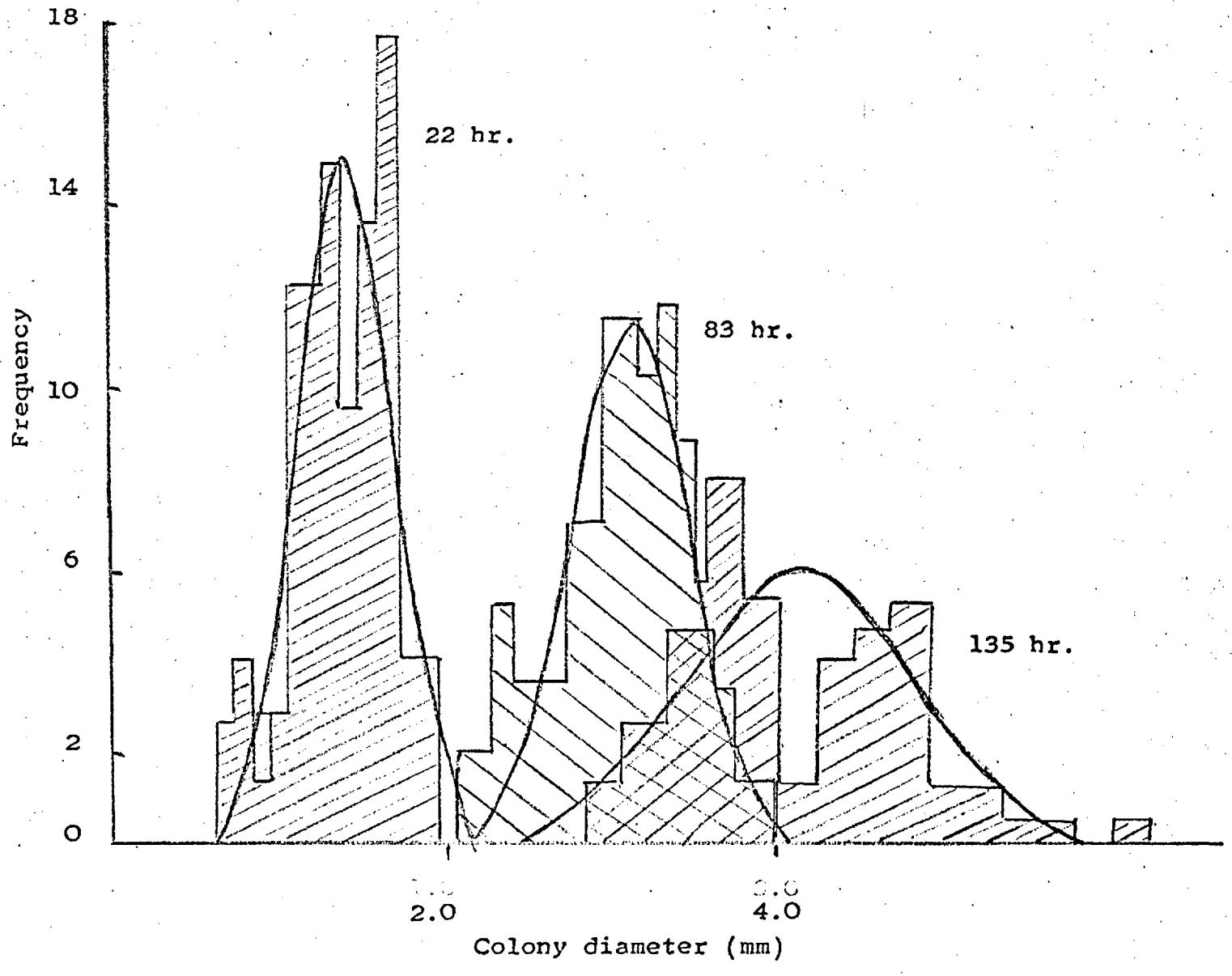
Statistical spread of Colony Areas:

Some typical histograms of colony sizes are shown in Figure (3-11). A precise mathematical treatment would be very complicated but the following semi-quantitative discussion shows the main principles.

When N, the number on the plate, is large and the colonies are small, distances are such that diffusion does not limit supply rate and growth is determined by slower internal processes. All colonies will cease to expand at the same time when, what is the equivalent of a stirred medium becomes unsuitable for further growth. The size variations will be mainly determined by differences in the individual lag times.

On a mean lag of about 10 hours there is a spread of about 15 per cent, that is about 1.5 hours. The shortest time of actual growth, that is the time from the end of the lag to the moment of size measurement, is also about 10 hours, and the variation in this will thus be 15 per cent also. It is, however, a constant absolute time and its proportional effect gets progressively less as time increases and the colonies become bigger. The final radius of the colony is

Figure (3-11).

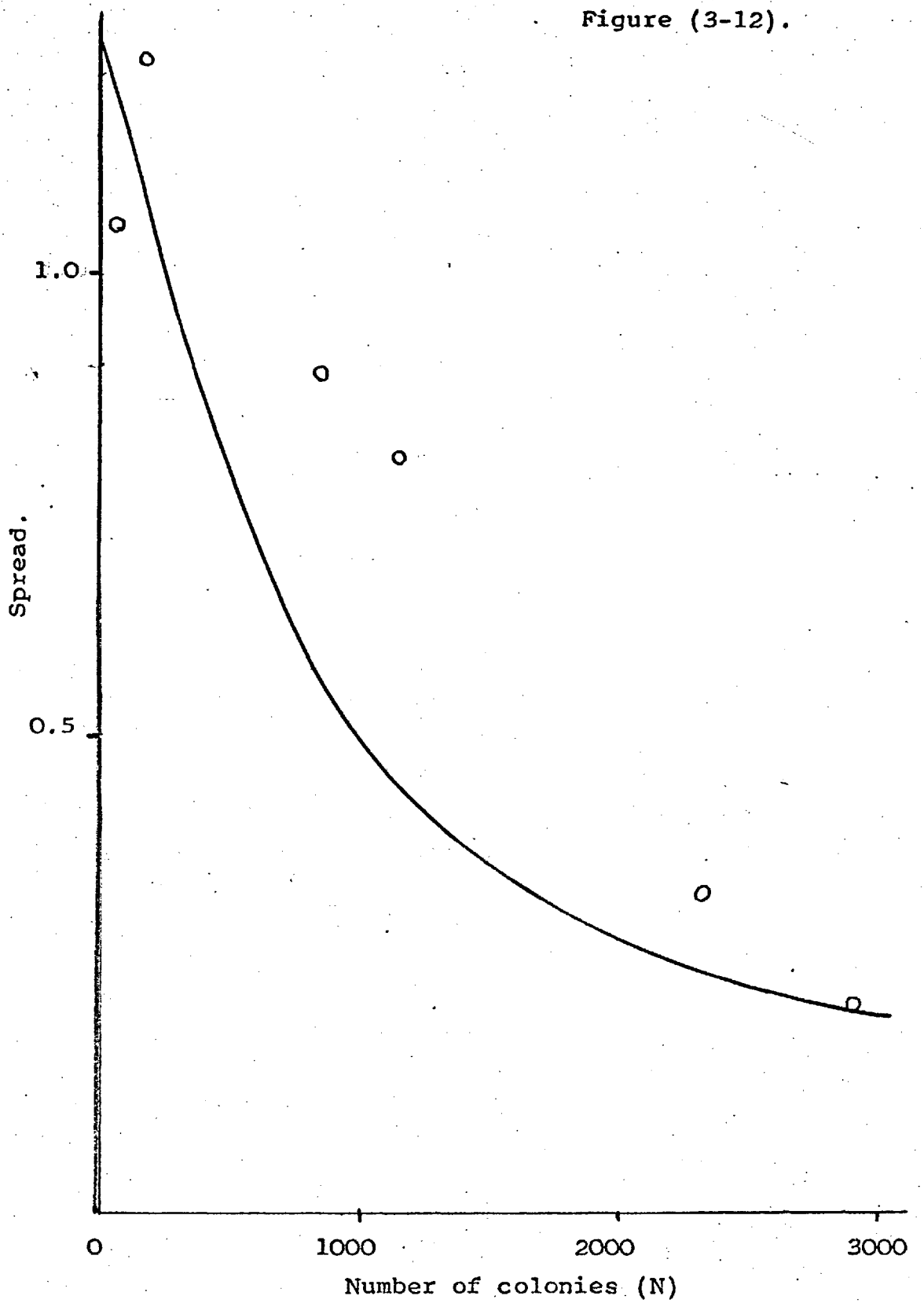

18
14
10
6
2
0
Frequency
22 hr.
83 hr.
135 hr.
2.0
4.0
Colony diameter (mm)

proportional sometimes to the growth time: in other circumstances the area rather than the radius increases linearly with time. In the first case the spread in area will be 30 per cent and in the second 15. We shall assume roughly that the measurements at about 10 hours will show a spread midway between these, that is 22 per cent. This will, however, increase if the lag is more prolonged. It will apply only to densely populated plates at a total time of about 20 hours. From this point on, the influence of the variable lag time will become relatively less and less important. The observed spread in area, however, increases considerably and this is due to the increasing influence of diffusion rate in determining growth, and consequent dependence on free area. When growth is entirely limited by diffusion the spread in area will be determined by competition between the randomly scattered growth centres. The considerations of the foregoing sections suggest a figure of 100 to 140 per cent for the spread due to this factor. Thus we might expect a change in the spread from about 0.22 or somewhat more to about 1.20 as the ratio of diffusion time to inherent growth time increases.

This ratio increases as the number of colonies on the plate decreases and for a given number of colonies it increases as the plate moves towards exhaustion.

The variation of the spread for fully grown colonies with the number is compared with expectation in Figure (3-12). The variation from the early to the late stages of growth is

Figure (3-12).



implicit in Figure (3-10) where the dependence on free area, which implies dependence on diffusion, is shown to increase very markedly with the stage of growth (for a plate containing about 150 colonies).

A precise theoretical treatment of the variation of spread with the number of colonies on the plate is scarcely possible, each colony being surrounded by a variable number of neighbours all at different distances. But a relatively crude model will suffice to show that the observed behaviour is consistent with the order of magnitude of the diffusion times.

Consider the case of two sinks where the concentration is kept at zero in a medium where the initial concentration is c_o. Let the sinks be one cm^2 in area and the distance h apart. The integration of the diffusion equation gives

$$c/c_o = \frac{4}{\pi}\left[\sin\frac{\pi x}{h}\, e^{-(\frac{\pi}{h})^2 Kt} + \frac{1}{3}\sin\frac{3\pi x}{h}\, e^{-(\frac{3x}{h})^2 Kt} + \frac{1}{5}\sin\frac{5\pi x}{h}\, e^{-(\frac{5x}{h})^2 Kt} \ldots\right.$$

c being zero at $x = 0$ and $x = h$. From this x can be plotted at points from 0 to h. A graphical integration easily gives the total amount of material which has disappeared into the sinks at time t and this can be calculated for various values of h and t. A plot can then be made of the time needed for the residual amount of material in the medium to drop below, say, 5 per cent as a function of h.

Now h the distance between the sinks might be crudely compared to the average distance between colonies, or to twice the radius of the 'free area' round the average colony. On this basis and with K taken as 10^{-4} cm^2 min^{-1} we have with N = 400 a diffusion time of about 1050 min and at N = 50 of about 4700 min. If the times taken for colonies to reach 95 per cent of the final size at these populations are calculated by the radius law we find they would have been 1550 and 4400 min. respectively. The former is substantially greater than the diffusion time and the latter slightly smaller. Thus at N = 400 radial law growth could be maintained for most of the time, whereas in the case of N = 50 it would have been scarcely possible to maintain it. We might conclude that on rather densely spread plates almost complete growth could occur without limitation by diffusion while with sparsely covered plates diffusion would have played a limiting role for a considerable part of the time.

The correspondence between the system of colonies on the plate and the two sinks being only rough it is hardly possible to estimate more closely where the transition would occur, but it is clear that the right general region is predicted. One further consideration is, however, relevant. Owing to the random distribution of colonies on the spread plate, the law of which is given approximately in the previous section, the radius of the 'free area' around about one in eight of the colonies will be greater than the average by more than a third

(calculated by assuming a Gaussian distribution). This would have corresponded to a diffusion time of 7000 min., so that for these, growth would have been even more delayed by diffusion. Nevertheless, the interplay of the various diffusion fields will be highly complex and, for a proportion of colonies at the last stages of growth, slow communication with distant parts of the plate will be of significance.

Later Stages of Growth:
Instability of Perimeter.

After the earliest stages, when the colony is still below the threshold of visibility to the naked eye, it can for some time be treated as a regular circular disc of approximately constant thickness. With a considerable number on the plate, growth has usually reached a point near its limit before there is any perceptible distortion of this circular pattern. But as the number diminishes so that growth can be protracted and a large area reached, new phenomena make their appearance. They begin with an instability of the perimeter, which loses its smooth circular form and develops undulations. These become more and more exaggerated as time goes on, but according to a pattern depending upon the strain of organism, the composition of the solid substrate and the buffer concentration. The morphological changes will be discussed in the next chapter, but first further evidence that this prolonged growth is regulated by the diffusion of nutrients or byproducts will be presented.

Figure (3-13) shows the growth rate of a single colony on a circular plate and the rate at which the required material could be supplied by diffusion if this limited growth. The experimental growth rate for a colony of the methylene blue-resistant strain is also shown. The calculation is made with $K = 10^{-4}$ cm^2 min^{-1} and is an approximate one only, by the method of finite differences applied to successive 0.5 cm. zones. It is not very accurate, but it is evident that only after several days would diffusion be slow enough to limit growth and it is just about at this time that the change of appearance in the colony sets in.

To apply a sharper test, a special experiment was made in which normal agar, 0.5 cm. deep, was contained in a long narrow rectangular dish and inoculated with a layer of cells across one end. The progress of the front along the trough was then measured and compared with the diffusion rate, which could now be calculated from the linear diffusion law. The results are given in Figure (3-14); for this calculation diffusion of buffer was taken as the most important quantity. It can be seen that there is quite good agreement between the theoretically predicted and the experimentally observed rates of advance.

Figure (3-13).

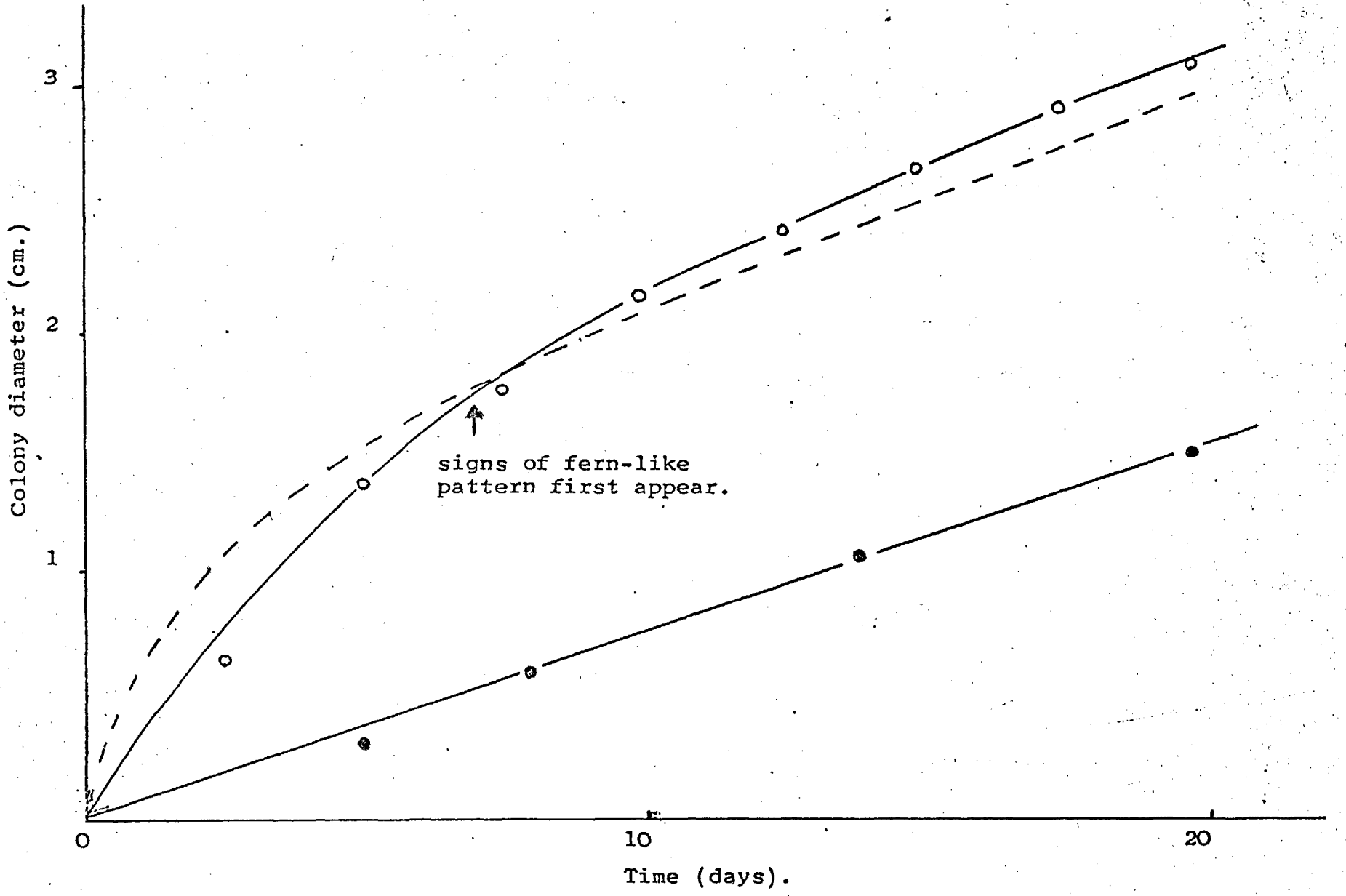
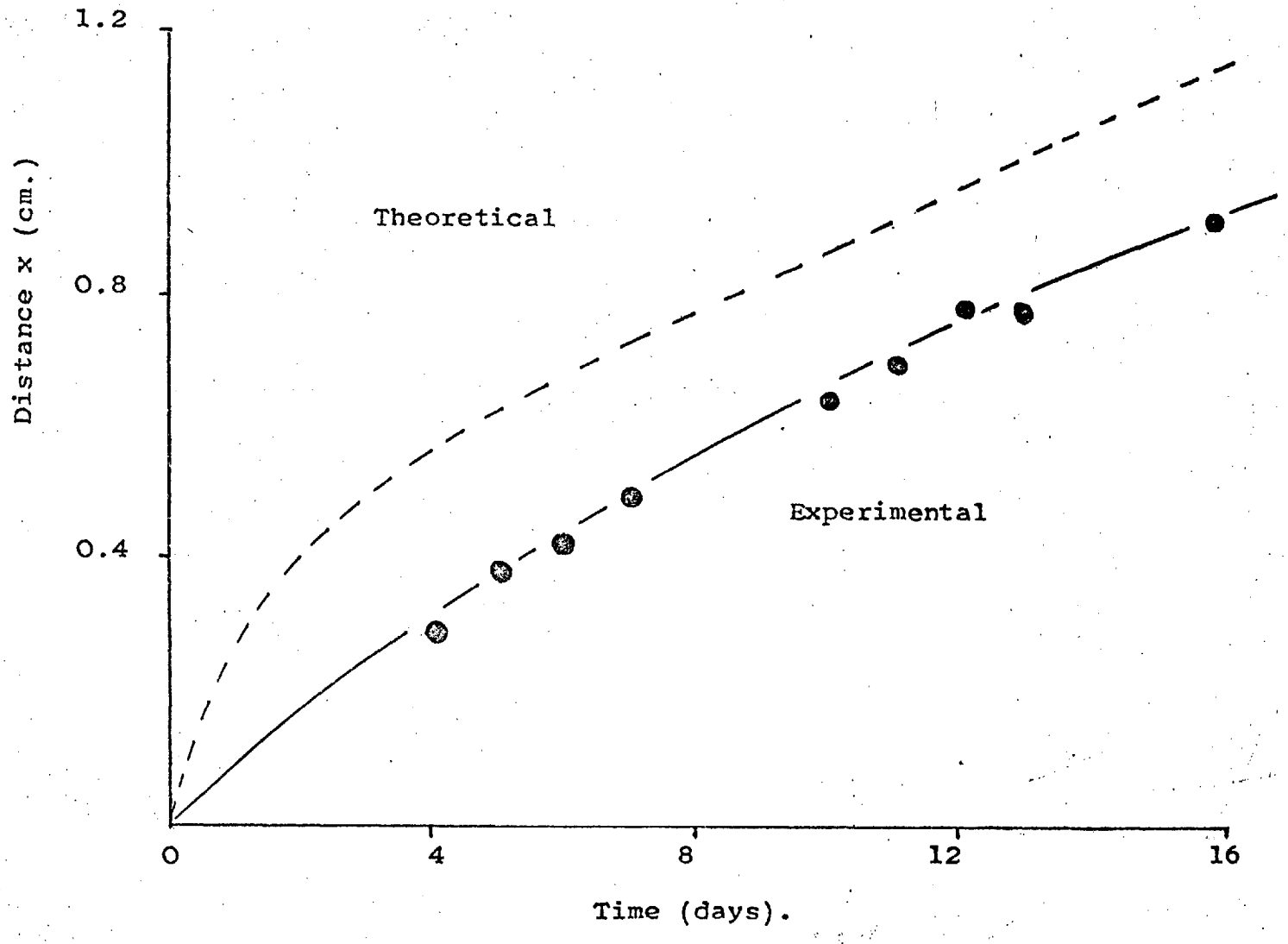

Colony diameter (cm.)
3
2
1
0
10
20
Time (days).
signs of fern-like
pattern first appear.

Figure (3-14).


1.2
0.8
0.4
Distance x (cm.)
Theoretical
Experimental
0
4
8
12
16
Time (days).

Chapter 4.
Morphological Changes.

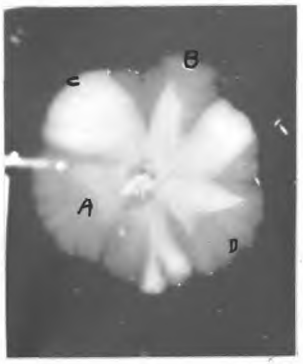
In Chapter 3, it was shown from theoretical and experimental studies, that for a single colony on an agar plate the diffusion of nutrients and by-products becomes important at about two days after inoculation. Thus, the rate at which the colony increases in area after this time (page 45) depends upon the concentration of buffer and glucose in the agar and hence upon the rate at which these substances can diffuse towards the colony and inhibitory by-products of growth away from it. In this section the morphological changes which take place in a colony of Aerobacter aerogenes during this prolonged growth, will be considered.

The brand of agar powder used to make the gel has quite an important effect upon the growth of a large colony, this is discussed in Chapter 5, but for the present studies, one brand of agar was used throughout, namely "Difco Bacto Agar" (see Chapter 2 for more detail).

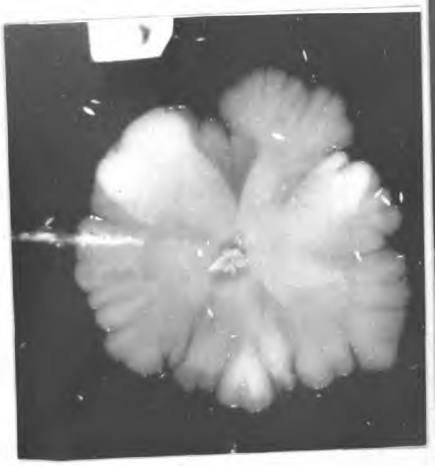
Plate (4-1) shows the development of a typical colony formed by the normal strain of Aerobacter aerogenes on normal minimal agar, and in Figure (4-1) the perimeter of such a colony at different times during its growth is illustrated. From this figure it can be seen that during the first 5-6 days of growth the small irregularities in the perimeter caused by the inoculation are reproduced, sometimes being slightly decreased, and the perimeter remains basically convex.

Plate (4-1)

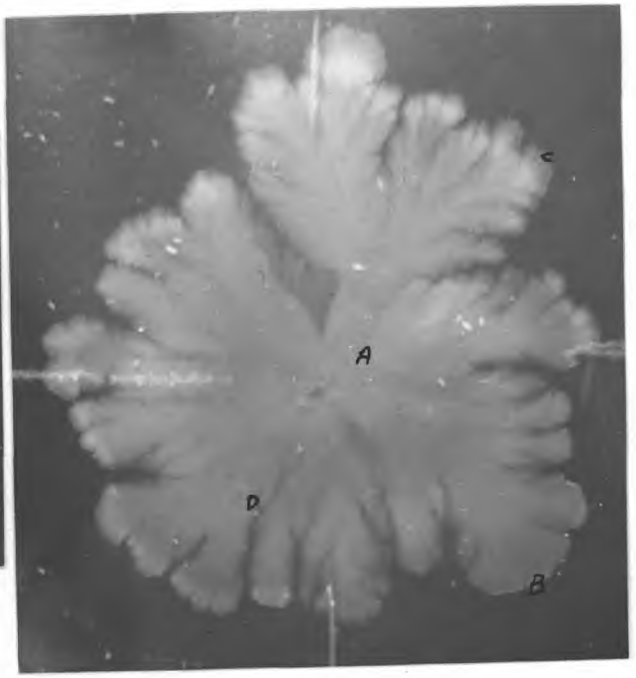
3 days



7 days



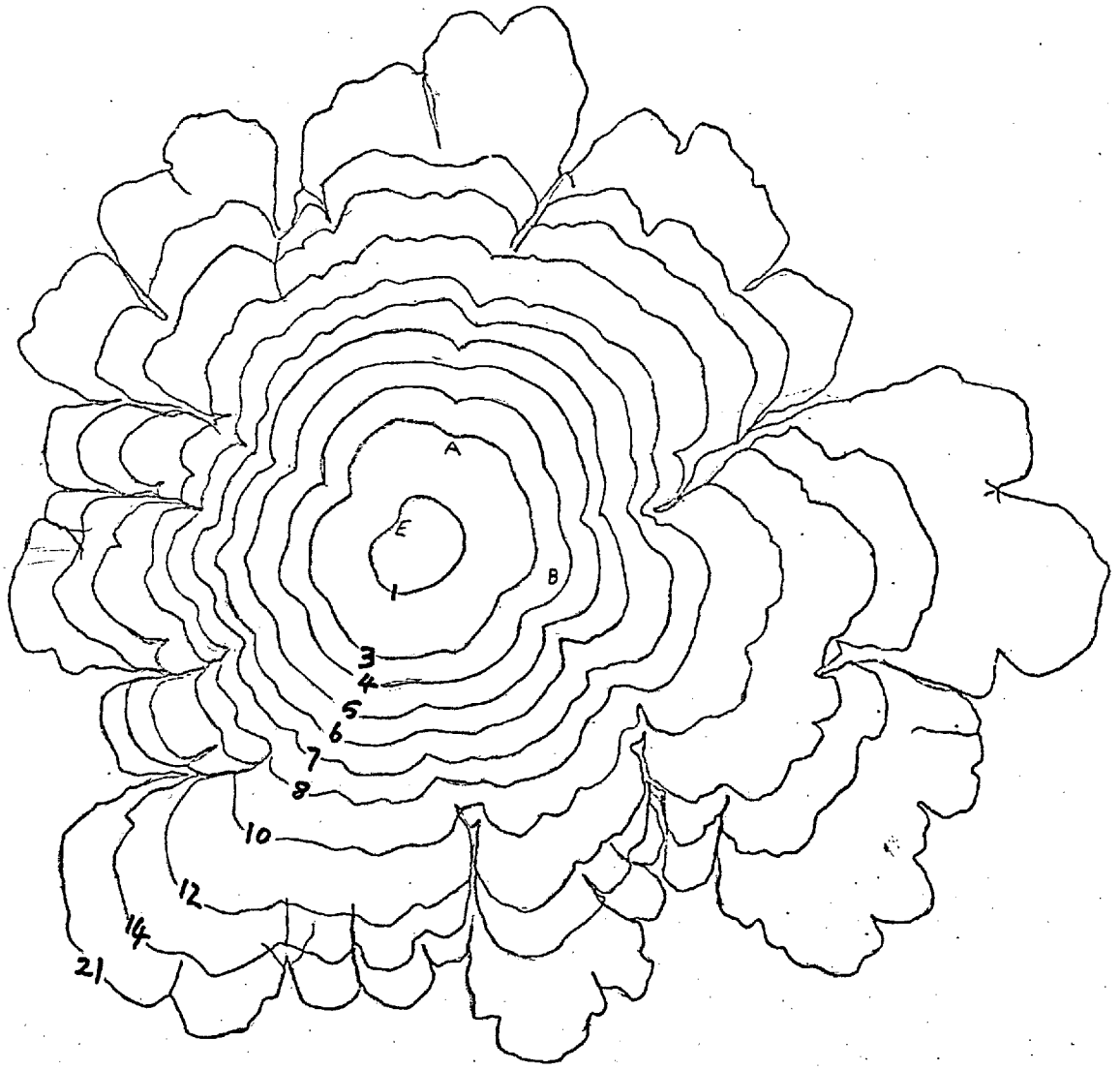
12 days



21 days

(All 3 x actual size)

Figure (4-1)



Colony perimeter at various times.
(Numbers refer to the age of the colony in days).

6 x Actual size.

At about 7 days points at the centre of the hitherto small indentations on the perimeter (such as A in Figure (4-1), slow down considerably in their outward growth and virtually cease growing altogether soon afterwards. Such points can be seen to form the origins of the fjord-like indentations observed in 3-week old colonies. The slight protuberances in the perimeter between these indentations continue to advance, but later they too may become flattened and subdivided by the formation of inlets, (see for example point B in Figure (4-1).

An inspection of Figure (4-1) also shows that the pattern of colony growth develops essentially because some parts of the perimeter slow down and stop growing, while other regions continue at approximately the same rate as they did in the first week of growth. It is evident from Plate (4-1) that the frequency with which inlets form in the perimeter increases in the later stages of the growth of the colony.

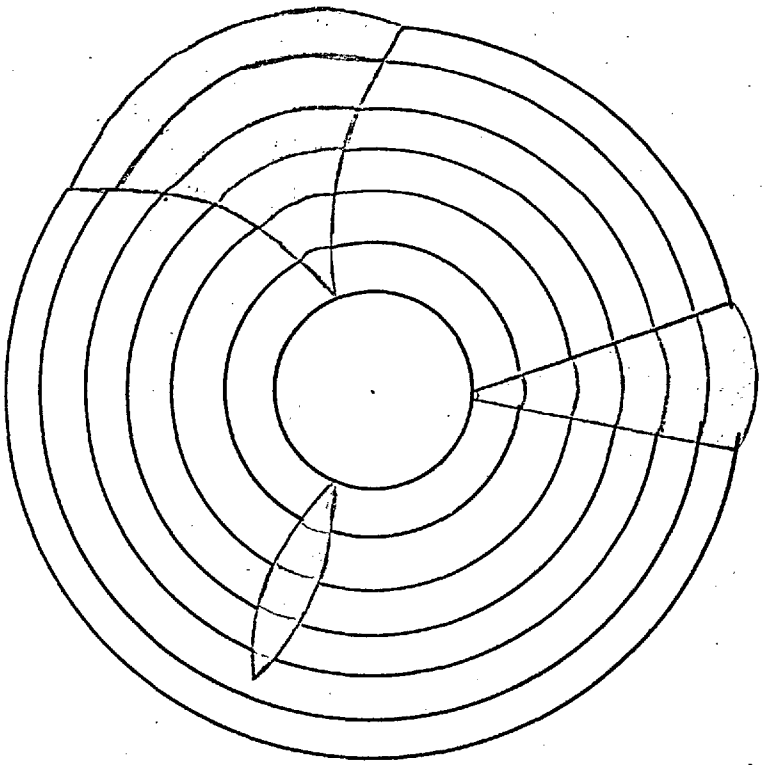
The whiter sectors which appear after about 7 days of incubation (Plate 4-1), are regions where the colony has a greater vertical thickness. This is readily detected by viewing the surface of the colony by reflected light. These regions are very similar to some reported to occur in colonies of Penicillium notatum and other moulds, by Portecorvo and Gemell (1944), who stated that the sectors occurred spontaneously, following the irradiation of a colony with a sublethal dose of x-rays or by the mixed inoculation of two

strains of organisms. The types of sectors obtained by Pontecorvo and Gemell and which were classified by them are illustrated in Figure (4-2). They interpreted the various shapes of sectors in terms of mutants whose relative advantages sometimes varied with the changing conditions in the colony during its growth. Thus the 'whiter looking' sectors whose sides open at an increasing angle and terminate in a bulge at the perimeter were considered to be most probably regions in which the cells were growing faster than those in the rest of the colony. A mutant whose initial advantage over the parent strain became a disadvantage as the conditions changed during the growth of the colony, was postulated to be responsible for the formation of the lens shaped sectors shown in Figure (4-2).

Portecorvo and Gemell succeeded in imitating the various sectors by plating mixed inocula of various strains. Although they did not report subculturing cells picked from the sectors and showing that they had intrinsically higher growth rates, which would be the most direct proof that these sectors originated from mutants, the evidence which they present is nevertheless quite strong. They do not report any development of the colonies into more complex shapes than those shown in Figure (4-2).

The similarity in appearance between the mould colonies described above and the colonies of Aerobacter aerogenes during the first week of growth, suggested that an investigation of the possible role of mutants in the

Figure (4-2).



Colony perimeter at different times.

latter colonies should be undertaken.

From the work on spread plates, which was described in Chapter 3, it would appear that the strain of Aerobacter aerogenes used in these experiments remains essentially homogeneous, in the mode of culture employed - at least as far as lag time and rate of growth of cultures or rate of increase in colony diameter are concerned. Thus histograms of the lag times of cells inoculated on to an agar plate are uni-modal suggesting that the population is composed of one type of cell, (Chapter 3, and also found by a more direct method by J.B. Woof and Sir Cyril Hinshelwood, F.R.S. 1960).

The histograms of colony diameter obtained are also uni-modal in contrast to those which are obtained with a population containing mutants. In these circumstances histograms of the type shown in Figure (4-3a) are obtained. However, even when the distribution is definitely bi-modal this is not in itself unambiguous evidence for the presence of mutants under all conditions, Figure (4-3b) shows a histogram which has been derived theoretically for a homogenous population of cells, and Figure (4-3c) shows an experimental histogram obtained with a population that was probably homogenous (Dean and Hinshelwood 1966, page 308).

Later in this Chapter experiments will be described in which the perimeter of a colony of Aerobacter aerogenes became irregular under conditions of very adverse pH. ($\simeq$4). It has been shown (Eddy and Hinshelwood 1953) that a

Figure (4-3a).

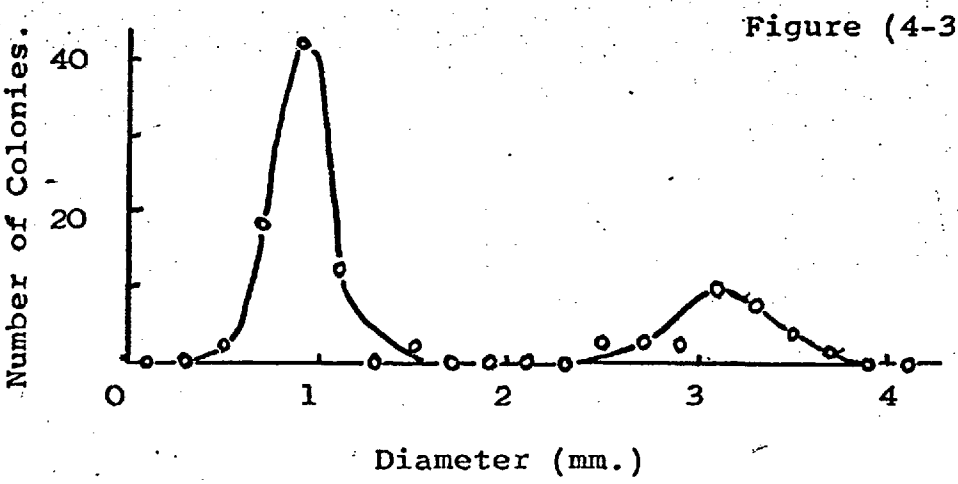


Figure (4-3b).

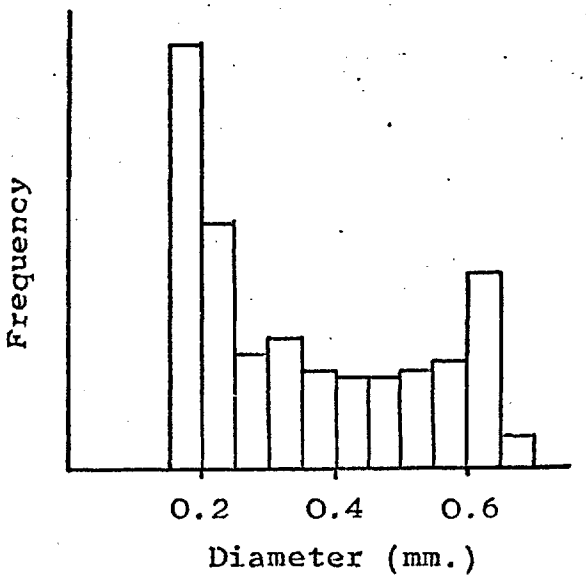
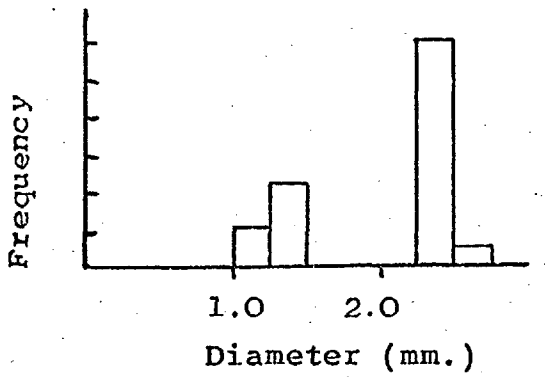


Figure (4-3c).



population of cells of Aerobacter aerogenes does not become more tolerant to low pH levels and so the development of peripheral irregularity just mentioned cannot be explained simply in terms of selection of mutants.

Peripheral irregularity can also develop due to a shortage of glucose and here again the production of a mutant which can grow faster or more efficiently than the normal strain seems unlikely since the latter has been grown in a glucose medium for a great many generations, hence selection should have already taken place. Moreover, the optimum rate of growth in glucose medium has not changed during serial sub-culture carried out over the last thirty years.

The conclusion appears inescapable, therefore, that a selection of mutants cannot account for all of the morphological changes observed in large colonies of Aerobacter aerogenes, but, nevertheless, as a more direct test, the following experiment was also performed. Small portions were picked with the tip of a fine platinum wire from different regions of single colonies of 6 and 19 days of age and inoculated on to fresh plates - the location of the samples being shown by th points such as A B C D in Plate (4-1). It was found that all the samples exhibited the same type of developmental patterns as the parent colony. The growth rates of the various colonies produced in this way are shown in Table (4-1); each reading is usually the average from 2 or 3 colonies. No general trend is discernable and the samples which would be expected to yield the fastest growing colonies if mutant cells

TABLE (4-1).

Age of parent colony when sub-cultured. (days)	Growth rate of sub-cultured colony (sq. mm/day) and region from which the sample was taken. (See Plate (4-1)			
	(A)	(B)	(C)	(D)
6	47.4 ± 2.5	40.8	44.0 ± 2.9	47.9 ± 5.9
19	41.5 ± 5.7	41.0 ± 3.7	38.3	42.2 ± 7.5

See Plate (4-1) for an explanation of the terms A, B, C and D

were producing the 'sectors' and irregularities in the parent colony, do not in fact do so.

Another method of investigating this phenomenon is to grow a colony on a permeable membrane resting on the surface of a reservoir of stirred liquid medium. This may be achieved by using a membrane which is permeable to both nutrients and by-products whilst retaining the cells.

The apparatus used was kindly lent by Dr. J. Russell and was originally described by Lougheed (1958). For the present purpose it was used in a modified form which is shown dismantled in Plate (4-2a) and completely assembled in Plate (4-2b). Figure (4-4) shows diagramatically, the construction of the growth cell.

A black 'Millipore' filter, (type HABG 14200), proved a satisfactory membrane when used upside down to prevent the grid lines on it from modifying the colony pattern, (Chapter 5). This filter was fragile while it was dry, but was considerably stronger when wet and was thus kept moist with sterile distilled water prior to filling the cell with normal minimal medium. The stirring system was adjusted so that the surface of the liquid, when the lower half of the cell was completely filled, moved slowly and did not form a vortex. In this way good contact between the liquid and the filter was assured.

Plate (4-2a)

See page 86 for key.

Plate (4-2b)

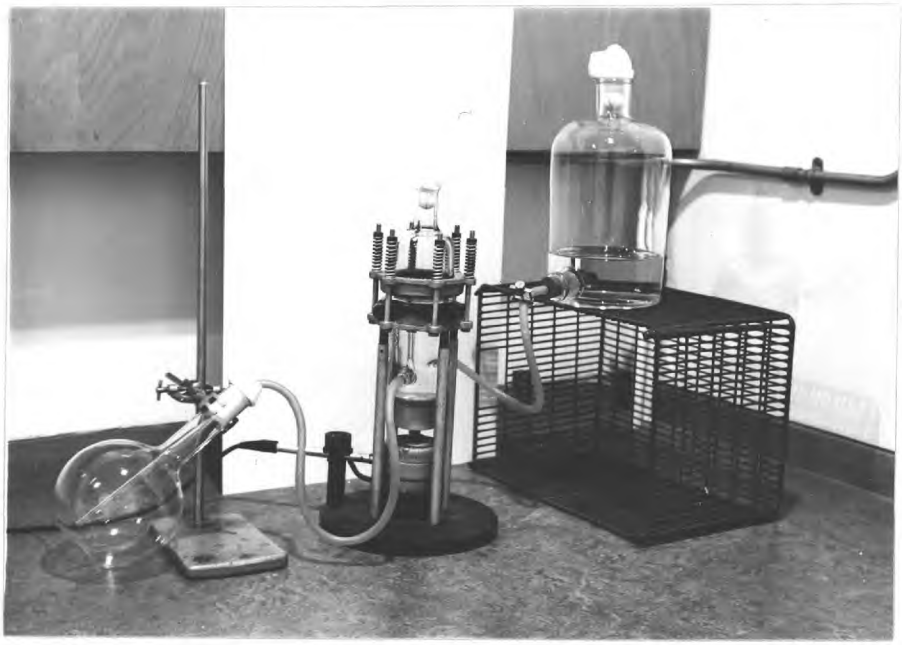
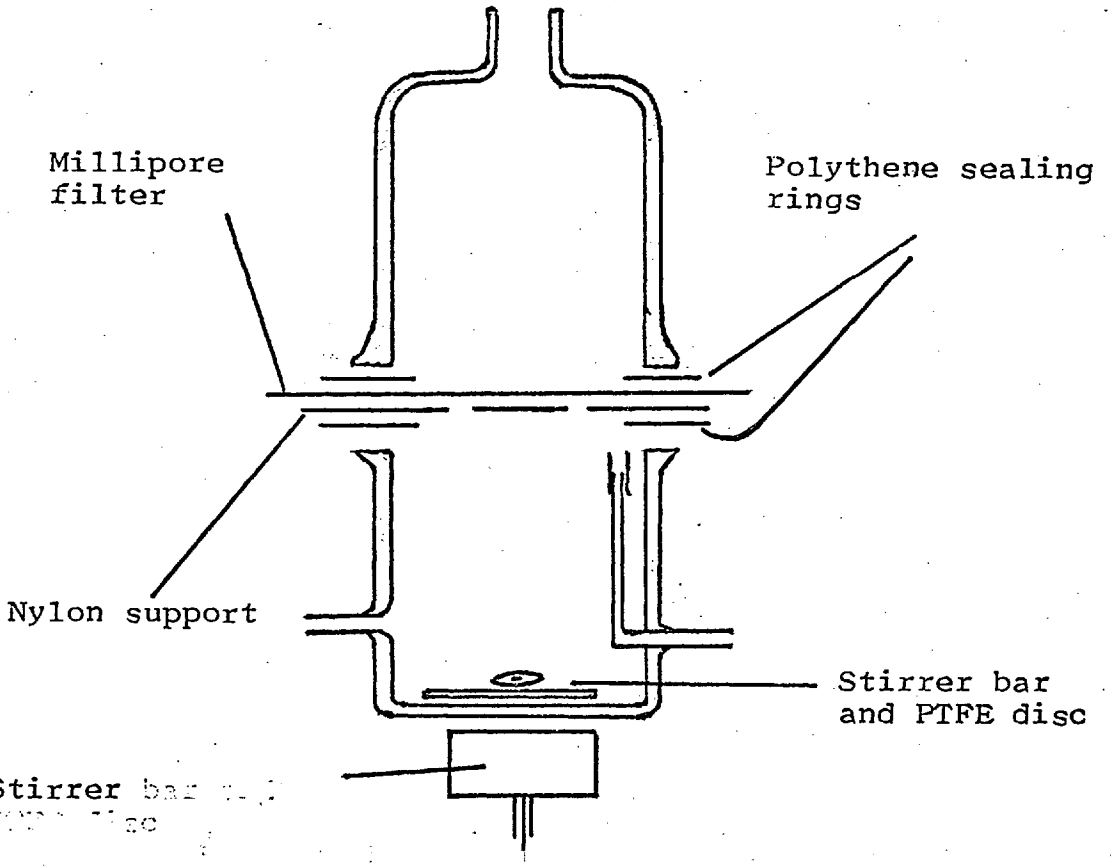


Figure (4-4).



Key to Plate (4-2a).

A = PTFE disc and glass stirrer bar
B = Polythene sheet seals
C = Millipore filter
D = Nylon sheet (2 holes) to support Millipore filter
E = Overflow
F = Inlet pipe

The growth cell was completely assembled, connected to its two subsidiary vessels and autoclaved; sudden changes in pressure were avoided. When cool, normal minimal medium was mixed aseptically in the reservoir and admitted slowly to the cell. For simplicity, the medium was not allowed to flow through the cell continually, but the level was adjusted at 12 hr. intervals. The temperature of the vessel was maintained at about 33°C.

Plate (4-3) shows the appearance of a colony produced under these conditions following the single inoculation with cells of the normal strain of Aerobacter aerogenes. The colony attained the size shown after 5 days of growth at which time cells from the colony penetrated the filter and multiplied rapidly in the liquid beneath. Due to the production of unfavourable growth conditions in this way the colony ceased growing and retained the appearance shown for the next two days after which the growth cell was dismantled.

On comparing Plate (4-3) with the appearance of a normal colony developing on agar, (Plate (4-1)), it can be seen that the colony produced over stirred minimal medium exhibits no 'sectors' and a practically circular perimeter, (the small protrusion in the perimeter was found to be caused by growth around a fibre, probably from the cotton wool plug, (c.f. Chapter 5).

Thus it appears that the presence of uniform concentration of growth by-products beneath a growing colony is insufficient

Plate (4-3)

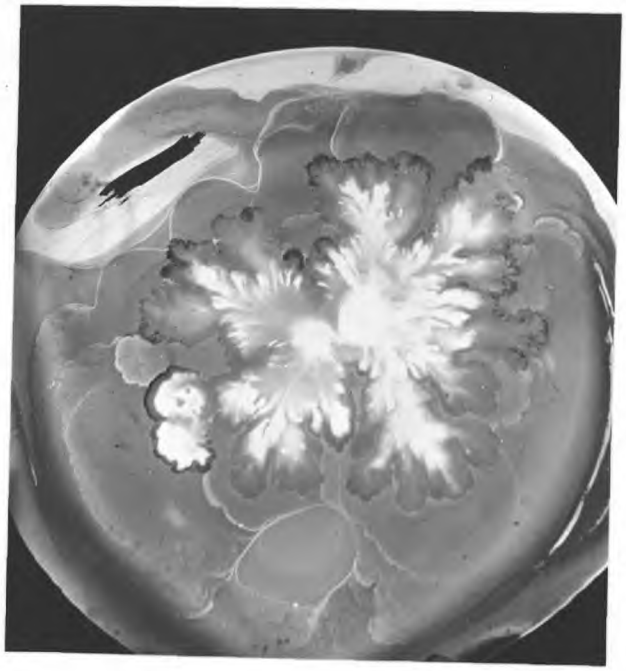
(3 x Actual size)

to cause it to form 'sectors' or develop peripheral irregularities which strengthens earlier conclusions that mutant cells are not responsible for the development of the different regions in a colony of Aerobacter aerogenes.

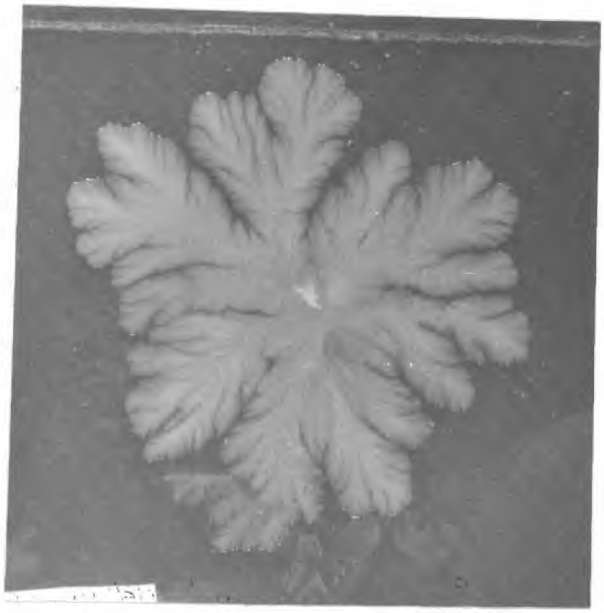
Another possibility is that in some way the micro-structure of the agar gel directs the growth of the cells. In this connection the growth of blue-green algae and of myxobacteria on the surface of the agar plates has been shown (Stanier 1947, Stanier 1942) to be orientated along the lines of stress in the gel. Weiss (1929) has shown that fibro-blasts grow preferentially parallel to a stress in a gel rather than normal to this direction. Thus the development of irregularities in a colony of Aerobacter aerogenes might indeed be closely related to regions of changing stress in the gel, such as stress patterns set up as the gel solidifies. Alternatively radial lines of stress could arise from hydrolysis of the agar beneath the colony by the acids produced as by-products of the growth of the cells.

To study the effect of the structure of the agar gel on colony development, a sterile cellulose acetate 'Millipore' filter type HA, 0.45 mμ. pore size was placed on the surface of a solidified agar plate and some cells of Aerobacter aerogenes inoculated on to the top of the filter with the tip of a platinum wire. The appearance of the colony at 30 days after inoculation is shown in Plate (4-4) together with a colony grown directly on the surface of an agar gel as a

Plate (4-4)



Millipore filter



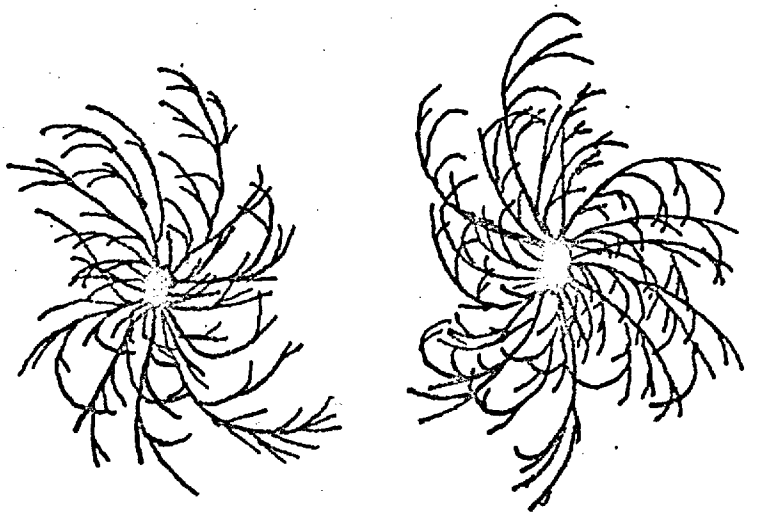
Control

(Both 2x Actual size)

control. The growth of the colony on top of the 'Millipore' filter was slower than in the control test, but the essentially similar character of the two patterns suggests that the structure of the gel is not of prime importance in controlling the form of colony development. Nevertheless, its influence in a secondary capacity must not be disregarded, since markedly different colony patterns are obtained with different brands of agar (Chapter 5). This may be partly due to physical differences but local differences as, for example, in diffusibility from one region of the gel to another, might also be involved even in the colony growing on the filter.

A third possible factor which may influence the development of the morphology of a large colony of Aerobacter aerogenes is the existence of some sort of more or less specific interaction between two dividing cells. Such a mechanism seems to be important in the growth of Bacillus mycoides on agar surfaces. The type of colony obtained is shown in Figure (4-5), (Gause G.F. 1940). Gause attributes the formation of this pattern to a reaction between a spiral twisting action as the cells divide and the agar surface. Thus the pattern is not obtained if the gel is too soft. This organism, it is claimed, exists in two genetically different types which are characterised by sinistral and dextral colony forms, Figure (4-5). However, as far as Aerobacter aerogenes is concerned, observations on the division of cells (Dean and Hinshelwood 1957) do not suggest that any

Figure (4-5).



L D

Colony patterns formed by sinistral (L) and dextral (D) strains of Bacillus mycoides.

consistent orientating effect operated.

Thus, it would appear that none of the three mechanisms discussed above, each of which has been shown to be mainly responsible for the morphological pattern in the specific examples, can be nominated as the prime agent in the present one.

In Chapter 3 the conclusion was drawn that the rate of growth of a single colony one week old is dependent on the rate at which nutrients can diffuse towards it and by-products away from it. Experiments will next be described which suggest that the morphological development of such a colony is also closely related to the diffusion of nutrients and by-products. Some general comments on the problems involved must first be made however.

The effect of the composition of the agar gel on the morphology of colonies of Aerobacter aerogenes could, in principle, be demonstrated by comparing the appearance of colonies of the same age on agar gels of varying composition. The development of a colony of the normal strain which was described earlier in this Chapter, is,however, a continuous and complex process and hence it is difficult to say readily that one colony is "more developed" than another. The formation of various mathematical functions, such as the average deviation of the colony radius from the mean value, as a measure of the development of a colony, will be discussed in Chapter 8. The choice of such criterion is, however, somewhat

arbitrary and one simple mathematical function is unlikely to characterise all the changes taking place. Thus the applicability of some numerical function to describe the development of a colony is a topic in its own right, (see Weiss (1955) regarding the development of an embryo), and so for the present slightly modified systems will be considered in which the colony development is more ontogenic and hence facilitates a comparative study of the effect of changes in the concentration of nutrients in the agar.

Regarding the actual experiments themselves, they were carried out with a strain of Aerobacter aerogenes resistant to methylene blue (Chapter 6) and they show directly that peripheral irregularity can result from the reduction of the flux to or from the colony of some nutrient or by-product, to a certain critical level. In them, a strain of Aerobacter aerogenes resistant to methylene blue, was stabbed on to the surface of minimal agar containing 10, 20, 30 or 40 per cent of the standard buffer concentration (Chapter 2). In other respects the composition of the gel was unchanged. The colony which formed had a smooth perimeter with only a few gentle indentations (Plate 4-5a) throughout the time interval in which its area increased linearly with time. Later the rate of growth of the colony slowed down abruptly and after this had occurred the perimeter became irregular over a further period of two days, (Plate 4-5b). It has been shown (page 55)

Plate (4-5a)

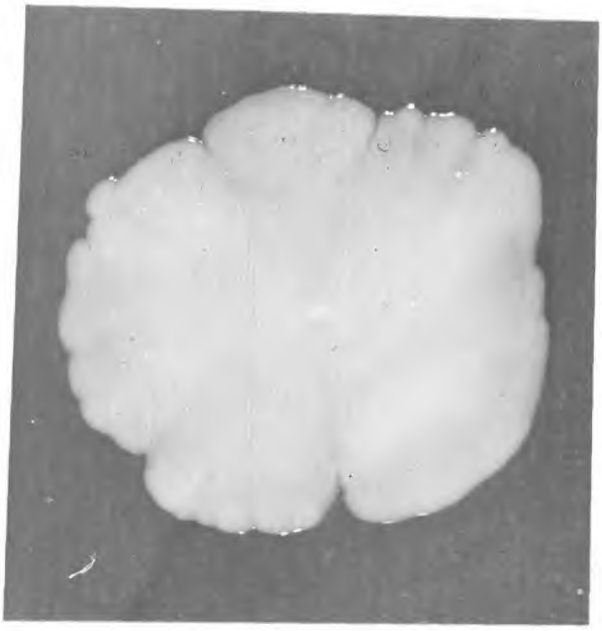
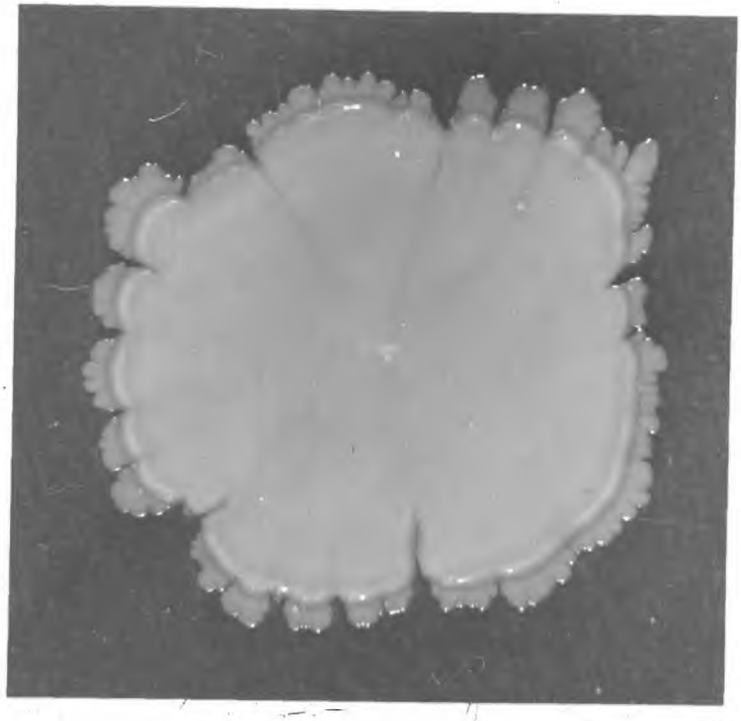


Plate (4-5b)



(Both 4 x Actual size)

that the average rate at which material can be supplied to a colony by diffusion over a given period of time is proportional to the basal concentration in the agar of the substance in question. This conclusion was reached by assuming that the concentration of a nutrient was maintained at zero level in the gel immediately beneath the colony and such an assumption led to the formation of a suitable model based on the application of simple diffusion equations to the system. This model of the colony would be open to criticism if it was used to calculate absolute values of fluxes reaching or leaving a colony, but in comparative studies these objections are largely overcome. In Figure (4-6a) the time of growth before the peripheral frill appeared on the colony is plotted against the concentration of buffer in the agar, while in Figure (4-6b) the colony area at the time the peripheral frill appeared is related to the concentration of buffer in the agar. Both of these plots show directly that the peripheral frill develops when the declining flux of buffer reaching the colony perimeter falls to a certain critical level.

This example of peripheral irregularity seems to be regulated by the balance between outward diffusing by-products of an acidic nature and inward diffusing buffer. A colony can also be induced to develop an irregular perimeter if conditions are so arranged that the flux of nutrient reaching the perimeter declines sufficiently. Plate (4-6) shows the

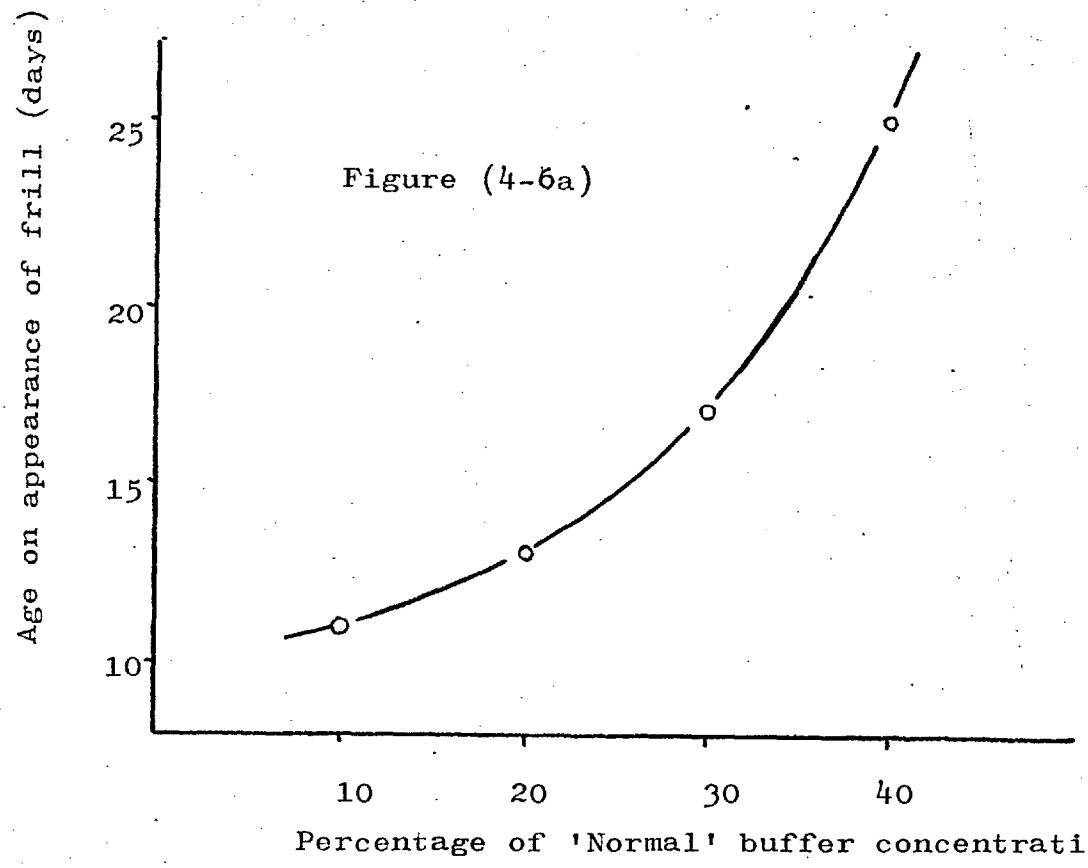


Figure (4-6a)

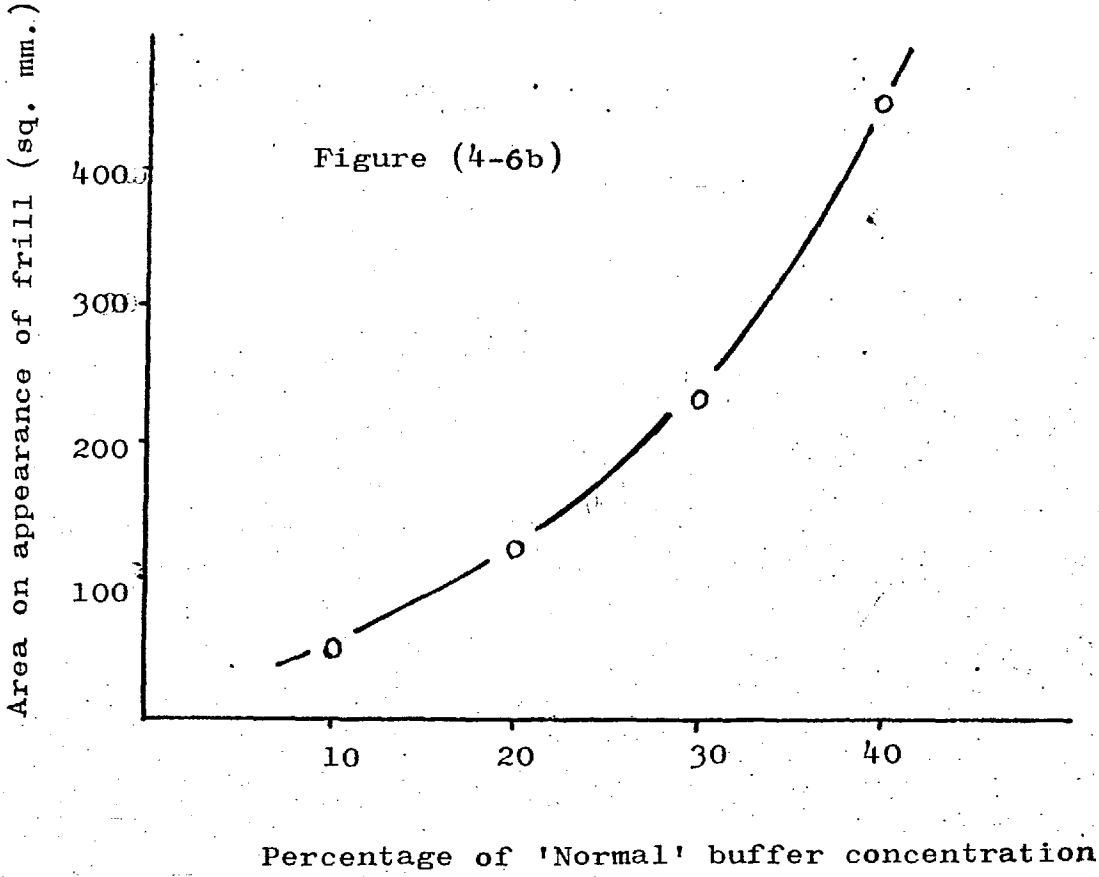


Figure (4-6b)

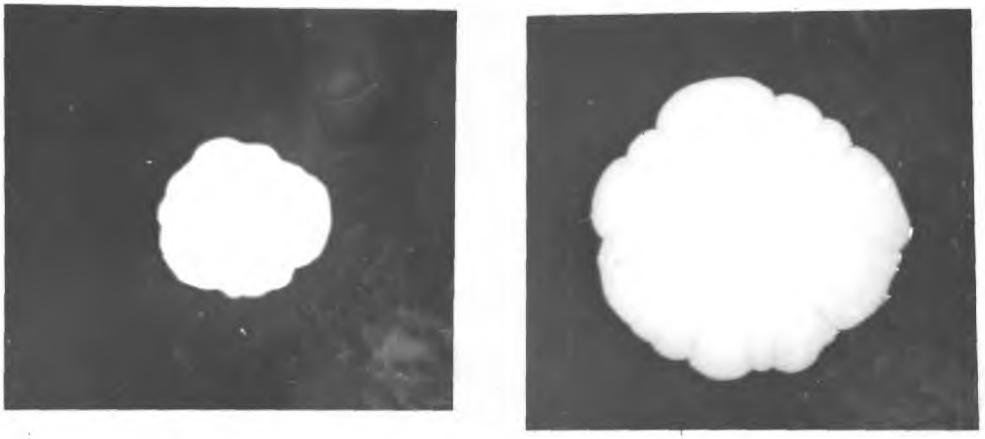
appearance of 10 and 18 days after inoculation of colonies of a strain of Aerobacter aerogenes, resistant to methylene blue, on drug free plates containing decreasing concentrations of glucose. It can be seen that at a given time, the colonies become progressively more irregular as the concentration of glucose in the agar decreases. The use of a mathematical function to describe this irregularity is discussed in Chapter 8. It has been mentioned that for colonies on spread plates, doubling the glucose concentration in the agar, led to a severe inhibition of the growth of the colonies. Such an effect (compare Pirt 1967) could result from the fermentation of the glucose reaching the centre of the colony leading to the formation of excessive quantities of toxic by-products although the conditions are such as to inhibit the division of the cells. It might, therefore, be suggested that the development of an irregular perimeter which has been ascribed to a shortage of glucose is in fact a consequence of excess of the by-products produced by the cells themselves. That this is not so is shown by the finding that when low concentrations of glucose were used and hence smaller quantities of by-products would be expected to be produced, peripheral irregularity developed earlier.

When large numbers of cells (3,000) are spread on the surface of an agar plate a confluent film of growth is obtained. In such a situation many cells start growing very

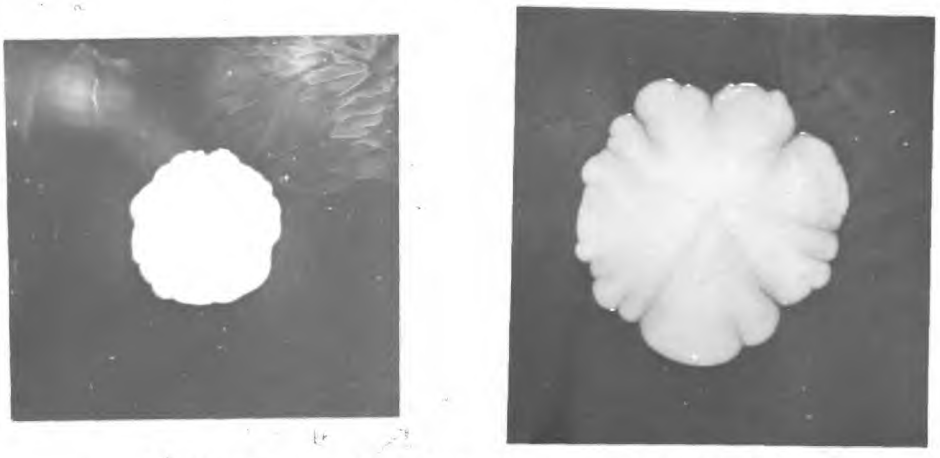
Plate (4-6)

10 days

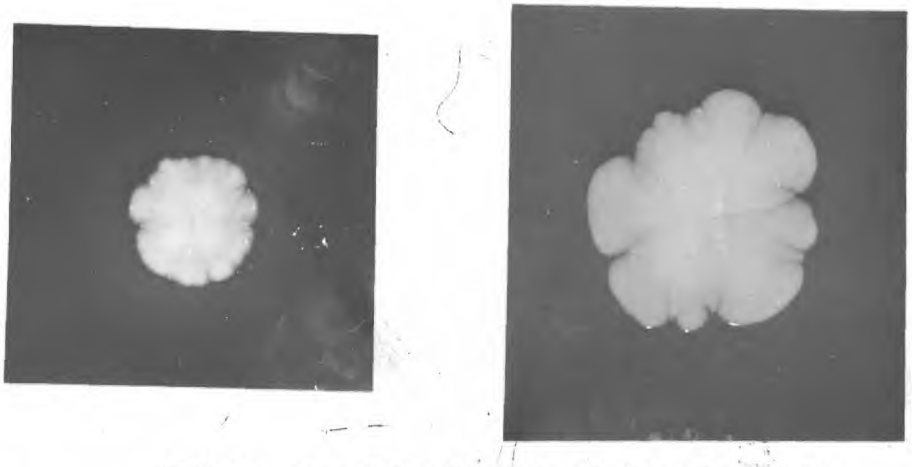
18 days



2.0 x normal glucose concentration



1.0 x normal glucose concentration



0.8 x normal glucose concentration

(All 2 x Actual size)

close to each other and the film produced is much thinner than the vertical height of isolated colonies. (The factors which regulate the vertical height of a colony are discussed in Chapter 7). On the other hand, when the inoculum contains less than 3,000 cells there is still plenty of free agar surface around the colonies when growth ceases. Since it has been shown in Chapter 3 that the growth stops due to the exhaustion of the gel, the formation of isolated colonies indicates that unit area of gel can support less than unit area of colony. Thus all colonies on a spread plate should some time in their growth pass through a position of instability like that described for a single isolated colony. No irregular colonies were found on plates containing more than about 600 colonies since here diffusion is effectively faster over the shorter inter-colony distances on these densely inoculated plates. Thus the time interval between the phase at which an unstable perimeter would be expected and the cessation of growth due to the complete exhaustion of gel, is about comparable to the growth rate of the perimeter. With a decreasing number of colonies on a plate this time interval becomes longer and progressively irregular colonies develop.

The view of a developing colony which has been reached, therefore, is that after the first two days of growth any subsequent progress becomes dependent upon the rate at which nutrients or by-products can diffuse to or from the colony. The amounts of nutrients consumed and by-products produced may be regarded, to a first approximation, as being proportional

to the area of the colony and the rate at which these materials can enter or leave the colony as proportional to the length of the perimeter. For a circular colony, therefore, the increase in the demand for nutrients and in the production of by-products are both proportional to the square of the time of incubation. However, the escape of metabolites is only linearly related to the time. This is certainly a simplified and rather empirical approach to the problem, but it illustrates another factor which increases the instability of the expanding circular colony.

As a result of this initial growth, gradients of nutrients towards the colony and of by-products away from it arise and the rate of diffusion of any one of these substances may become the growth regulating factor. Under these conditions any slight irregularities present in the perimeter resulting from the method of inoculation with a platinum wire or from slight heterogeneities in the surface of the gel will become exaggerated. Any regions of cells which protrude slightly further out from the colony than their neighbours will be in an environment which is more suitable for growth since not only will nutrients be more plentiful, but also the concentration of toxic metabolic products will be lower. These small groups of protruding cells will, therefore, tend to grow faster than those in neighbouring regions, and in so doing will tend to reinforce their advantage. Moreover, not only are small peninsulae of growth self-amplifying, but also by their production of toxic by-products and their consumption

of inwardly diffusing nutrients, they will also inhibit growth in the intervening regions. The development of an irregular perimeter in a colony is thus encouraged for several reasons. This developmental process repeats continually at various points on the perimeter as the various peninsular regions become unstable in the same manner as the original circular colony did.

These ideas are borne out by an examination of the perimeter of colonies at different stages during incubation (see Figure 4-1). Initial small irregularities in the perimeter, probably, as already pointed out, resulting from the inoculation with a platinum wire, are reproduced with little or no change in shape in the subsequent perimeter arising over the first few days of growth (Figure (4-1), points E,F. for example). These small irregularities are the origin of the main large peninsulae observed in the three weeks old colony. It can also be seen from Figure (4-1) that the rate of advance of any region of the perimeter of the colony never rises above that occurring during the first few days of growth. The pattern develops since, as mentioned earlier, some regions of the perimeter can maintain approximately their earlier rate of growth, while at points in between growth slows down and then stops.

This mode of development of a large single colony of Aerobacter aerogenes resembles in some ways the dendritic growth of crystals. Crystal growth is a complex phenomenon, but for the purpose of the present somewhat limited comparison

a distinction may be made between two types of growth. In one, the crystal grows by the more or less uniform deposition of material on to its faces; in this way the geometry of the crystal is maintained although in some cases certain faces will grow faster than others leading to a distortion of the basic shape. This type of growth may be likened to the early stages in the growth of a colony in which the perimeter is stable and maintains its basic circular shape. The second mode of crystal growth, which is of interest in the present context, is the 'dendritic' type. An example of the dendriti crystal growth is shown in Plate (7-2b). Cabrera and Coleman (Gilman 1963) describe dendritic growth as follows:- "From the experimental point of view dendritic growth appears to be favoured by the following conditions:- (1) a volume diffusion field operates which favours the non-singular surfaces, and (2) high supersaturation and high equilibrium concentration producing a high rate of growth in such a way that the growing surface does not wait for the condensing atoms to diffuse to it but goes forward to catch them". They suggest that the situation is actually more complicated and state that no serious theoretical analysis of whether the above two conditions are sufficient to explain dendritic growth exists, but they conclude that these factors must be important.

Thus, apart from the obvious differences which are dictated by the need for the crystal to conform to certain

geometrical limitations, the (basic) driving force for the development of a dendritic crystal and a large colony of Aerobacter aerogenes would appear to be very similar. Both systems remove 'nutrients', (e.g. glucose in the case of colonies and molecules in the case of a dendritic crystal), from their immediate surroundings at a sufficiently high rate to maintain a diffusion gradient of this material. The resultant pattern represents the preferred growth of certain regions of the growing front, due initially to a slight positional advantage in the case of a developing colony and probably to rather more complex reasons in the case of a crystal, at the expense of their neighbouring regions. Both patterns represent the fastest way of gathering material from a given area for each system. Under normal conditions a large colony of Aerobacter aerogenes bears little resemblance to a dendritic modified crystal. Plate (7-2a) shows, however, that under slightly modified conditions, a colony very similar in form to a dendritic snow crystal can arise. The reasons for this difference are discussed in Chapter 7.

Chapter 5.

General Investigation.

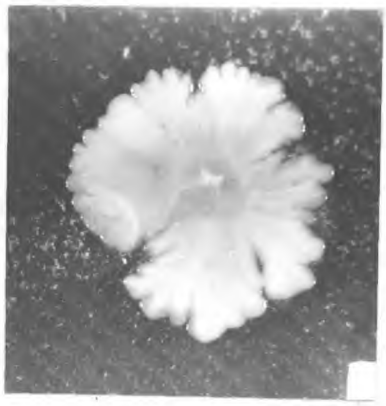
In Chapter 4, experiments were described which showed why a smooth circular colony of Aerobacter aerogenes should develop into an irregular 'floral' pattern with prolonged growth. In this chapter, experiments of several different types which give information about the conditions inside such a colony, are discussed.

Brand of Agar Powder:

The growth of a single colony of the normal strain of Aerobacter aerogenes on normal minimal medium solidified with 1.25% w/v of different brands of agar powder has been studied. Plate (5-1) shows the appearance of such colonies at 20 days after inoculation; (fuller details of the brands of agar powder used are given in Chapter 2). It can be seen that a characteristic pattern develops on each agar. Figure (5-1) shows the area of these colonies plotted against time, from which it can be seen that an approximately linear relationship was obtained, (see Chapter 3), on all agars. Growth rates, which are the average of readings on at least two colonies are reported in Table (5-1). Although the rates of colony growth vary consideribly on different brands of agar, the morphologic development proceeded along similarly general lines in each case and this, together with the fact that the kinetics of growth are the same, suggests that the basic mode of growth is common throughout.

Plate (5-1)

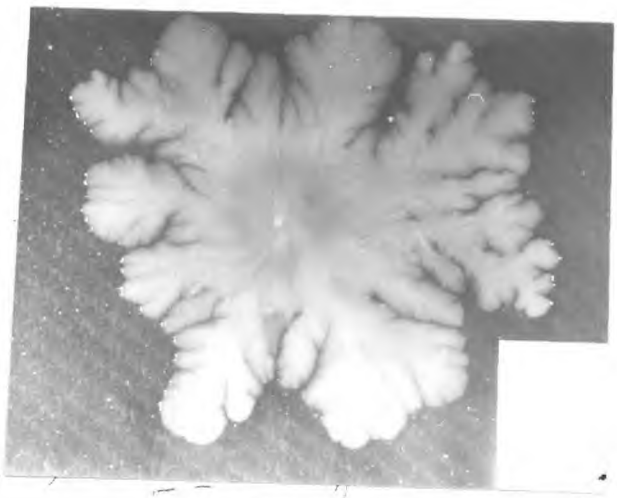
Normal



Nutrient

Difco Purified

Oxoid ion



Oxoid 3

All 2 x Actual size

Figure (5-1).

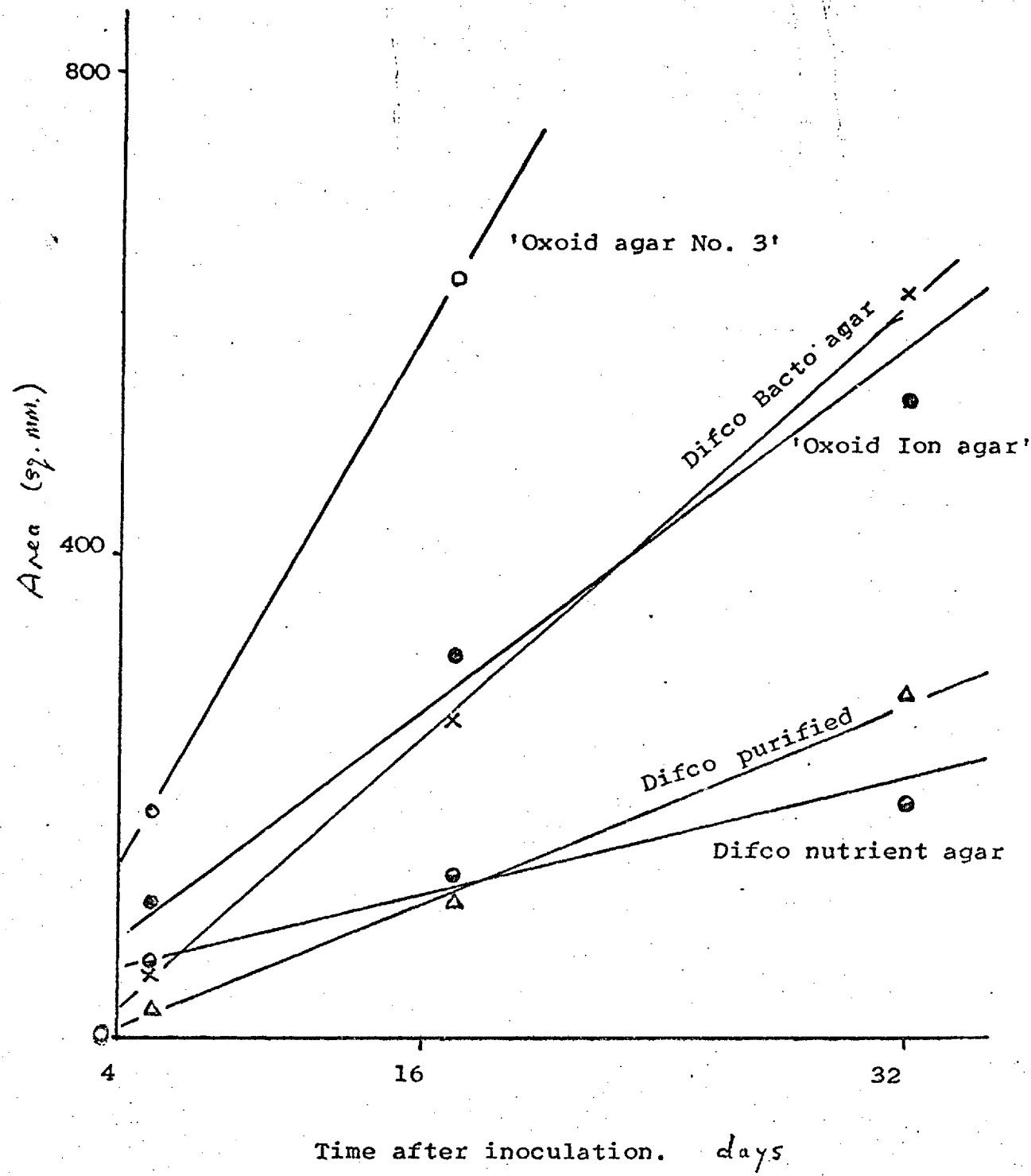

800
400
0
Area (sq. mm.)
'Oxoid agar No. 3'
Difco Bacto agar
'Oxoid Ion agar'
Difco purified
Difco nutrient agar
4
16
32
Time after inoculation. days

TABLE (5-1).

Agar.	Growth Rate (sq. mm./day).
Oxoid agar No. 3	45.1
Difco Bacto agar	22.2
Oxoid Ion agar	19.1
Difco Purified agar	10.3
Difco Nutrient agar	6.3

To account for the variation in the rates of colony growt on the various agars, two types of differences in the gels, namely physical and chemical, may be cited. The physical nature of the surface does not seem to be of prime importance, since a colony of Aerobacter aerogenes growing on a sheet of porous cellulose acetate resting on an agar plate is very similar in appearance to its control colony, (Plate (4-4)) (page 90). The former colony is slightly slower growing than a normal one, but this is not necessarily a direct consequence of the difference in the surface, but may, for example, be due to lower diffusion coefficients within the cellulose acetate membrane, which from the considerations advanced in Chapter 3, would be important. A physical factor which could exert a direct effect on the rate of growth of a colony is the variation of the diffusion coefficients of substances important in its growth, from one gel to another. It has been shown that the diffusivity of a gel decreases only slightly with increasing concentration of gelling agents (e.g. K for NaCl at 40°C in 1% and 2% agar gels is $2.143 \times 10^{-}$ $cm.^2\ sec^{-1}$ and 2.084×10^{-5} $cm.^2\ sec^{-1}$ respectively). Thus it seems that differences in, say, the water content of the various powders will not contribute much to differences in the diffusivity of the final gel and hence, from the consideration of Chapter 3, to the rate of growth of the colony.

Variations in the gel structure due to differences in the methods of manufacture of the powder, probably exert little influence on the diffusivity of the gel. Stiles and Adair (1921) found that the diffusion coefficient of sodium chloride in similar gels made with completely different gelling agents, (agar and gelatin), were very similar, e.g.

4% aqueous gelatin gel, 20°C, K(NaCl) = 1.242×10^{-5} $cm.^2\ sec^{-1}$

4% aqueous agar gel, 20°C, K (NaCl) = 1.290×10^{-5} $cm.^2\ sec^{-1}$

This latter result would be expected from the conclusion that diffusion coefficients in dilute gels (1.25% agar), are very similar to those in pure water and hence that the gelling agent exerts little effect on diffusion (Chapter 3).

Three physical properties of gels which could have influenced the development of colonies growing on them, have been considered. They are:-

1. The physical nature of the gel surface.
2. The effective concentration of the gel connected with variations in the 'purity' of the agar powders.
3. The modification of the gel structure due to differences in the various methods of manufacture.

From what has been said, however, it would appear that in this case all of these factors can exert, at most, a rather secondary effect.

The differences observed in colony growth rate are probably due to chemical variations in the agar powders. Variations in the concentrations of metal ions present as impurities could have a profound effect. Ions such as Pb^{++} or Cr^{+++} are toxic even in quite small concentrations while others, such as Mo^{+} are essential for growth. The extremely low rate of colony growth which was observed on 'difco Purified Agar' (which is a much refined product), may have been caused by low concentrations of essential metal ions that are present in adequate amounts in the less refined agars. Another possibility is that the agar powders contain certain biologically active organic compounds, either of a growth accelerating or retarding nature. Oxoid Agar No. 3 may contain traces of amino acids, since the rate of growth of Aerobacter aerogenes on this agar was similar to that which was obtained when small amounts of meat broth were added to normal minimal agar (page 134).

Thus the use of agar powder introduces rather unknown factors into a study of the growth of large bacterial colonies. There are two alternative gelling agents, gelatine and sodium silicate. Gelatine would in principle be suitable since it is not liquified by Aerobacter aerogenes as is the case with some organisms. It is, however, no more precisely defined in its chemical composition than agar powder and is a relatively weak gelling agent. Moreover, a 15-20% solution would be necessary and the temperature of incubation reduced

to 25°C. Similarly sodium silicate, although a more potent gelling agent than gelatine, has many disadvantages. The gels, which are rather tedious to prepare, (Jackson et al 1949,) , exhibit a tendency to crack and have a rather high ionic strength. They proved toxic to some of the modified strains. Also sodium silicate is not very well defined chemically, e.g. "About 18% w/w Na_2O and 36% w/w $Si\ O_2$".

Although colony morphology and growth rate can vary considerably because of its rather ill-defined chemical composition and hence 'purity', from one brand of agar to another and quite probably between batches of the same brand, it is still the most suitable gelling agent. However, since one batch of 'difco Bacto' agar has been used throughout this work to keep the 'unknown variables' as constant as possible and in conjunction with control experiments this has permitted general trends to be detected.

The Effect of Aeration:

In this section the possibility that all the oxygen in the air over the gel in a Petri dish may be consumed during th growth of a colony is considered.

The stoichiometry of the complete oxidation of glucose ma be represented as follows:-

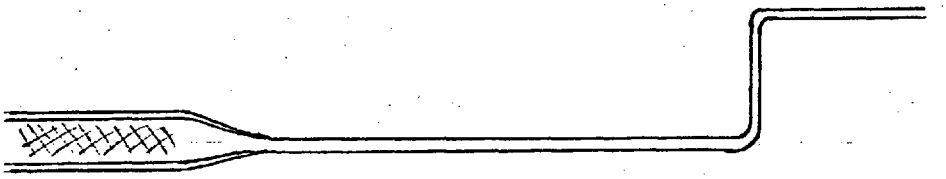
$$C_6H_{12}O_6 + 3O_2 = 6\ CO_2 + 6H_2O$$

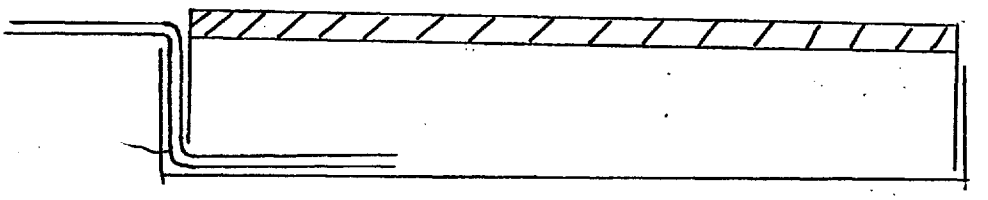
180 gms. 3 x 22.4 litres.

From this equation it can be calculated that enough oxygen is present in the air over the agar in a 9 cm. diameter Petri dish to oxidise about half of the glucose present in the usual volume, (15 ml.) of 'Normal minimal' agar. However, the exhaustion of oxygen can never by itself cause the complete arrest of Aerobacter aerogenes since growth can continue anaerobically at a decreased rate. Nevertheless, in anaerobic growth, the glucose is used less efficiently and more acid by-products are produced in the formation of a given number of cells (page 142) and so if oxygen did become exhausted in the latter part of the growth of a large single colony it would be expected, in the light of Chapter 4, that this would have some effect on the colony pattern.

To test the idea that anaerobic growth might produce a different colony pattern than aerobic growth, an experiment was devised in which a constant atmosphere, either of air or nitrogen could be maintained inside a Petri dish. For this a bent Pasteur pipette was used as shown in Figure (5-2). 'Oxygen-free' nitrogen was passed through a charcoal filter, (Chapter 2), and then through two Dreschel bottles in series containing glass distilled water and finally into the Petri dish through the bent pipette. A flow rate of about 25 c.c. per minute was maintained and the Dreschel bottles were placed inside the incubator in order to saturate the gas with water at the temperature at which the colonies were growing (35°C). Unless this precaution was taken the agar gel dried out rather quickly.

Figure (5-2).



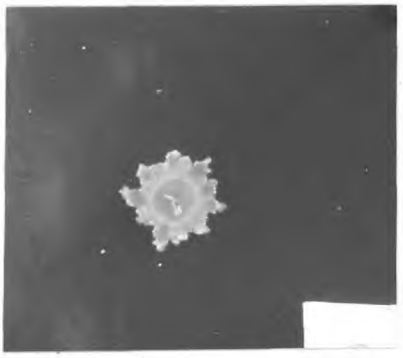


The appearance of a colony of Aerobacter aerogenes grown anaerobically, (i.e. in a nitrogen atmosphere), at 20 days after inoculation is shown in Plate (5-2a). It is clear that enforced anaerobic growth has considerably impeded the development. This result was verified by a duplicate experiment. It has been mentioned that Aerobacter aerogenes produces more acid when growing anaerobically than aerobically and hence it might be expected that some similarity would exist between the mode of growth of a colony under anaerobic conditions on 'Normal' minimal agar and that of one growing on agar containing a reduced concentration of buffer. In fact, the growth rate and morphology of an anaerobically grown colony are similar to that obtained under normal conditions on agar containing 10% of the standard concentration of buffer.

Plate (5-2b) shows a colony of Aerobacter aerogenes which had been grown in a Petri dish continually eluted with sterile air using the same procedure as described above for nitrogen. This colony is very normal in appearance, (c.f. Plate (4-1) and this result was verified in a duplicate experiment.

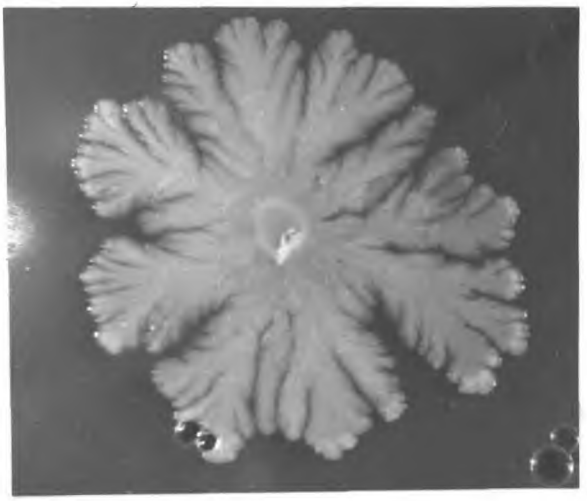
In conclusion, although the absence of oxygen produces distinct changes in colony development, this does not seem to occur during the growth of a colony under normal conditions since continually replacing the air over it produces no pronounced changes. This implies that either a Petri dish contains enough oxygen to oxidise the glucose consumed during

Plate (5-2a)



Anaerobically grown colony, 25 days

Plate (5-2b)



Aerobically grown colony, 25 days

(Both 2 x Actual size)

the growth of a colony or that adequate diffusion of air into the dish occurs.

Effect of varying Glucose Concentrations:

The prolonged growth of single colonies of Aerobacter aerogenes in minimal agar containing various concentrations of glucose was next studied. At all of the glucose concentrations employed (i.e. 0.33 - 6.67 g.p.l.) the area of the colony increased approximately linearly with time over the period from 4 to 25 days after inoculation. The variation of this growth rate with glucose concentration is shown in Figure (5-3a). Up to about 4.3 g.p.l. glucose, the colony growth rate increased in a linear manner in direct proportion to the glucose concentration and this fact has already been cited, (Chapter 3), as indicating that growth in this region is limited by the diffusion of glucose to the colony. At glucose concentrations greater than 4 g.p.l., however, a sharp decrease in the growth rate was observed and this was accompanied by a change in the appearance of the colony from the normal whitish colour to a light brown. Similar inhibition of growth has been reported earlier for spread plates containing high glucose concentrations (Chapter 3) and also by Pirt (1967) who suggested that excess glucose was being fermented to produce toxic by-products. A similar effect is produced in a single colony when, due to the higher basal concentration in the gel, the flux of glucose

reaching the colony is sufficiently high so that it is not all consumed by the actively dividing cells at the perimeter, (page 46), thus allowing some to reach the centre regions of the colony. There, although the cells are unable to divide due to the presence of high concentrations of various toxic by-products, (page 140), fermentation of glucose can occur, thereby polluting the conditions at the edge of the colony, and so inhibiting its growth.

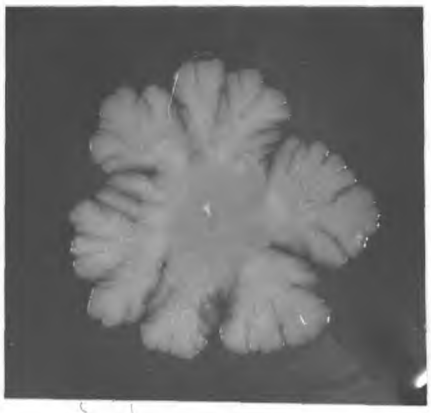
Plate (5-3) shows the appearance of typical colonies at 20 days after inoculation on agars containing glucose at the various concentrations tested in Figure (5-3a). It can be seen that the colonies in the range where the diffusion of glucose limits the growth rate are very similar. The transition from this state to the one in which growth is regulated by the outward diffusion of toxic by-products, is accompanied by a distinct change in the morphological pattern. This is discussed more fully in Chapter 8.

Effect of varying Buffer Concentrations:

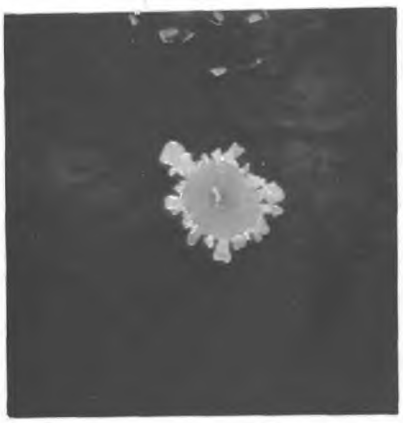
The effect of varying the buffer concentration in the agar, (not the initial pH), on the prolonged growth of a singl colony has also been investigated. At concentrations between 10% and 200% of the normal buffer concentrations, the colony area increased linearly with time over a period of about 3 weeks. Colonies growing on 10% buffer agar gave growth curves with a slightly stepped structure and this behaviour is discussed in Chapter 9.

Plate (5-3)

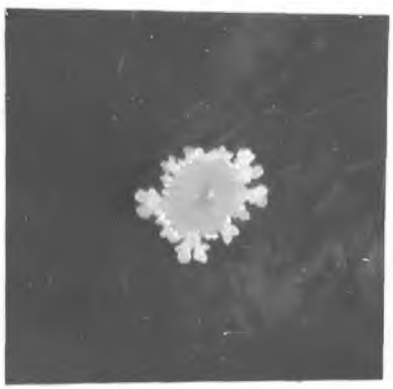
0.5 x normal glucose concentration



0.8 x normal glucose



1.5 x normal glucose concentration



2.0 x normal glucose concentration

(All 2 x actual size and 20 days old)

Figure (5-3a).

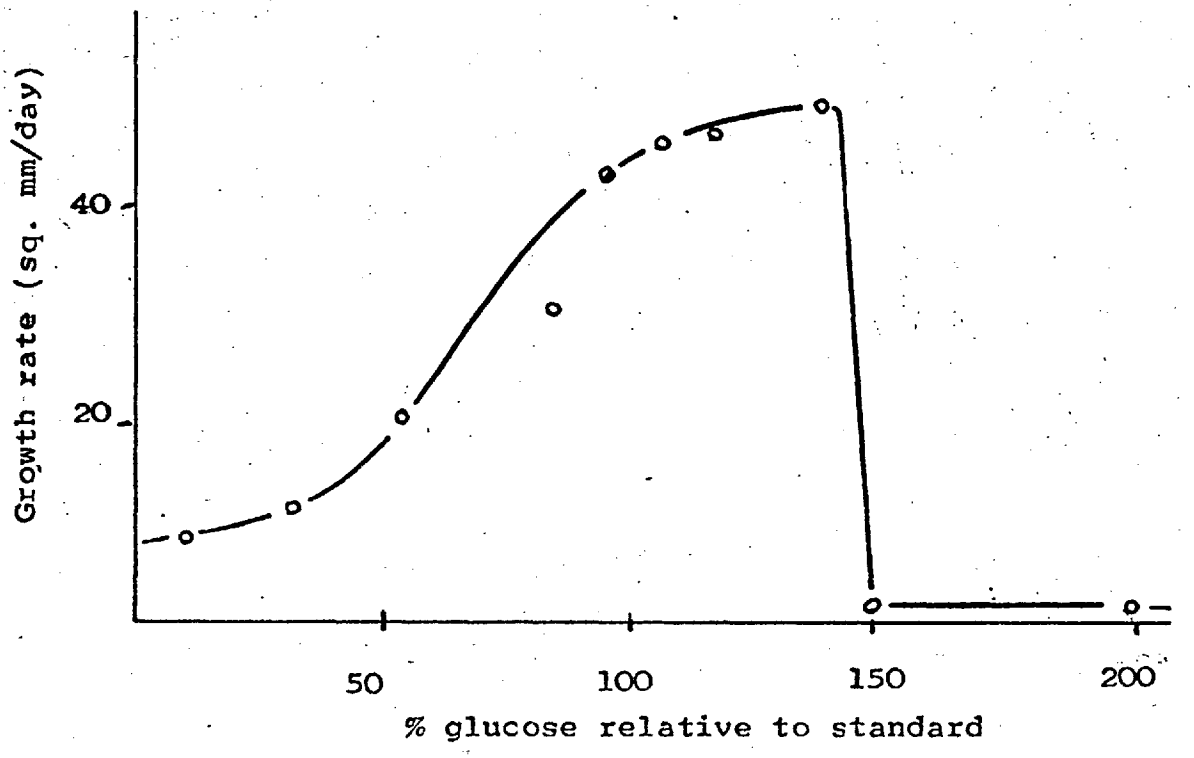
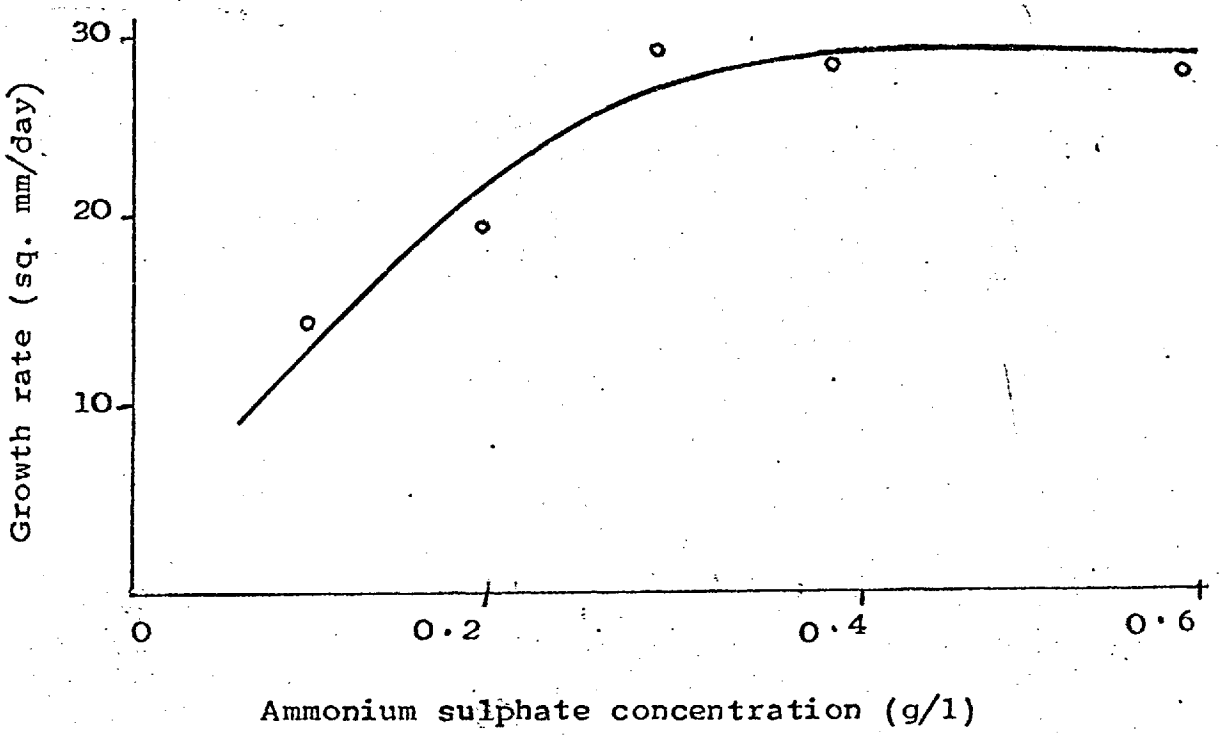
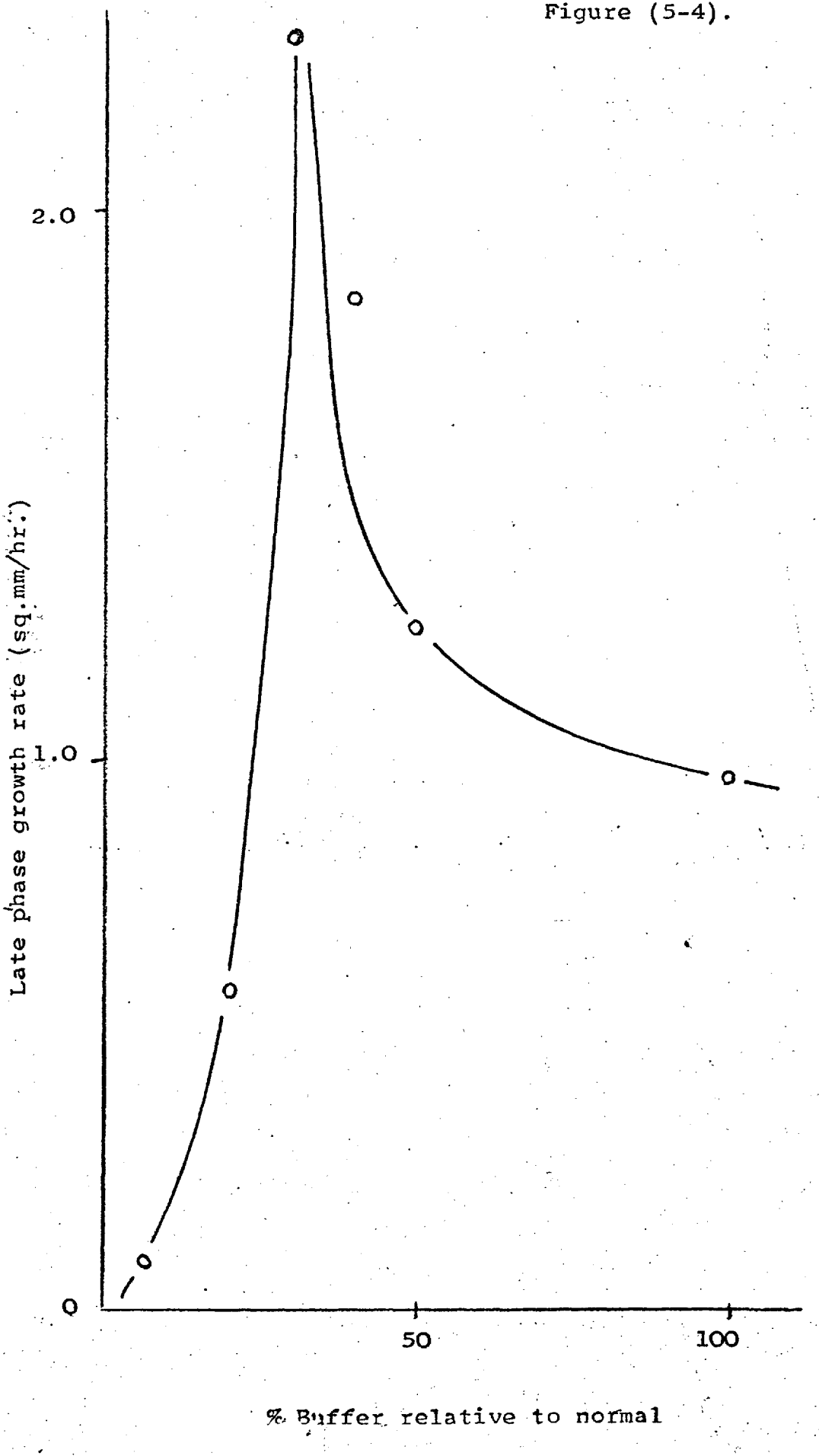


Figure (5-3b)



A plot of the colony growth rate for the normal strain, (average of 2 plates), against the buffer concentration in the agar is given in Figure (5-4). Work to be described later in this chapter, shows that the pH inside a colony growing on 10% buffer agar is near to 4, a value that has been found, (Lodge & Hinshelwood) to be the lowest at which Aerobacter aerogenes will grow. It seems, therefore, that the inward diffusion of buffer is the rate controlling factor in this case. The linear increase in the rate of growth from 10% to 30% of buffer (Figure 5-4) appears to be caused by an increasing flux of buffer reaching the colony and so permitting faster growth. At concentrations of buffer greater than 30% of normal the rate of colony growth decreases progressively. This general type of behaviour was also observed when the glucose concentration was varied, (previous section), and it seems probable that a similar explanation applies to both cases. At the maximum rate of growth, (i.e. when the buffer concentration was about 30% of normal), the dividing cells at the perimeter of the colony are being kept supplied with adequate buffer to maintain their environment suitable for growth, while the pH in the centre of the colony is sufficientl low to inhibit the fermentation of the small quantities of glucose which arrive there when the normal concentration of glucose is employed. If excess buffer is present, however, the pH could conceivably be maintained at a level allowing fermentation to proceed with the consequences already described in the last section.

Figure (5-4).


2.0
1.0
0
Late phase growth rate (sq.mm/hr.)
50
100
% Buffer relative to normal

Effect of varying the Concentration of the Nitrogen Source:

Figure (5-3b) shows the variation in single colony growth rate as the concentration of ammonium sulphate in normal minimal agar is altered. It can be seen that up to 0.4 g.p.l. ammonium sulphate, the rate was proportional to the concentration and hence the diffusion of this substance was limiting growth. At concentrations greater than 0.4 g.p.l. the growth rate was independent of the ammonium sulphate concentration and hence regulated by other factors.

pH Changes:

The pH values at points in the agar gel in and around a growing colony are not in themselves of great interest, but when they are compared with data obtained from the growth of Aerobacter aerogenes in various minimal liquid media they can provide interesting information about the conditions inside a colony.

For the purposes of this present study, the factors which may cause Aerobacter aerogenes to stop growing may be classified as follows:-

1. Nutrient exhaustion, e.g. glucose, ammonium sulphate Mg^{++} etc.
2. Adverse pH.
3. Inhibitory concentrations of toxic by-products other than H^{+}.

This classification is somewhat arbitrary since, for example, adverse pH may be regarded as caused by excessive by-product formation or limitation by nutrient exhaustion (buffer). The categories, however, are convenient in view of the types of measurements that can readily be made on colonies or suspensions of cells, for example, the effect of pH or dependence of final population on the concentration of, say, glucose. Also, a more precise specification of the reason for the cessation of growth may in fact be rather difficult to give, because of the complex interaction of possible individual causes. Thus, for example, certain by-products are known to become more toxic at adverse pH, (Albert, 1951), and also the operation of an effect known as 'Substrate accelerated death', (Postgate & Hunter (1964)).

In the latter, the addition of a substrate, say glucose, to cells which have stopped growing because of the exhaustion of some other nutrient, increases the death rate of these cells. Various effects observed in liquid cultures will now be described. Their relevance to colony growth will become apparent later in this section.

First, it has been shown, (Lodge and Hinshelwood (1939); Dagley, Dawes and Foster (1953)), that for Aerobacter aerogenes growing in liquid media, decreasing the initial pH from its optimum growth value near 7.0 to 4.0, decreased

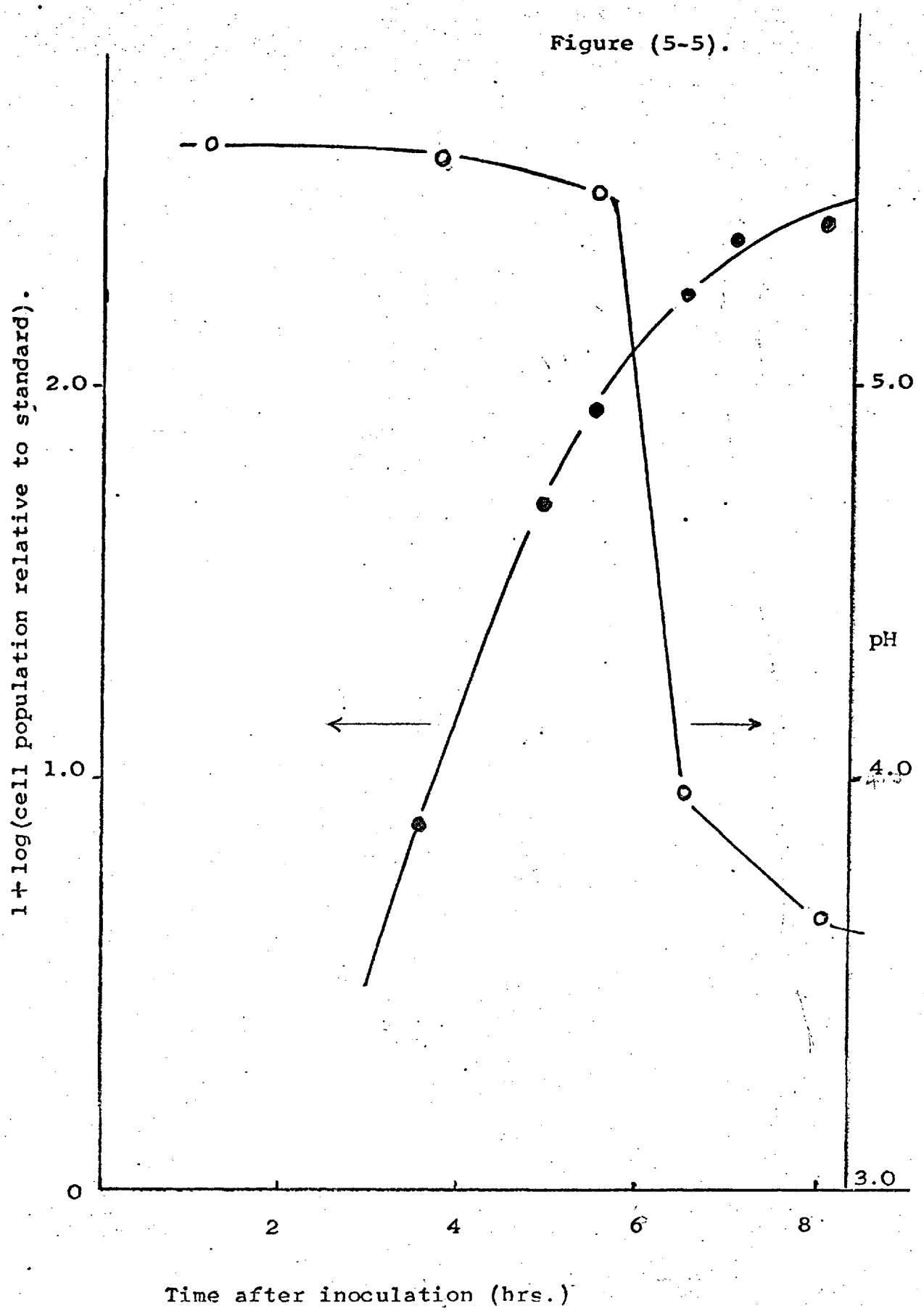
the maximum population, (cells per ml.), attained in a given batch culture. Such changes in the initial pH of the growth media had little effect on the mean generation time of the organism until pH 3.8 - 4.0, when no growth took place.

Secondly, in normal minimal medium containing concentrations of glucose up to 1 g.p.l., growth ceases due to glucose exhaustion since the final pH is still quite favourable for growth, (6.8), the final cell population being proportional to the concentration of glucose in this region (Chapter 6).

Thirdly, the growth of Aerobacter aerogenes in aerated liquid batch culture ceases because of adverse pH when the glucose concentration is raised to 10 g.p.l. and the buffer concentration kept the same, but its initial pH adjusted to 5.5 (Chapter 2). This effect is illustrated in Figure (5-5) in which the variation of the pH of the culture with time is also displayed. In this medium, the final population of cells is independent of slight variations in the glucose concentrations, (Chapter 6), and this, together with the sharp drop in pH which occurs at the end of the logarithmic phase suggests that adverse pH is here causing the organisms to stop growing. The slope of the growth curve (i.e. the growth rate), in Figure (5-5) did not decrease appreciably until the pH of the medium had fallen below 4; this is in agreement with the findings of previous workers, (Lodge and Hinshelwood 1939; Dagley, Dawes and Foster 1953), see page 22.

When the initial pH of the above medium was raised to

Figure (5-5).


1 + log (cell population relative to standard).
2.0
1.0
0
5.0
4.0
3.0
pH
2
4
6
8
Time after inoculation (hrs.)

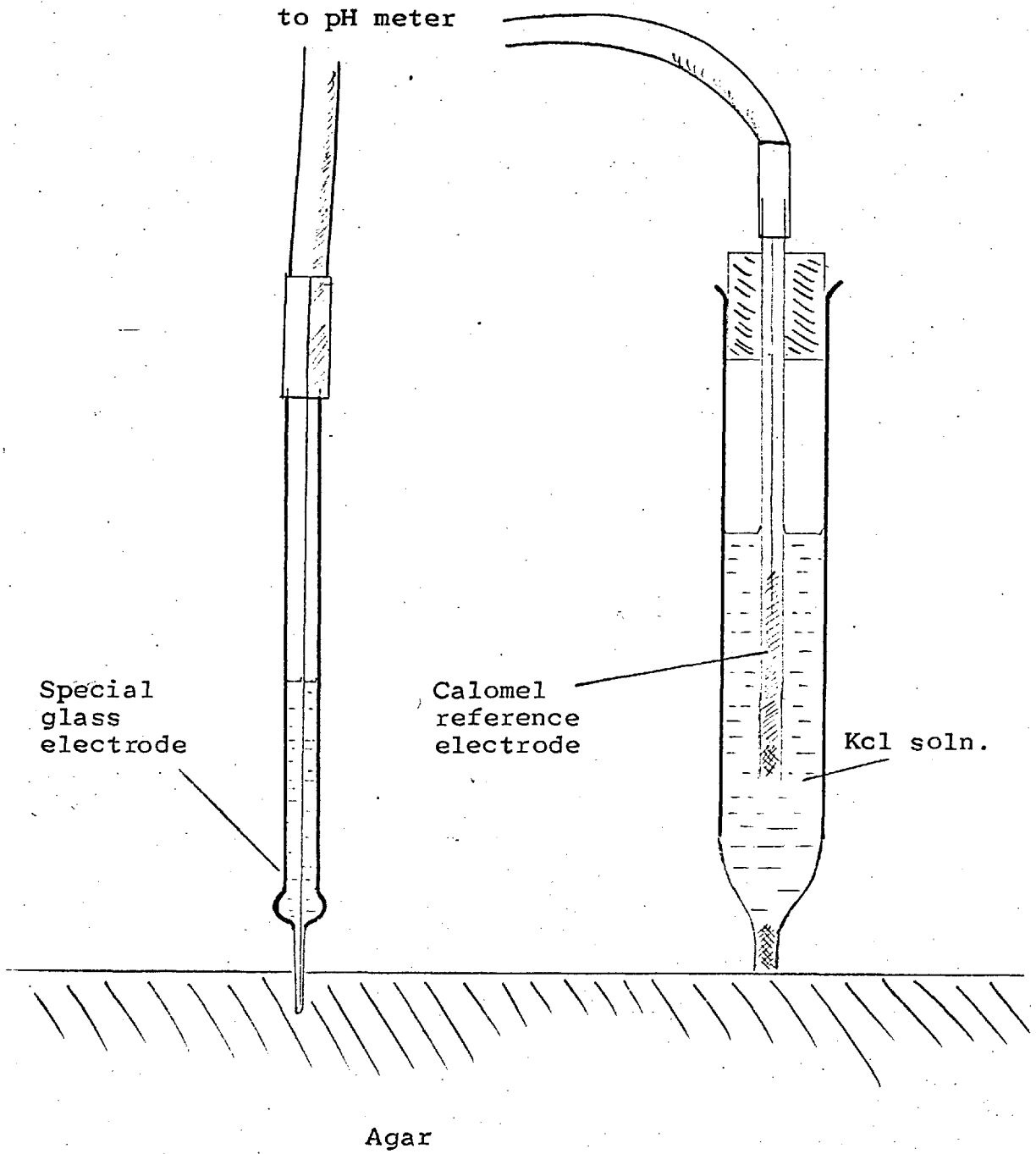
7.1 (i.e. normal minimal medium), a similar form of curve to Figure (5-5) was obtained, but the fall in the pH which accompanies the end of the logarithmic phase was less steep and it seems likely that other toxic by-products are also playing a part in inhibiting growth.

When a culture of Aerobacter aerogenes which has ceased growing at a pH of about 4, is left to stand for 2 or 3 days, the pH of the medium rises due to the breakdown of the acids which were initially produced as by-products, (Dr. A.C.R. Dean - personal communication). This could be due to the adaptation of the cells to these acids and subsequent growth or to the disruption of dead cells releasing components which neutralise or break down the acids.

Changes in the pH observed in bacterial colonies will now be discussed.

While chemical indicators can be used to give a rough idea of the pH in the agar gel around a growing colony, (methyl red), it was felt that the use of a glass electrode and pH meter would yield the most accurate information. Figure (5-6) shows how a glass electrode and a calomel reference electrode were used to investigate the pH on an agar plate. The glass electrode, ('Soft Tissue' type No. 42593) and the centre of the calomel reference electrode (No. 42529) were obtained from Cambridge Instruments Company Ltd. The outer vessel for the calomel electrode was made from a drawn out length of glass tubing,

Figure (5-6)


to pH meter
Special glass electrode
Calomel reference electrode
Kcl soln.
Agar

the end of which was plugged with cotton wool through which the saturated solution of potassium chloride percolated slowly to make electrical contact with the gel surface. It was established that the pH readings obtained in this way were independent of the distance between the two electrodes and the reproducibility was found to be about 0.1 of a pH unit.

The variation with time of the pH in a plate of normal minimal agar containing 10% of the normal buffer concentration and on which about 1,000 cells had been spread is shown in Figure (5-7). The average diameter of the colonies after 2 days of incubation, when they have virtually finished growing, is well below that obtained on 'normal' minimal agar, (normal buffer) and the pH of the gel is very close to a completely inhibitory value. Evidence has already been presented (Chapter 3), suggesting that the exhaustion of the buffer limited the size of the colonies on minimal agar containing 10% of the normal concentration of the buffer and these changes in pH are in accord with this. The pH of the gel rose from 4.1 at 4 days to 4.7 at 6 days and this is similar to the changes in liquid culture discussed earlier.

The sequence of events on a plate of normal minimal agar (normal buffer) spread with about 1,000 cells is shown in Figure (5-8). A pattern similar to that found on 10% buffer agar (Figure 5-7) was in evidence, in that an initial sharp drop followed by a gradual rise and levelling out took place.

Figure (5-7).

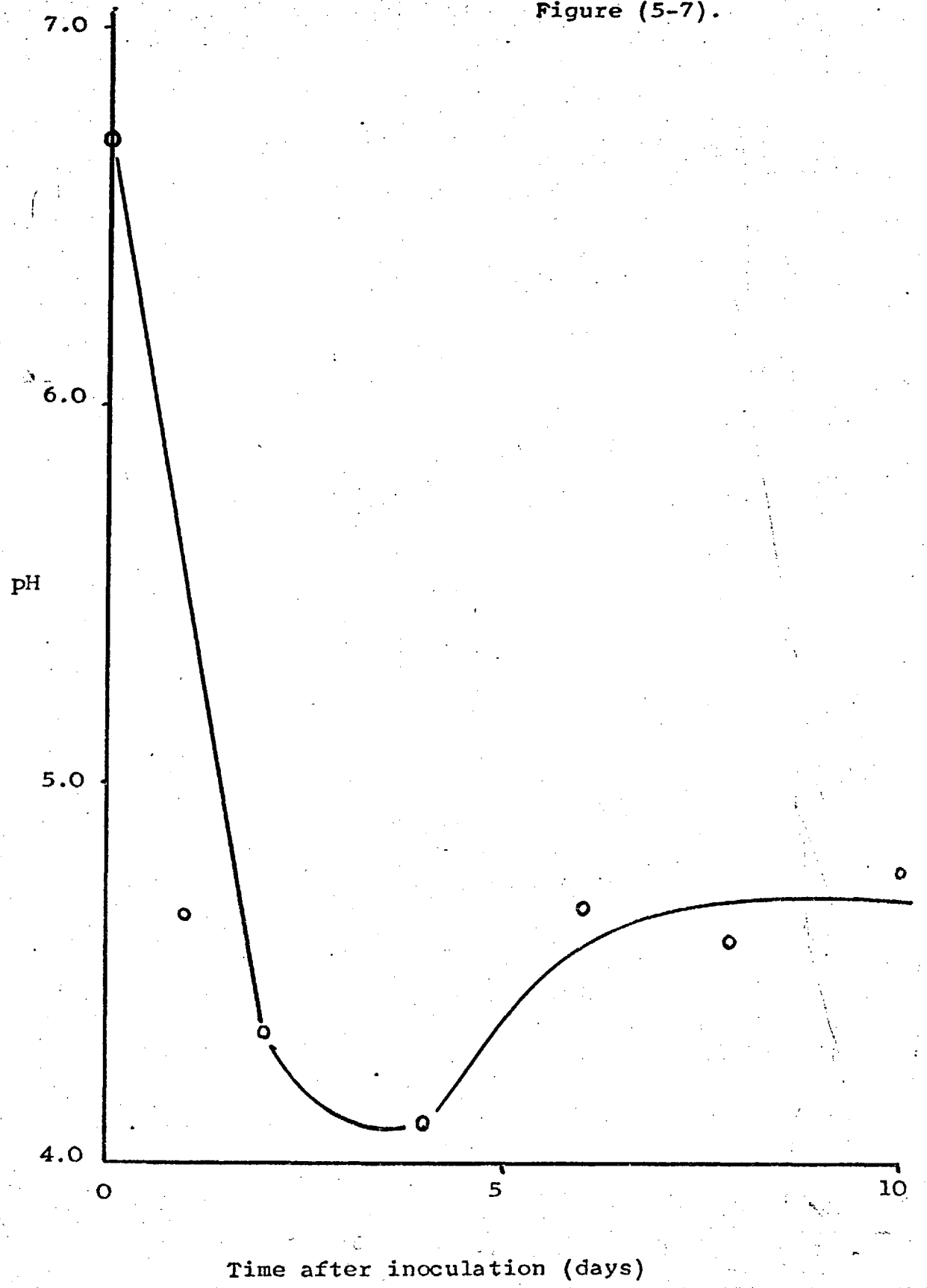
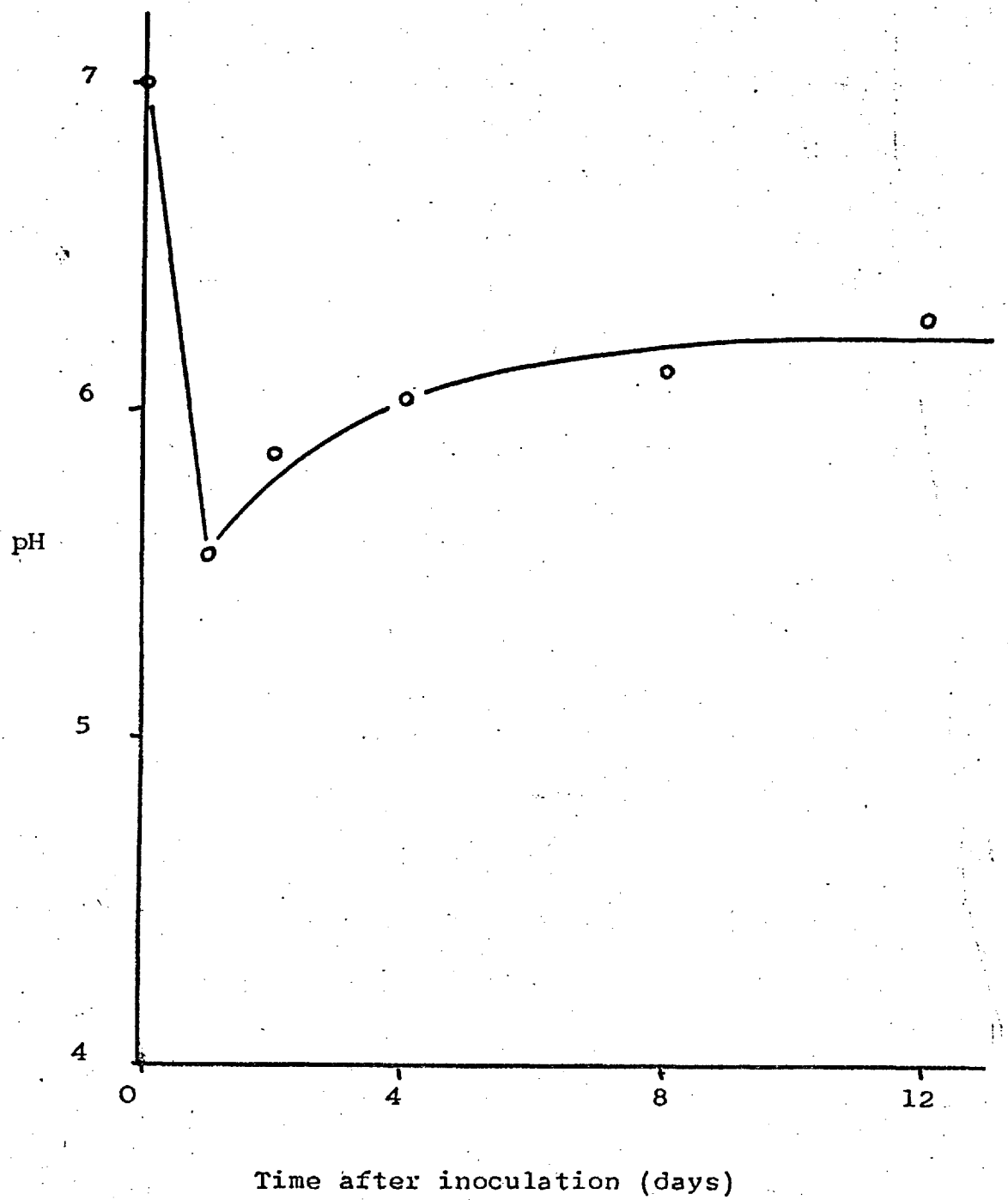


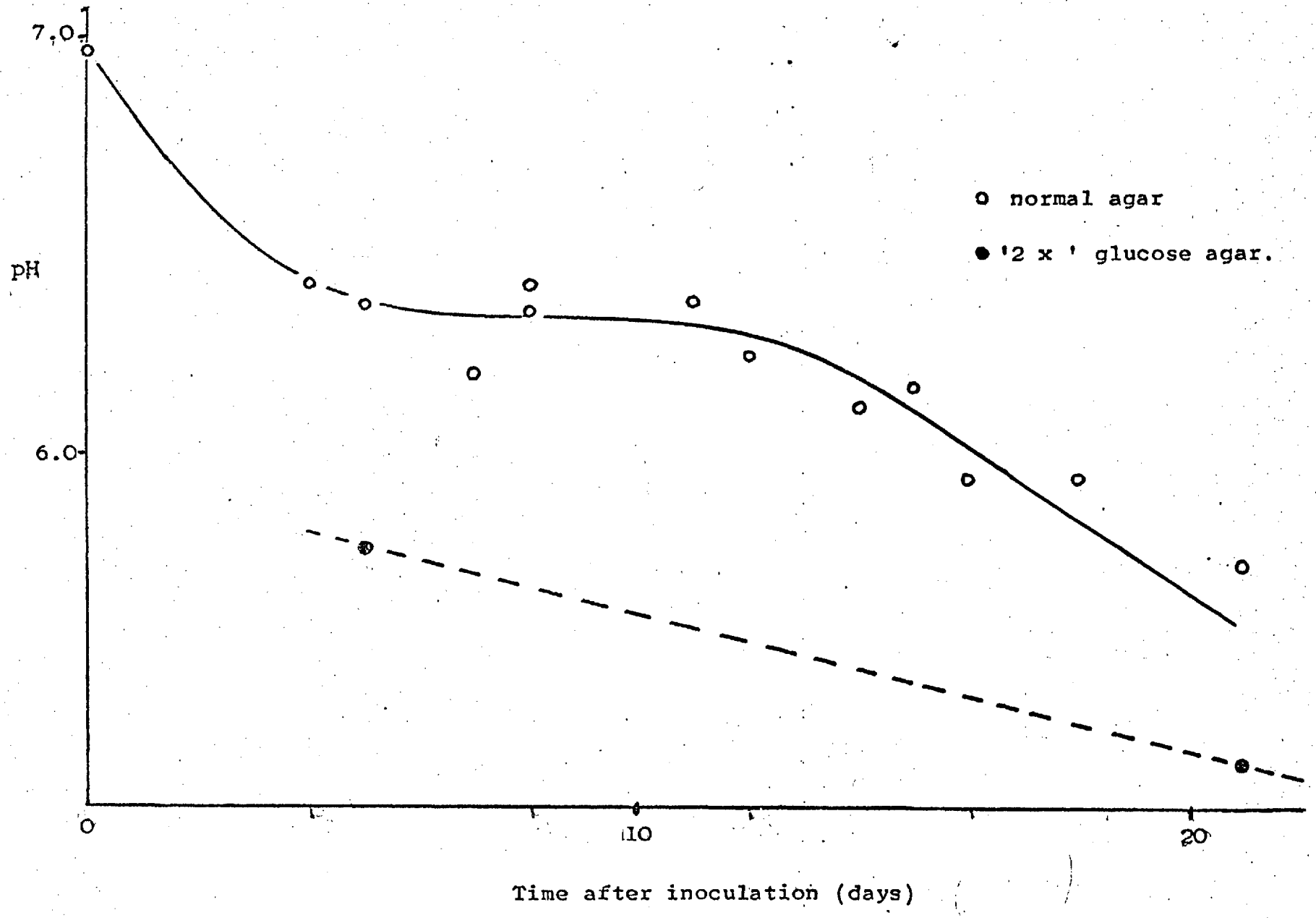
Figure (5-8).



The minimal value observed, about 5.5, is not in itself strongly growth-inhibitory, but the dependence of the total area of colonies, ($\propto C\,ND^2$), upon the buffer concentration from 10% to 200% of the normal level, as described in Chapter 3, indicates that the cessation of growth is closely related to buffer exhaustion. These pH measurements suggest that the effect of buffer exhaustion in limiting growth is an indirect one, since a direct inhibitory effect would require a pH as low as 4. It may be that some growth by-product is the limiting factor, but that this only becomes toxic when the pH falls to a certain low level. Another possibility is that the reduced pH of 5.5 limits growth in much the same way as it reduces the total population attained in batch liquid culture, which was reported earlier in this section.

Figure (5-9) shows the variation of the pH at the centre of a single colony on one plate with time. The general downward trend which is produced by growth on glucose is similar to that which was observed for a spread plate with the same agar. The lowest pH recorded (5.7) was reached after 21 days, whereas on a spread plate (Figure 5-8) this same value was reached after 1 day. This more gradual decrease in the case of the single colony correlates to some extent with its slower and more prolonged growth. The horizontal portion in the middle of the curve in Figure (5-9) is probably due to a tendency for the pH in the centre of the colony to rise,

Figure (5-9).


7.0
pH
6.0
o normal agar
● '2 x ' glucose agar.
0
10
20
Time after inoculation (days)

while acid is still being produced by the actively growing cells at the perimeter. This idea is supported by the pH values observed at different points in a colony since the level at the perimeter was found to be slightly lower than at the centre. Although this evidence by itself does not reveal anything about the glucose concentrations in the centre of the colony, it does suggest that the conditions there are rather similar to those in an old liquid culture.

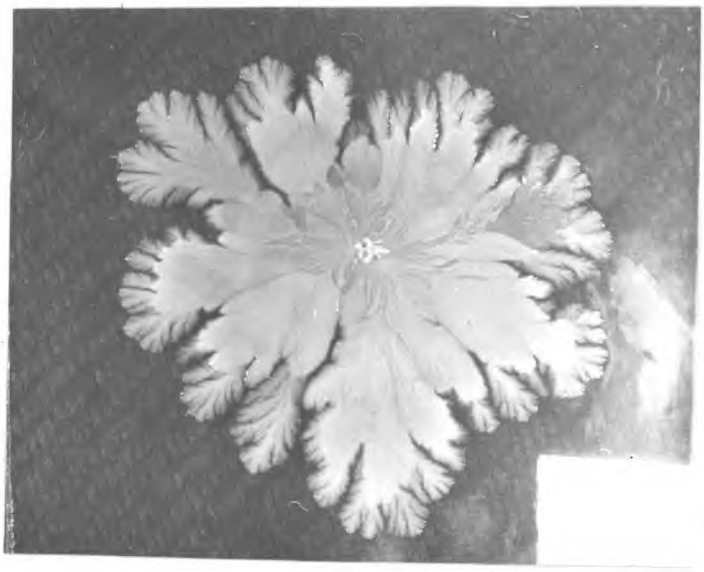
Growth on other Substrates:

Replacing the glucose in the normal minimal agar medium with the same concentration of glycerol had little effect on the appearance of the colony (Plate 5-4a). Nor did the addition of 1 ml. of meat broth to 15 ml. of the normal (glucose) minimal agar result in any pronounded changes in appearance (Figure (5-4b)), although, as would be expected, the rate of growth was augmented considerably.

~~A discussion of the morphology of these colonies in growth rate will now be discussed.~~

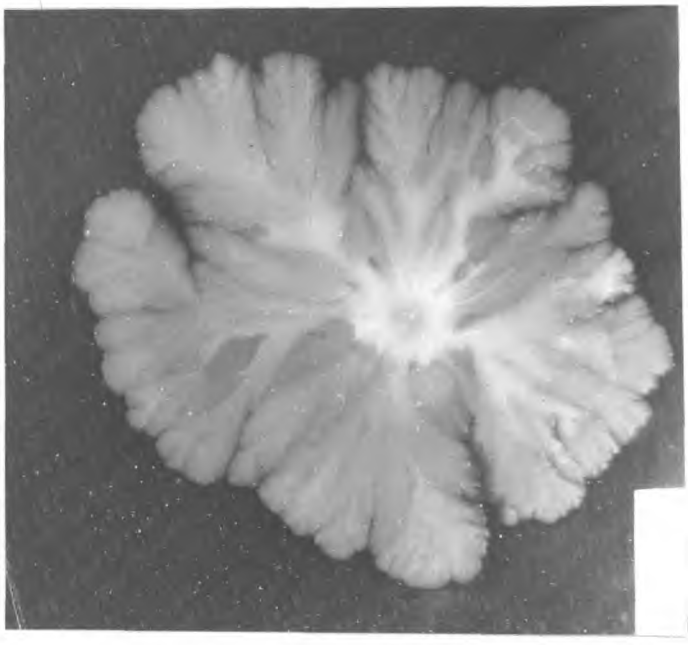
The addition of meat broth to normal minimal agar produce an increase in colony growth rate which was much greater than the corresponding effect in liquid media. The M.G.T. (Chapter 2), of Aerobacter aerogenes in normal minimal liquid medium is about 32 minutes and this falls to about 20 minutes with the addition of meat broth, (Stephens and Hinshelwood (1949)), which represents a 1.6-fold increase in growth rate. An experiment comparing large single colonies of Aerobacter

Plate (5-4a)



27 days after inoculation

Plate (5-4b)



15 days after inoculation

(Both 2.5x actual size)

aerogenes showed a rate of growth of 27.8 sq. mm./day on normal minimal agar and 64.0 sq. mm./day on normal minimal agar (15 ml.), containing meat broth (1 ml.) i.e. a 2.3-fold increase. This increase probably results from a combination of two factors. First, the addition of meat broth to normal minimal medium will increase the growth rate of the cells by a factor of about 1.6, as in liquid medium, but, since the colony growth is diffusion controlled, (Chapter 3), raising the basal nutrient concentration will also increase the growth rate by an additional factor. This is feasible when meat broth is the nutrient, since the by-products of growth on it are less acid than those produced from glucose, (Wilson and Miles, 1957, page 116).

Direct Physical Means of controlling Colony Shape:

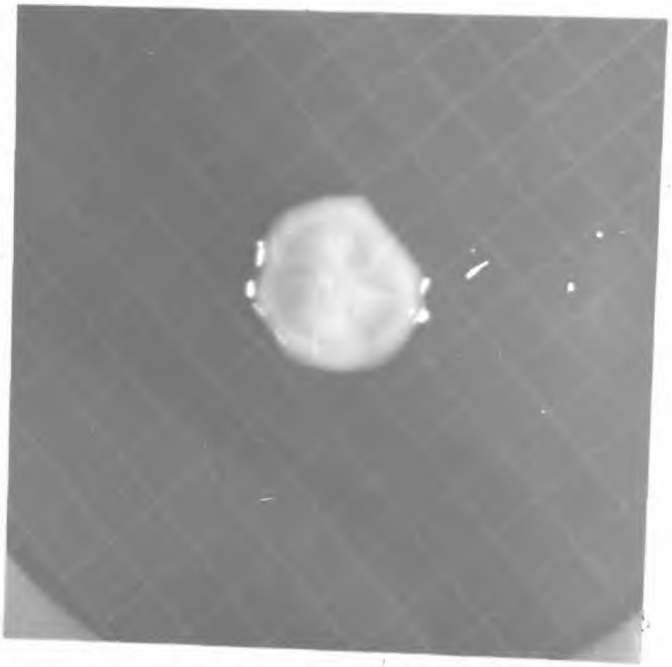
Two physical methods of influencing colony shape were investigated during this work and they are described below.

Plate (5-5a) shows the way in which a fine plastic fibre resting on the surface of the gel can 'lead' the perimeter of a colony of Aerobacter aerogenes. This effect is very probably connected with the channel of liquid produced by the formation of a meniscus against the fibre. Aerobacter aerogenes is non-motile and the work of Armstrong, Adler and Dahl (1967) suggests that the movement of such bacteria by diffusion is not rapid. It seems probable, therefore, that the cells in the liquid channel advance by

Plate (5-5a)

15 days after inoculation , 2·5×.

Plate (5-5b)



17 days after inoculation , 2·5×.

dividing, much as the cells on the neighbouring gel surface do, but that this process is faster in the liquid, perhaps because the packing of the cells is less close.

Plate (5-5b) shows a colony of Aerobacter aerogenes growing on a 'Millipore' filter, (Type HABG, Chapter 2), resting on the surface of a plate of normal minimal agar. It can be seen that although the colony can cross the grid lines ruled on the filter, it does not do so readily. This seems to be connected with the fact that the grid on these filters is impermeable.

Chapter 6.

Modified Strains.

It was thought that an investigation of the metabolism and colony formation of various modified strains of Aerobacter aerogenes would be interesting in its own right. Such studies might also suggest possible factors which are important in the regulation of colony growth generally or illustrate certain ideas which have been developed from the work carried out on the normal strain.

The modified strains were prepared by sub-culturing the organism in minimal media containing progressively higher concentrations of a particular drug or dye. In this way strains which were able to tolerate the toxic agent in much higher concentrations than the original strain were obtained. Details of the strains and their preparation are given in Chapter 2. A strain was also prepared by prolonged sub-cultur in minimal medium under anaerobic conditions in view of a report (Warburg 1956), that continual culture in the absence of oxygen caused the degeneration of some yeasts.

There has been considerable controversy over whether the acquisition of resistance to a particular agent by a strain of bacteria takes place by the selection of a small number of mutant cells or by a modification to the majority of the population, (for example, Wolstenholme and O'Connor (1957); Dean and Hinshelwood, (1966). For the present purpose, however, we are only interested in correlating the changed

biochemical properties of the resistant strains with changes in their colony formation and are not concerned with the mechanism by which these modified cells have been produced.

Growth Rate in Liquid Culture:

The exponential growth rates (Chapter 2), in normal minimal medium free of any drug or dye, are shown in Table (6-1). Generally, drug-or-dye-resistant cells have a lower growth rate in minimal medium than the normal strain, since the latter is the fastest growing system by virtue of its evolutionary selection and modifications to its metabolism to resist the action of some toxic substance, however incurred, will be accompanied by deviations from this optimum pattern.

It has been suggested that strains of organisms prepared by exposure to agents such as acridines and cationic dyes, grow by enforced anaerobic metabolism, (Ephrussi 1949; Gause 1966). Data for the normal strain under anaerobic culture conditions has, therefore, been included in this and subsequent tables for comparison with the resistant strains.

Glucose and Ammonium Sulphate Utilisation:

The efficiencies of glucose utilisation, in the absence of any drug or dye, of several modified strains of Aerobacter aerogenes have been determined by growing the strains in minimal media containing varying concentrations of glucose and measuring the concentration of cells when growth ceased. Figure (6-1a) shows the final cell population relative to a

TABLE (6-1) GROWTH RATES IN LIQUID MEDIUM.

Strain resistant to	Mean generation time in liquid medium (min).		Inverse ratios i.e. relative rates.
(Normal)	33,34,34,35	Av34	1.0
5-Aminoacridine	49,50	Av49.5	0.8
Janus black	75,76,77,80	Av77	0.44
Anaerobic strain	35,39	Av37	0.92
Normal strain (anaerobic growth)	45	Av45	0.75

Figure (6-1a).

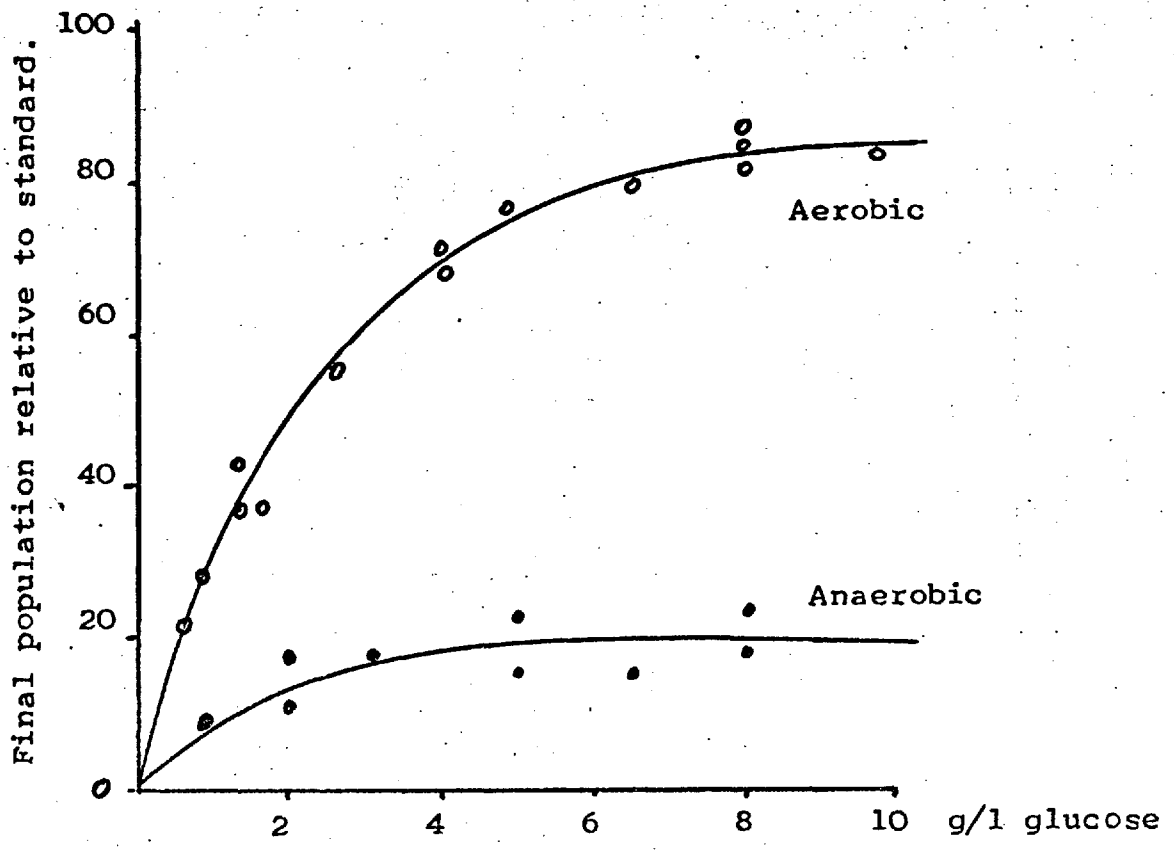
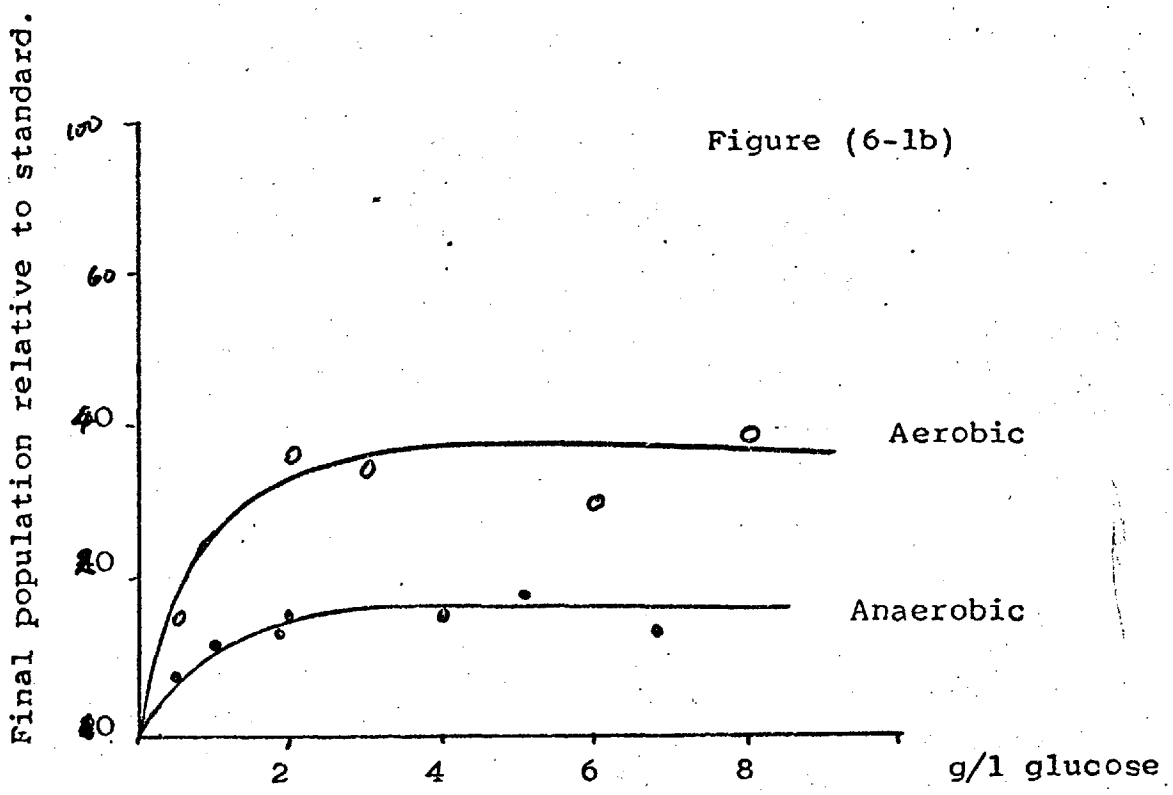


Figure (6-1b)



standard culture, (Chapter 2), for the normal strain in aerated tubes, as a function of glucose concentration. The form of this curve has been discussed in Chapter 5 with the factors which can cause a culture of Aerobacter aerogenes to stop growing, (page 123). In the first part of the curve, glucose exhaustion is the dominant factor, since in this region the limiting population of cells is proportional to the glucose concentration and the final pH level, (6.5), is still suitable for further growth. At higher glucose concentrations the accumulation of toxic by-products, together with associated low pH levels limit growth, since here the final cell population is independent of the glucose concentration and the final pH (4.0) is quite adverse for growth.

The limiting population for the normal strain grown under anaerobic conditions, (tubes eluted with nitrogen), is shown in Figure (6-1a) as a function of the glucose concentration. It can be seen that at a given concentration of glucose, the limiting cell density under anaerobic conditions is always much less than that attained aerobically. This demonstrates the reduced efficiency with which the cells can utilise glucose by anaerobic metabolism.

The curves for aerobic and anaerobic cultures are of the same form, however, which suggests that for both conditions, the exhaustion of glucose was limiting the growth at low glucose concentrations, whilst at higher concentrations the accumulation of toxic by-products was the limiting factor.

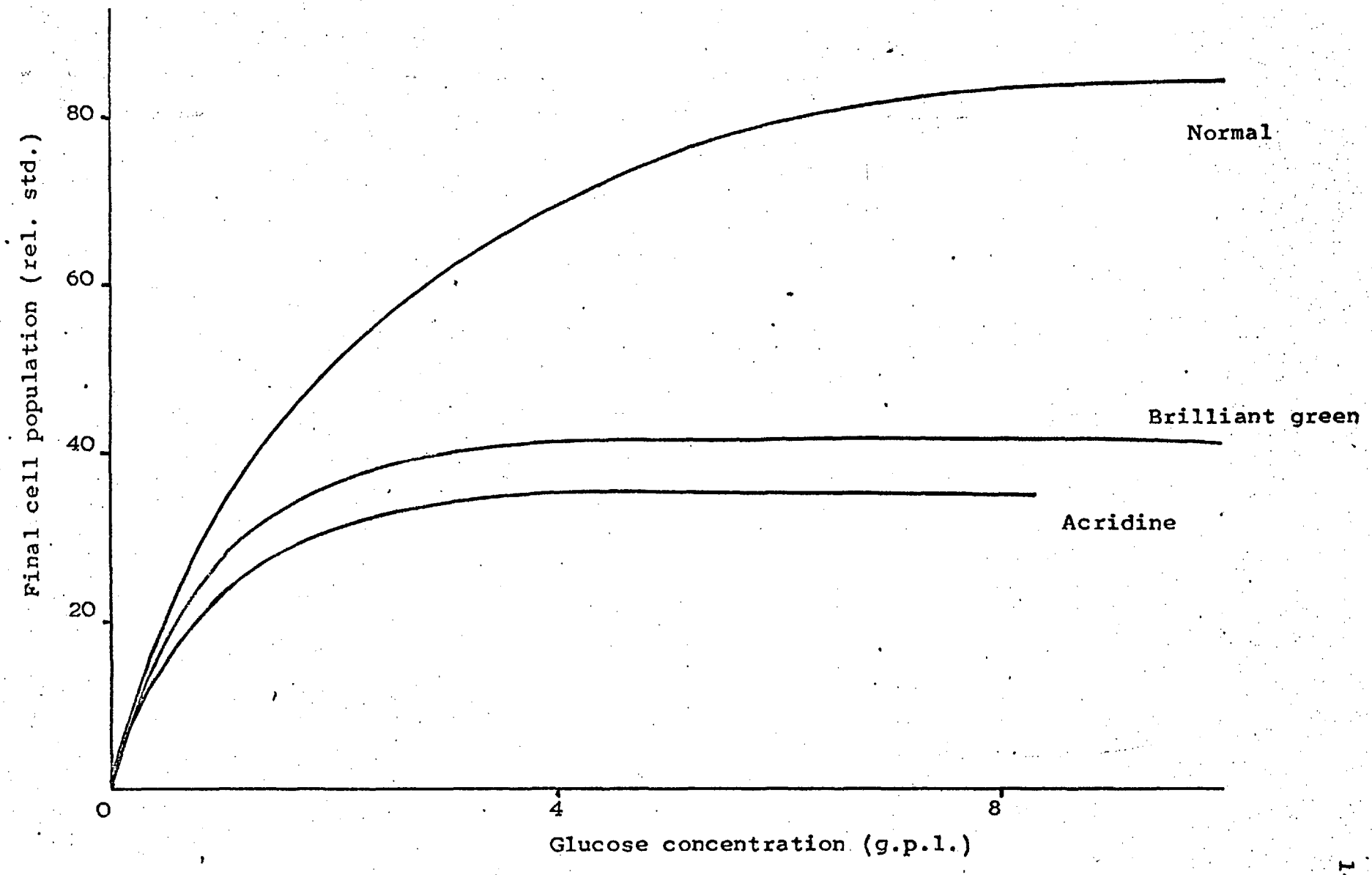
The final cell population as a function of the glucose concentration, obtained under aerobic and anaerobic conditions, with a strain of Aerobacter aerogenes resistant to 5 — aminoacridine is shown in Figure (6-1b). It is interestin that while the efficiencies of the normal and the acridine-resistant strain are similar under anaerobic conditions, they differ considerably in aerobic culture. Thus the normal strain utilises glucose approximately four times more efficiently aerobically than anaerobically, but for the acridine-resistant strain this ratio is about two.

The suggestions of various workers that strains of organisms resistant to drugs, in particular acridines, have an impaired respiration and thus can only metabolise glucose by anaerobic routes have already been referred to. That this mode of growth could be operating in the acridine-resistant strain of Aerobacter aerogenes is suggested by its generally lowered efficiency of utilisation of glucose under aerobic conditions and its similarity, in glucose utilisation, to the normal strain under anaerobic conditions. The metabolism of glucose by the acridine-resistant strain does not, however, proceed entirely by the anaerobic route, since glucose is utilised with an efficiency which is distinctly higher than that found in anaerobic cultures of the normal strain. In addition, differences in the efficiency do exist between the aerobic and anaerobic cultures of this resistant strain, which would not be so if complete anaerobic metabolism was operating.

In Figure (6-2), the glucose utilisation curves obtained under aerobic conditions, with the normal strain of Aerobacter aerogenes, a strain resistant to Brilliant Green and a strain resistant to 5 - aminoacridine are shown. Although the strains differ in the efficiency with which they utilise glucose it is evident that the curves are all of the same form. The principle source of experimental error in the individual points seems to be due to variations in the rate of supply of air to the individual tubes. If the flow to a particular tube falls sufficiently or stops altogether for a while, anaerobic growth will take place and a lower final population will be obtained. The scatter is particularly noticeable in the horizontal parts of the curves where it has been shown that toxic by-products are causing growth to cease. Some of these products are volatile and it is possible to remov them by vigorously aerating a culture, (A.C.R. Dean, personal communication). Variable rates of aeration could thus affect the final population.

The efficiency with which glucose was utilised was also measured for other modified strains. Rather than construct complete curves, however, a simpler procedure was adopted. In it, measurements were made of the limiting cell population reached in several duplicate tubes of minimal media containing 1 g.p.l. and 10 g.p.l. of glucose. It can be seen from Figure (6-2) that such points are characteristic of the whole curve. The value obtained at 1 g.p.l. is a measure simply of

Figure (6-2).



(Experimental points omitted for clarity (see Figure (6-1)

the efficiency with which the cells can utilise glucose, since growth in this region is limited by its exhaustion. The final population obtained at 10 g.p.l., however, depends upon the efficiency of glucose consumption and also on by-product production.

The data obtained in this way is given in Table (6-2), columns 2 and 3; each result is the average of at least two duplicate tubes. The high values recorded for the barbitone-resistant strain are in agreement with the work of Dean and Moss 1967. In general, the efficiencies are less than that of the normal as would be expected from similar arguments to those used in the section dealing with growth rates in liquid media. Nevertheless, there appears to be no correlation betwe the growth rates in liquid media and the efficiency with which glucose is converted into bacterial substance, presumably because the great variety of different processes within the cell which determine these two properties are not necessarily all common to both.

For the various strains studied, the results obtained when 1 g.p.l. of glucose was used showed an 18% average deviation from the mean, while the corresponding figure for culture in 10 g.p.l. glucose media was 41%. This 18% variation is a measure of the differences in the efficiency of glucose utilisation between the various strains. On the other hand, the value of 41% obtained when glucose was present in excess

TABLE (6-2).

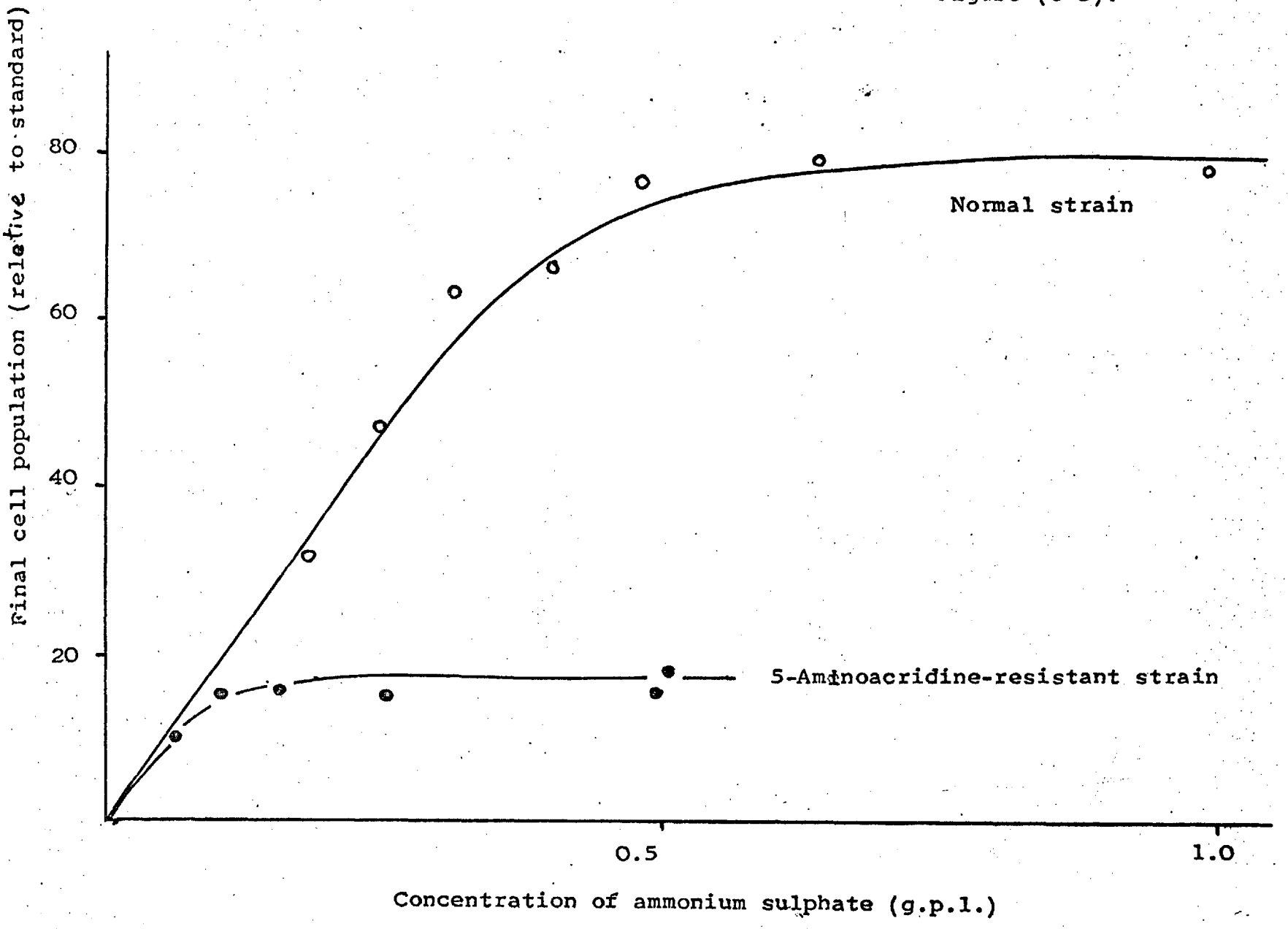
Strain resistant to	Population in liquid medium relative to Standard. 1 g/l glucose.	10 g/l glucose. final pH in brackets.	ND^2 ($mm.^2$/plate)	ND^2 relative to normal.
5-Aminoacridine	0.67	0.35 (4.7)	499	0.26
Chloramphenicol	0.90	0.55 (4.3)	1122	0.58
Crystal violet	0.75	0.56 (3.3)	1862	0.96
(Normal strain)	1.00	1.00 (3.75)	1946	1.00
Barbitone	1.05	1.30 (4.65)	2220	1.14
Brilliant green	0.90	0.40 (4.5)	2910	1.50
Methylene blue	1.35	1.25 (6.2)	-	-
Janus black	1.25	0.66 (6.3)	-	-

of requirements, suggests partly a greater error in the experimental results, as discussed earlier, but possibly also that the strains vary in their production of by-products or in their susceptability to these substances. The variation in the final pH values reported in Table (6-2) support the latter suggestions.

The efficiencies with which the normal and the acridine-resistant strains utilised ammonium sulphate under aerobic and anaerobic conditions were compared in a similar manner to that just described for glucose. These results are given in Figure (6-3) which shows that the relative values for the limiting populations of these two strains were similar to those in Figure (6-1), where the glucose concentration was varied.

It has already been pointed out, (Chapter 5), that for large single colonies of the normal strain of Aerobacter aerogenes growing on normal minimal agar, ammonium sulphate was not growth limiting and, furthermore, that the growth of the colony could in most circumstances be interpreted in terms of glucose, buffer or by-product regulation. It can be argued, therefore, that for the purposes of the present study, the efficiencies of utilisation of NH_4^+ by the various modified strains are probably of secondary importance and for this reason they have not been included.

Figure (6-3).


Final cell population (relative to standard)
80
60
40
20
Normal strain
5-Aminoacridine-resistant strain
0.5
1.0
Concentration of ammonium sulphate (g.p.l.)

Colony Formation on Spread Plates:

It was stated in Chapter 3 that with the normal strain, when more than 1,000 colonies were present on a plate, the final total area of the colonies was constant and independent of the number, N. This was shown by the horizontal portion in the plot of ND^2 against N, (Figure (3-2) page 37), where D is the average colony diameter. It was also suggested that the observed result was a consequence of the cessation of growth due to the exhaustion of some nutrient in the gel or to the accumulation there of some by-product up to a critical concentration. This was supported by the fact that the limiting value of ND^2 was found to be proportional to the volume of the gel and also to the initial buffer concentration.

The limiting values of ND^2 obtained with some of the modified strains on normal minimal agar, (no drug or dye present), are given in Table (6-2), columns 4 and 5. For the normal strain on normal minimal agar, the limiting value of ND^2 is a measure of the maximum number of cells, in the form of colonies, which the normal volume of the gel will support. It might be expected, therefore, that there would be a proportionality between the values of ND^2 for the various strains and the efficiency with which they utilise glucose in liquid culture. For this to be so, it is necessary that all the colonies are of approximately the same vertical height. It was observed, that while this criterion was satisfied by the colonies of the majority of the strains, those formed by the

Brilliant Green - resistant strain were significantly thinner. This could provide an explanation for the relatively high value of ND^2 observed in this case. (The factors which regulat the vertical height of a colony are discussed in Chapter 7).

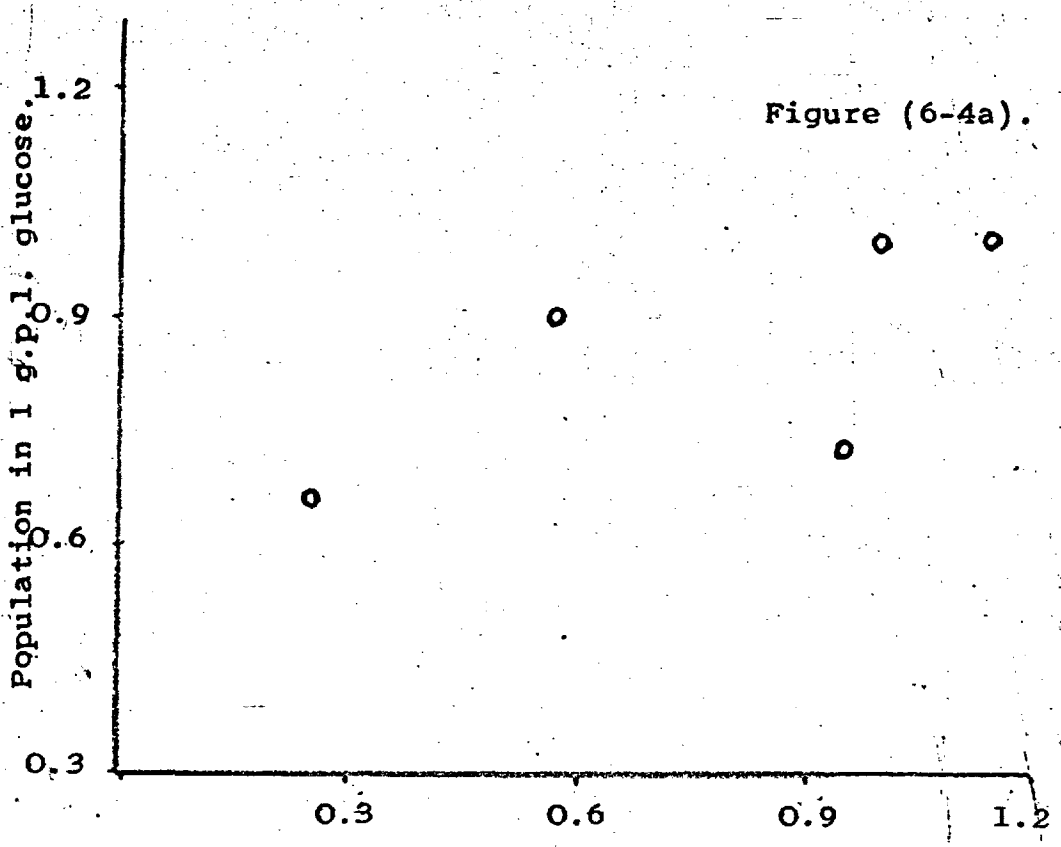
The values of ND^2 for the various strains listed in Table (6-2), (except the Brilliant Green-resistant), are plotted against the corresponding population densities obtained in liquid minimal medium containing 1 g.p.l. glucose, in Figure (6-4a). The postulated proportionality, is, however, not in evidence. Figure (6-4b) shows a similar test, but in this case the values of ND^2 for the various strains are plotted against the limiting population reached in minimal medium containing 10 g.p.l. of glucose. Here, in contrast, a rough dependence of the two functions is observed.

This rather approximate dependence suggests that while the basic relationship exists, other factors are also in operation. The fact that some sort of proportionality is detectable in Figure (6-4b) and not in Figure (6-4a) also suggests, that as with the normal strain, toxic by-product accumulation rather than glucose exhaustion limits growth on such plates.

Colony Growth Rates:

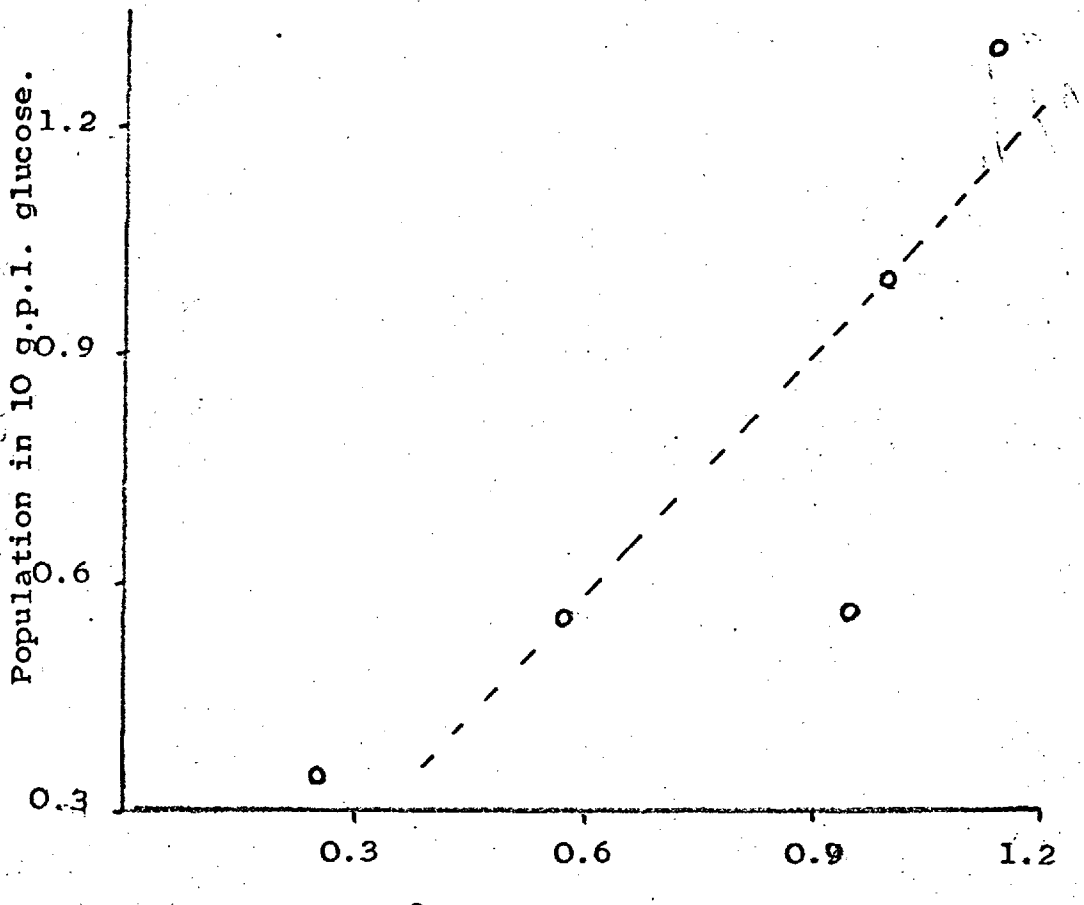
The kinetics of colony growth during the period immediately after the initial division of the inoculated cell have not been studied in these experiments. It is reasonable to assume, however, that it is exponential and if so, the growth rate of the colony at this stage should be directly

Figure (6-4a).



(see below)

Figure (6-4b).



related to the reciprocal of the mean generation time of the particular strain in uniform liquid media. As the colony grows, physical or chemical interactions between the cells slow down the rate, (Hoffman 1964), and when the diameter is 0.4mm. the 'linear' phase (below), is reached.

The diameters of colonies of Aerobacter aerogenes on sparsely spread plates, increase linearly with time in the range 0.4 - 4.0mm., (Chapter 3). It was concluded that the most likely explanation for this was that growth was confined to the cells in a narrow annulus at the perimeter of the colony. No measurements have been made on the growth rate of colonies of the modified strains of Aerobacter aerogenes in this early phase, but the work of Pirt, (1967), using slightly different conditions, is interesting in this respect. Pirt produced colonies by forming a small pool of a suspension of cells of various organisms on the surface of an agar plate. Confluent growth took place rapidly within this area and the diameter of the colony which formed was found to increase linearly with time in the range 2.0 - 5.0 mm. Whilst such a colony does behave in some respects in a similar manner to a colony formed from a single cell, there are certain differences as discussed in Chapter 3.

Pirt varied the intrinsic growth rate of cells of E. coli by adding sulphanilamide to the agar gel and observed that the linear rate of growth of the colony was proportional to the square root of the growth rate of the organism in liquid medium

The precise reason for this relationship is not clear, but it seems likely that factors other than the intrinsic growth rate of the organism are becoming important.

The rate of colony growth of some of the modified strains of Aerobacter aerogenes in the phase where the area increases linearly with time are shown in Table (6-3). The colony growth rates show no proportionality to those obtained in liquid medium, quite probably because growth in this phase is primarily limited by the diffusion of nutrients and by-products, as with the normal strain, (Chapter 3). If this were so it might be expected that colony growth rates would be proportional to the final populations reached in the glucose utilisation tests or to the values of ND^2 obtained with spread plates. No such relationship is exhibited, however, although the 5 - aminoacridine-resistant strain is noteworthy in the low values both of its colony growth rate and of ND^2. The methylene blue and Janus black-resistant strains, which show particularly low colony growth rates, also exhibit morphological peculiarities which will be discussed later.

In conclusion, only a rough correlation between the properties of a particular strain in liquid culture and its behaviour on solid media has been found. When more than 1,000 colonies are present on the plates and the final size of the colonies relatively small, ND^2 has been shown to be approximate proportional to the yield of cells obtained in liquid medium

TABLE (6-3) RATES OF COLONY GROWTH.

Strain resistant to	Rate of growth i.e. d(area)/dt relative to normal strain.
5-Aminoacridine	0.06
Chloramphenicol	0.92
Crystal violet	0.60
Barbitone	0.87
Brilliant green	0.67
Methylene blue	0.22
Janus black	0.39
Streptomycin	0.97
Streptomycin) Sulfhanilamide) Chloramphenicol)	0.69
Normal strain	1.00
Normal strain - anaerobic growth	0.05
Anaerobic strain - aerobic growth	0.67

containing 10 g.p.l. of glucose. The growth rates of the large colonies obtained on sparsely inoculated plates remain difficult to relate to other parameters observed in liquid cultures.

The colony growth rates in the phase of growth where the area increases linearly with time have been measured for some strains growing on different agar gels. The results are summarised in Table (6-4) and it can be seen, that although the rates differ considerably on normal agar, they are very similar on agar containing one-tenth of the normal buffer concentration. The most probable explanation would appear to be that when the basal concentration of buffer in the gel is low the rate of its diffusion towards the colony is the principal rate-controlling factor for all the strains, the various metabolic differences between them becoming of secondary importance. A similar effect, produced by the excessive formation of by-products may exist on the 'double glucose' agar plates, although since this agar was toxic to the methylene blue - and Janus black - resistant strains, some caution is necessary in drawing conclusions.

It is of interest to note here that some of the modified strains, particularly those resistant to methylene blue and to Janus black, appeared to be sensitive to media of high osmotic strength. Thus, with the above strains the viability on '1/10 buffer' agar was higher than on the normal agar and they would not grow at all on minimal agar containing double

TABLE (6-4).

Strain resistant to	Large Colony Growth Rates (sq. mm./day). Normal agar.	'1/10 buffer' agar.	'2 x glucose' agar.
(Normal)	34, 37	3.6, 2.8	2.7, 1.3
5-Aminoacridine	1.1, 1.3	2.4	2.0, 2.3
Methylene blue	7.8	2.3	-
Janus black	13.8	2.4	-

the normal glucose concentration or on normal minimal agar to which sodium chloride, (≈ 0.5% w/v), had been added.

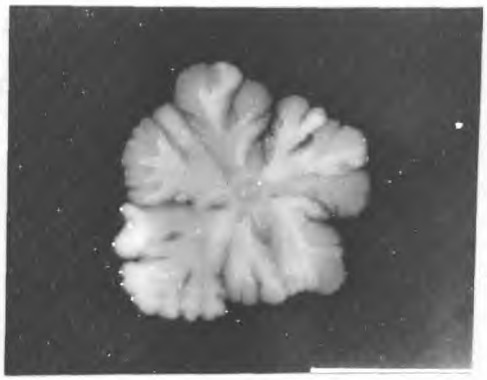
The faster growth rate of the 5-aminoacridine-resistant strain on '1/10 buffer' and on 'double glucose' agar relative to its performance on the normal gel is surprising, (Table (6-4)), but its colony behaviour in general seems rather anomalous.

Colony Morphology:

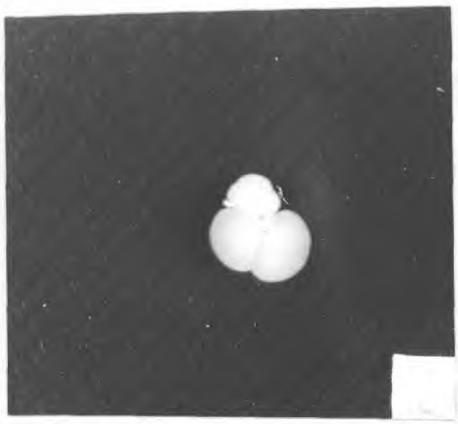
Colonies of the various strains on spread plates do exhibit some variation in morphology, for example, the Brilliant Green-resistant strain forms colonies which are particularly thin, but this is much less striking than the differences observed in colonies which have been produced by inoculation with a platinum wire in the centre of a plate and subsequently allowed to grow for about two weeks.

Plate (6-1) shows colonies of some of the various strains at 15 days after inoculation on normal minimal agar. For a given strain, two colonies were of a recognisably similar pattern although they were not identical to the extent of being superimposable. Also, the pattern characteristic of one strain can in general, be distinguished from that of another. It was stated in Chapter 1 that different species of microorganisms produced characteristic colonies, but from the above, it appears that even modified strains of the same organism can produce distinguishable patterns. The colony

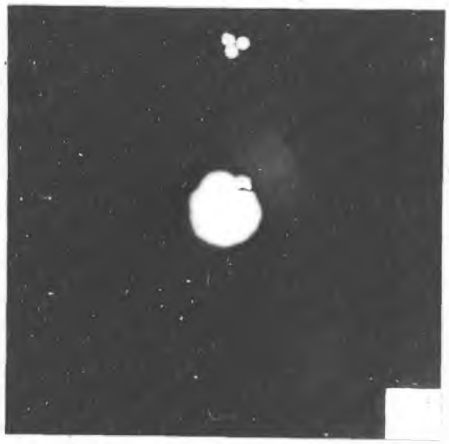
Plate (6-1)



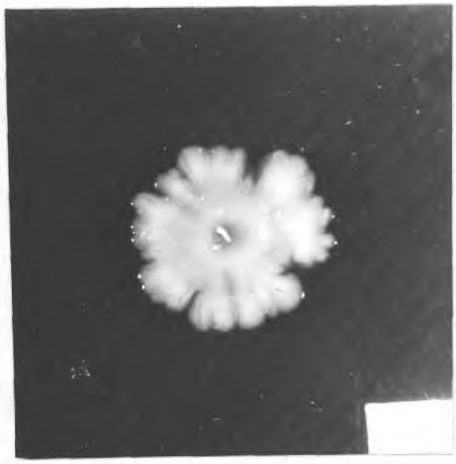
Normal Strain



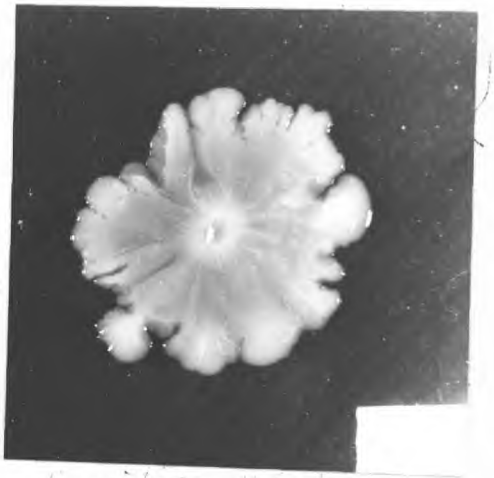
Methylene blue-resistant



Janus black-resistant



Crystal violet-resistant



Barbitone-resistant

5-aminoacridine-resistant

(All 2 x actual size and 14 days after inoculation)

morphology, in this prolonged phase of growth, is thus very sensitive to the metabolism of the particular cells. In this connection, it has already been mentioned that colony morphology is influenced by the composition of the agar gel, (Chapters 4 and 5).

The morphological development of the normal strain has already been described, (Chapter 4). The colony was observed to pass from a circular form with a uniform internal density, to one having a highly irregular perimeter and of somewhat variable internal appearance. In general, there is good evidence that colonies of the modified strains developed according to the same general pattern and for the same reasons as the normal strain. In contrast with this mode, the colonies of the methylene blue and Janus black-resistant strains developed much more slowly, (Plate (6-1)), and this finds good correlation with their particular metabolism. The biochemical properties of these two strains were reported earlier in this Chapter. Both strains grew at a low rate in liquid medium, utilised glucose reasonably efficiently and had a low colony growth rate. Thefinal pH in liquid medium was high. In the light of the discussion in Chapter 4, of the factors which are important in colony development, it can be seen that each of the above characteristics is in accord with the slow development of the colonies of these strains.

The morphology of the acridine resistant strain is noteworthy, but mainly because of a very low growth rate which

has been discussed earlier. The colony patterns of the remaining strains differed from one another in rather more subtle ways than in the examples just given. For the purposes of the following discussion, colony morphology will be considered as:-

a) The patterns present in the interior of the colony.

b) The form of the perimeter of the colony.

It seems likely that a detailed and in some way quantitative analysis of the different types of internal patterns must be made before any general correlation can be expected between the biochemical properties of a strain and this aspect of its colony morphology. However, to classify in detail the characteristic patterns within the various colonies would be a considerable task and not really appropriate in the present study especially, as in its present state, the theoretical understanding is unlikely to be adequate to correlate fully the differences with metabolic factors.

The development of the perimeters of the colonies, on the other hand, is rather more amenable to mathematical analysis, both from a semi-empirical approach involving geometric considerations and theoretically, employing calculated concentration gradients of nutrients and by-products. One limitation of studying the irregularity of the perimeter as a measure of the development of a colony, should be mentioned.

Particularly in a well developed colony, the perimeter may not be clearly defined, since the small radial inlets are often of rather indefinite length and merge gradually with the internal structure of the colony. Thus, the two factors which were postulated above as characteristic of the morphology of a colony, namely internal patterns and the shape of the perimeter, are not completely independent. Nevertheless, it is possible to detect many interesting trends in the development of colonies using measurements made on their perimeters, which are of necessity, approximate. These studies are reported in Chapter 8.

Chapter 7.

Effect of Adsorbents.

In Chapter 4, evidence was presented which suggested that the development, with prolonged growth, of a smooth circular colony of Aerobacter aerogenes into a complex 'floral' pattern, was the result of an instability of the perimeter incurred by the decreasing rates of supply of nutrients to the colony or of removal of toxic by-products from it.

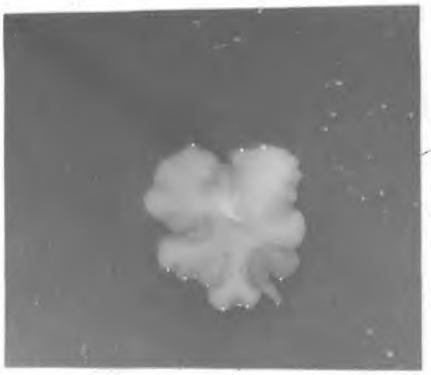
Various experiments have been performed with the intention of testing this hypothesis and clarifying the factors which regulate colony growth. One such idea, which has yielded interesting results and is described in this Chapter, is that by adding adsorbents to the agar, toxic by-products produced by the cells in the colony might be adsorbed and the colony development delayed or accelerated.

The adsorbent which produced the most interesting results was activated charcoal, (B.D.H. Ltd.). This was washed several times with hot glass-distilled water and dried at 150°C. It was added to the agar prior to autoclaving. Plate (7-1) shows the appearance of a colony of the normal strain of Aerobacter aerogenes at various times after inoculation on to normal minimal agar containing 1.0% w/v of activated charcoal. The appearance of a control colony growing on normal minimal agar, (no charcoal), is also shown. These photographs demonstrate that the addition of one per cent of activated charcoal to the agar reduces the rate of increase

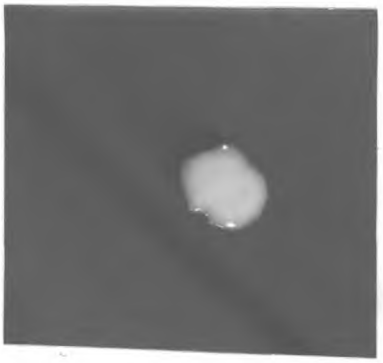
Plate (7-1)

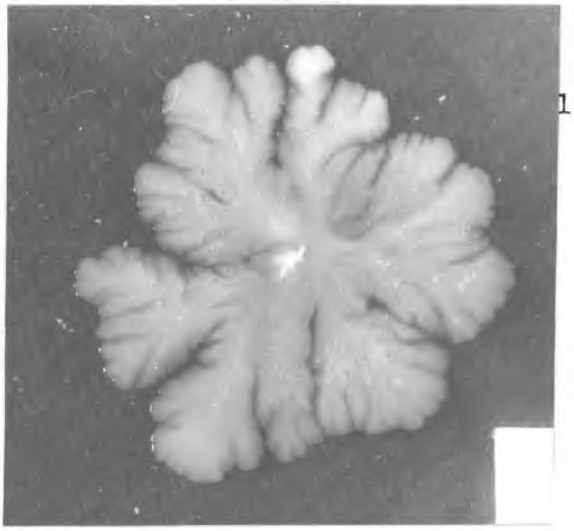
Normal agar

1% w/v charcoal-agar

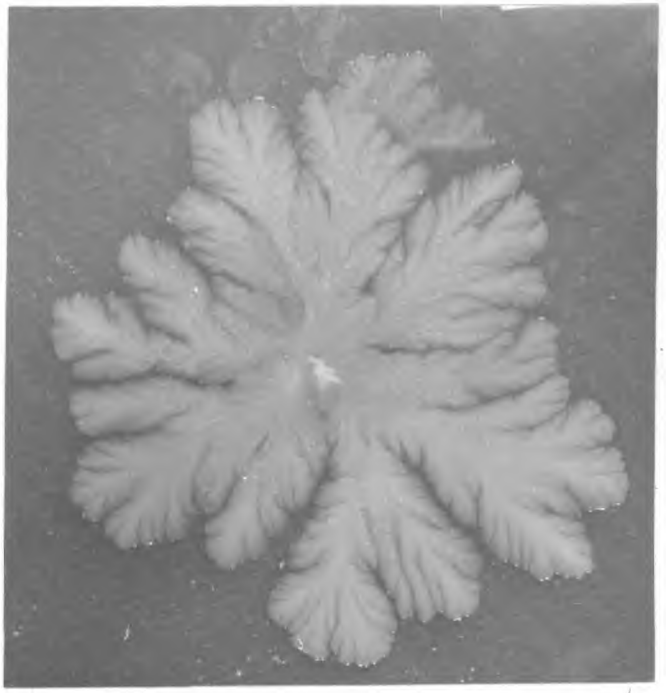


7 days





18 days



32 days

(All 2·5x actual size)

in the area of the colony and also modifies the shape considerably. Both conclusions were verified in duplicate experiments. The basic transition from a compact shape with a smooth perimeter into a highly irregular pattern, as incubation proceeded, was common to both agars.

The significantly reduced size of a colony growing on 1.0% charcoal-agar suggests that the charcoal may be inhibiting the growth of the cells. This could be due to some adsorption effect, (i.e. of diffusible intermediates, Dean and Hinshelwood 1966), or by the presence of traces of some toxic substance. The lag times between inoculation and the appearance of visible growth, however, were similar on both agars which does not support either mode of inhibition. A colony formed on 1.0% charcoal agar was observed to be considerably thicker than a colony on normal agar; an effect which also occurred, although to a lesser extent, with other adsorbents. These facts suggest that possibly the effect of charcoal upon colony growth is not simply to inhibit growth, but rather to modify the shape of the colony.

Further experiments were carried out using normal minimal agar containing 1.0% w/v activated charcoal which had been autoclaved and then allowed to stand for a few days. It was then heated until fluid, the charcoal removed by filtration and plates prepared. Cells of the normal strain of Aerobacter aerogenes were inoculated on to these plates and the colonies which formed were observed to develop in a very similar manner

to control colonies on normal minimal agar. Thus charcoal must be present in the agar to modify the growth and morphology of colonies. Also, charcoal does not produce its effect by permanently changing the gel structure, nor does it appear to release appreciable concentrations of toxic substances into the medium or to adsorb the various nutrients in the agar sufficiently to influence growth.

The effect upon colony growth of charcoal in the range of concentrations from 0.007% w/v to 5.0% w/v in normal minimal agar was next investigated. At concentrations of 0.007%, 0.033% and 0.330% the growth rates and morphology were similar to those obtained in its absence except that at the latter two concentrations some thickening of the colonies was observed. At concentrations of 2.0% and 5.0% w/v of activated charcoal, the low colony growth rates observed and the morphology, (normal strain), were similar to those reported earlier for '1/10 buffer agar', (Chapter 5). Moreover, on 2.0% charcoal agar the colonies showed a greatly reduced tendency to form the spiky irregularities observed at the 1.0% level and at the 5% level, 'spikes' were never observed. It appears, therefore, that the addition of various concentrations of activated charcoal to normal minimal agar modifies the growth rate and the morphology of a colony growing in such a gel. The type of development illustrated in Plate (7-1) can, however, only be produced over a relatively narrow range of concentrations of charcoal.

The growth of the normal strain of Aerobacter aerogenes on normal minimal agar, containing 1.0% activated charcoal, at the normal depth (Chapter 2), and two and three times this has also been investigated. It was found that the time of growth elapsing before 'spikes' appeared at the perimeter of a colony, (Plate (7-1)), was lengthened by increasing the depth of the agar, (10 days at the normal depth and 14 days at 2x normal). This will be discussed later.

Other Adsorbents:

The effect on colony growth of adding other adsorbents to normal minimal agar has been investigated by preparing gels containing microgranular cellulose powder, (Whatman, Chromedia CC31) and silica gel powder, (Whatman SG41), both at 1% w/v in the normal minimal agar. However, single colonies of the normal strain of Aerobacter aerogenes after prolonged incubatio on both of these agar gels were little different from those on minimal agar. A slight thickening of the colonies was nevertheless in evidence.

Clearly the most interesting modifications to colony morphology occurred when the normal strain of Aerobacter aerogenes was grown on normal minimal agar containing 1.0% w/v of activated charcoal. More detailed consideration of this type of growth will therefore be given.

As on the normal agar, colony development in the presence of 1.0% w/v of charcoal falls into two stages. There is an

initial phase of growth in which the colony remains uniform in appearance and its perimeter smooth and later, a period in which the colony develops into a complex shape. These findings, together with the experimental results reported earlier in this Chapter, particularly the fact that the onset of colony irregularity is delayed on deeper gels, suggest that although the colony growth rate and morphology are drastically changed from those on normal minimal agar, the basic mode of growth is the same in the presence and in the absence of charcoal.

On normal minimal agar, a colony of the normal strain of Aerobacter aerogenes remained of uniform appearance and had a smooth perimeter for about 2-3 days after inoculation, (Chapter 4). When 1% of charcoal was present a smooth perimeter and a uniform internal appearance were maintained for 7-10 days after inoculation, (Plate (7-1)). The development of the colony, as described in Chapter 4, has thus been retarded and it could be argued that this is a consequence of the adsorption by the charcoal of toxic growth by-products. However, the reduced size of a colony growing on 1.0% charcoal-agar requires some explanation before this hypothesis is acceptable.

Evidence has already been presented which suggested that charcoal did not reduce the colony area through a growth inhibiting effect, but rather modified the cross-section to

produce thicker colonies of smaller area than in normal circumstances. Pirt (1967), has suggested that the vertical height of the colonies of some organisms is regulated by the diffusion of nutrients through the colony to the actively growing cells at the surface. Cells of Aerobacter aerogenes can grow aerobically or anaerobically and in this case, therefore, there seems no reason, at least in the early stages of the colony's development, why growth should be confined to its surface. Immediately after the division of the initial cell inoculated on the gel a 'colony' may be regarded as expanding in the form of a hemisphere, a process which will most probably continue until a nutrient or by-product becomes growth limiting. This will occur first along the axis of the hemisphere, thereby regulating the vertical height of the colony which will now grow predominantly at its perimeter. Thus, if a toxic by-product did regulate the vertical height of a colony in this way, (which is quite feasible from the work described in Chapter 5), and if this substance was adsorbed on charcoal, a thicker colony would result. Through this mechanism, thicker and more compact colonies could be produced on 1.0% charcoal-agar than on normal minimal agar.

When small spike-like protrusions appear around the perimeter of a colony growing on 1.0% charcoal-agar, it seems that this is the start of the diffusion regulated or 'dentritic' type of growth, (Chapter 4). The reasons why the pattern should be so different from that obtained in the absence of

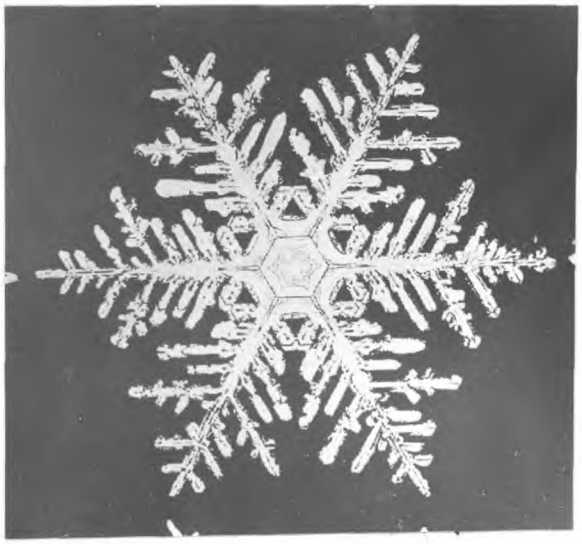
charcoal are considered below, but first a further modification to the pattern on 1.0% charcoal agar will be mentioned. Plate (7-2a) shows the appearance of a colony of the normal strain of Aerobacter aerogenes 8 weeks after inoculation on to 1.0% charcoal-agar of twice the normal depth, (Chapter 2). Thus, when prolonged growth is possible, as on deeper agar, the radially directed 'spikes' such as those in Plate (7-1), acquire branches at right angles. Plate (7-2b) shows a dendritic snow crystal, (Bentley and Humphreys, 1931), which is a common natural type. The similarity of these two patterns is most striking and correlates well with the suggestion advanced in Chapter 4 that the development of a large colony of Aerobacter aerogenes proceeds by a mechanism analogous to that of dendritic crystal growth. The similarity is limited by the crystal's geometry, but also these formations are quite different in size, (Plate (7-2)). However, the important morphology regulating factors such as the rate of growth, the diffusion coefficients in the surrounding medium and the volume ratio for the conversion of the diffusing material into substance of the growing pattern also differ considerably in magnitude. The fac that a snow crystal can form a dendritic pattern of a smaller size and in a medium of a higher diffusion coefficient than a colony does, may correlate with the much greater rate of growth of the former.

Since there is now good evidence that the 'spikes'

Plate (7-2a)

(2 x actual size, 56 days after inoculation)

Plate (7-2b)



(Approximately 70 x actual size)

produced by a colony of the normal strain of Aerobacter aerogenes cultured for a prolonged period on 1.0% charcoal-agar, (Plates (7-1) and (7-2)), are a form of the 'dendritic' type of colony growth described in Chapter 4, the nature of their formation will next be considered.

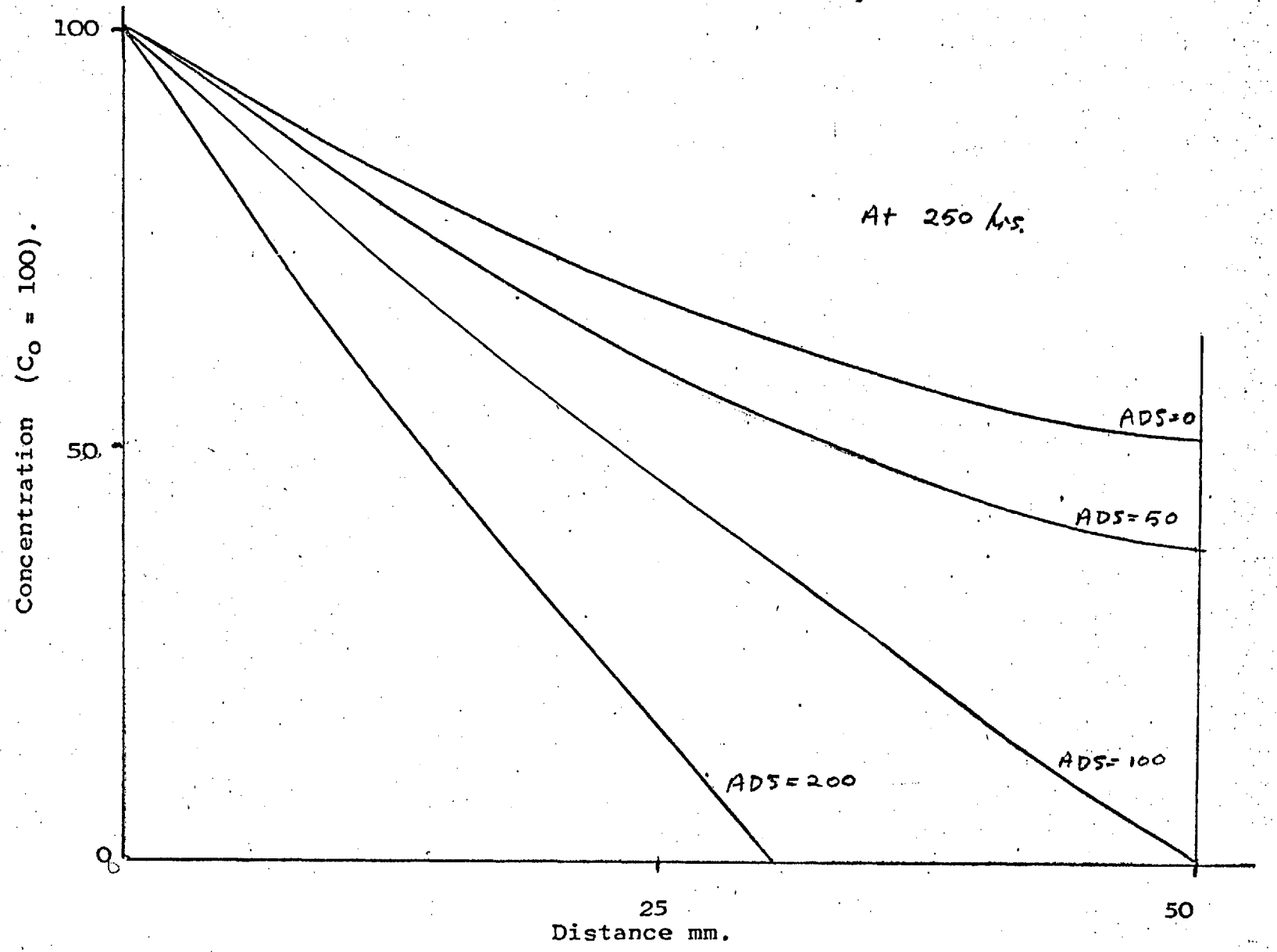
It was mentioned in Chapter 4 that the gradients of nutrients and by-products around a large single colony are instrumental in determining its morphological development. Thus, the characteristic pattern formed on 1.0% charcoal-agar may correlate with the different 'growth regulating gradients' in the presence of an adsorbent. Various calculations of the diffusion of nutrients towards and of by-products away from a growing colony, have been reported in Chapter 3. These results were based on a rigorous solution of the differential equation for a particular case of one-dimensional diffusion. As the present investigation progressed, however, it became clear that a more general procedure for calculating the relationship between concentration and distance was desirable. For example, using rigorous methods it is not easy to allow for the advance of the edge of the colony or for cases in which a nutrient is consumed or a by-product produced, at a constant rate. To this end, a computer programme was written by which diffusion concentration-distance curves could be calculated using a method of successive approximations, (c.f. Barrow et al 1947). This programme and various modifications of it to fit other cases are described in an appendix; a test to verify the

accuracy of the procedure is also given.

Using a modified form of this basic computer programme, it is possible to study the effect of the adsorption by charcoal on the diffusion of some nutrient or by-product. Since it is not known what growth regulating substance is being adsorbed, absolute computations of diffusion gradients cannot b made. However, as mentioned previously, the calculation of diffusion gradients around a growing colony is approximate for several reasons and in the present case particularly, the relative nature of the gradients with and without adsorbents, is the principal point of interest.

Figure (7-1) shows the concentration of some material diffusing into a finite cylinder 5 cm. in length, a distance which is approximately the radius of a Petri dish. The diffusing material is maintained at a concentration, C_o (g./ml.) at the origin, and 'ADS' is the concentration of adsorbed material in the tube as a percentage of C_o necessary to saturate the charcoal. This case corresponds to the outward diffusion of a growth by-product from a colony and it can be seen from Figure (7-1) that the presence of an adsorbent in the gel leads to the production of steeper diffusion gradients of these materials, if they are adsorbed. The gradients become steeper as the amount of material adsorbed per unit volume is increased. If an inwardly diffusing nutrient is being adsorbed, the same conclusions also apply and the concentration-distance curves are those obtained by

Figure (7-1).


100
50
0
Concentration (C_o = 100).
At 250 l/s
ADS=0
ADS=50
ADS=100
ADS=200
25
50
Distance mm.

inverting Figure (7-1) about the line c = 50.

In Chapter 4, it was stated that when a colony began to develop on normal agar, the cells in small protrusions in the perimeter had a slight growth advantage over those in the intervening regions, by virtue of their being at a higher concentration on a gradient of a nutrient or alternatively a lower concentration on a gradient of some toxic by-product. Thus the tips of such small irregularities tended to grow faster than the less favoured regions, thereby reinforcing any initial advantage and leading to the formation of the large peninsulae observed in developed colonies. On an agar gel containing 1.0% of charcoal, on to which the growth regulating substances are to some extent adsorbed, the concentration gradients which are responsible for the development of the colony will be steeper than in the absence of charcoal. It might be argued, therefore, that in presence of charcoal the tips of any small protrusions in the perimeter of a colony find themselves at such a great advantage over the intervening regions that they form the spike-like shapes shown in Plate (7-1 This mode of growth takes place with little lateral expansion because outward growth is much more favoured. However, from work to be described in Chapter 8, it seems that possible modifications to the various concentration gradients can only partly explain this spike-like form of growth. Calculations based on diffusion gradients suggest that the peninsulae around a developing colony should become slightly longer and

thinner in the presence of charcoal, but definitely not to the extent which is observed in practice.

Earlier in this Chapter it was pointed out that in the presence of charcoal in normal minimal agar the colonies were much thicker than in its absence and it was suggested that this effect could be explained by the adsorption of toxic growth by-products. This thickening effect was not, however, confined to the early stages of colony growth and in fact the 'spikes' which were produced on prolonged growth were considerably thicker than the peninsulae of a colony on normal minimal agar. It seems quite possible, therefore, that, just as the original colony growing on 1.0% charcoal-agar remained small in area due to its upward growth, the regions of irregular growth also tend to remain compact and the predominant horizontal expansion takes place at the favoured tips of the peninsulae. When the supply of nutrients to or the removal of by-products from these spikes falls, they too become unstable and branches develop at right angles, thereby repeating the earlier pattern.

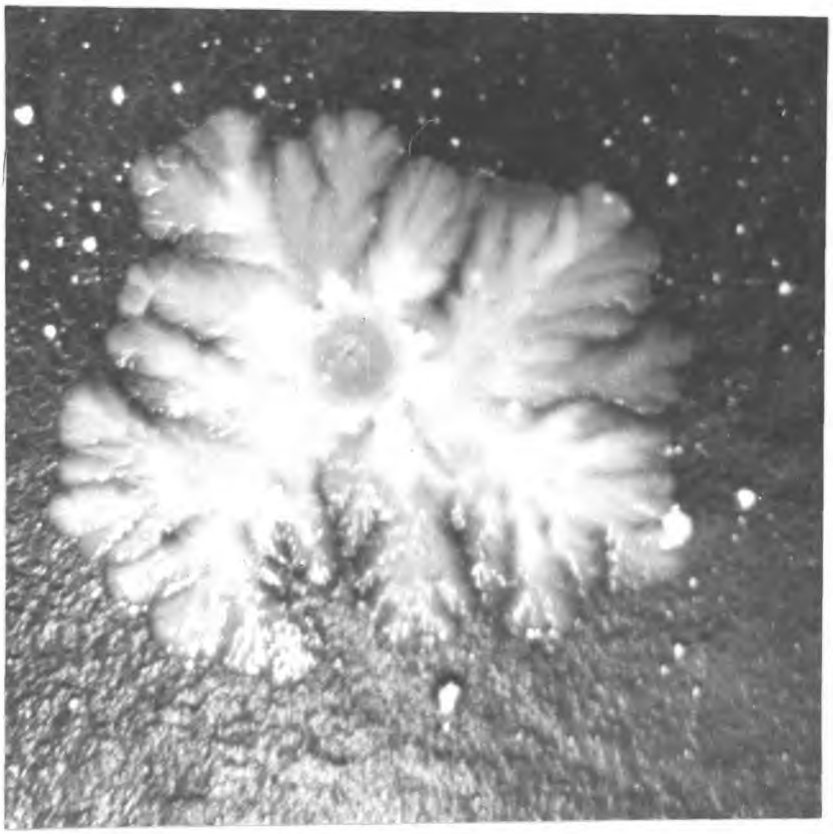
Although it seems most probable that the mechanism by which charcoal modifies the colony morphology is by the adsorption of toxic by-products, it is difficult to specify which substance is affected because of the enormous range of possibilities. However, in this connection, the literature pertaining to the 'swarming' of some Proteus species is interesting.

It has been observed that when cells of motile Proteus species such as Proteus vulgaris and Proteus mirabilis were inoculated on to the surface of suitable agar gels, they produced a small compact colony initially. A few hours later, strings of cells began to migrate, ('swarm'), from this colony and settled in a ring concentric with it. After further growth in this ring, more cells migrated outwards and formed another concentric ring; this process was repeated and led to the formation of several rings of growth. It was observed, (e.g. Lominski and Lendrum, 1947), that this phenomenon was induced by certain growth by-products of the cells. Later, it was found, (Alwen and Smith, 1967), that 'swarming' could be completely inhibited and a small compact colony produced, by the addition of 1% w/v of activated charcoal to the gel. These authors suggested that the by-products responsible for 'swarming' had been adsorbed on to the charcoal. It can be see that there is a similarity between the phenomenon of the 'swarming' of Proteus and the development of irregular perimeters in colonies of Aerobacter aerogenes.

Smith and Alwen suggested that the by-products which were responsible for inducing 'swarming' in Proteus were long-chain fatty acids. These substances were known to be produced by the cells, were toxic to them and would have been strongly adsorbed on charcoal. By these same criteria, long-chain fatty acids might possibly be the regulating substances in the

growth of a colony of Aerobacter aerogenes on 1.0% charcoal-agar. To investigate this hypothesis it was decided to add various fatty acids to normal minimal agar containing 1.0% w/v activated charcoal and study the effect on the morphology of colonies growing on such gels. Three acids, palmitic, stearic and myristic, which are abundant in the lipids of Aerobacter aerogenes, (Dr. A.M. James; personal communication), and are thus possible growth by-products, were employed. When they were present in concentrations of about 10^{-4} M. and 10^{-6} M., colonies with a growth rate and morphology somewhat similar to those obtained on '1/10 buffer' agar (Chapter 5), were produced. Plate (7-3) shows the appearance of a colony of the normal strain of Aerobacter aerogenes at 30 days after inoculation on to normal minimal agar containing 1.0% w/v activated charcoal and the above three acids at concentrations sufficient to saturate the gel and the charcoal. The acids, palmitic, (0.01M.), stearic, (0.004M.) and myristic, (0.002M.), in the proportions in which they occur in the lipid system of the cell, were added directly to the molten charcoal-agar which was then shaken immediately and also on several successive days after remelting the gel. A comparison of Plate (7-3) with Plate (7-1) shows that the colony which formed in these conditions bears a very strong resemblance to a colony growing in the absence of charcoal, although the growth rate of the former colony is reduced. The white specks visible in Plate (7-3) are composed of the excess of fatty acids.

Plate (7-3)



30 days after inoculation

(3 x actual size)

Although it is possible to inhibit the effect of charcoal on colony morphology by the addition of fatty acids as described overleaf, it cannot be claimed from this that long-chain fatty acids are necessarily the important colony growth regulating substances. In theory, any suitable adsorbate added to agar containing charcoal, in sufficient quantities to saturate the charcoal surface, should lead to the occurrence of relatively 'normal' looking colonies on the gel. Rather, the result of the experiment pertaining to Plate (7-3) should be interprete as supporting the conclusions advanced earlier in this Chapter that charcoal modifies colony morphology by its operation as an adsorbent and not through some modification to the gel structure or by some inter-cellular effect. This experimental result is not incompatible with the hypothesis that long-chain fatty acids could be important growth regulating substances in the development of a colony of Aerobacter aerogenes on normal minimal agar containing 1.0% w/v charcoal.

Modified Strains:

The growth of some modified strains of Aerobacter aerogenes (Chapter 2), on normal minimal agar containing adsorbents has also been investigated. Cellulose and silica gel powders (Whatman, see earlier in this Chapter), at a concentration of 1.0% w/v produced only a slight effect on colonies of the acridine-, barbitone-, crystal violet-resistant and the 'anaerobic' strains. The morphological appearance of these

colonies being similar to that of colonies grown on normal minimal agar although occasionally some vertical thickening did occur. Colonies of the methylene blue-resistant strain growing on agar containing 1.0% w/v cellulose powder were interesting in that a distinct yellow spot, about 2 mm. in diameter formed in the centre of the colony at about 5 days after inoculation. It is not clear why this should have happened, but it might have been a pigment analogous to the orange pigments produced by certain oxidation deficient strains of Staphylococci reported by Gause, (1966).

On normal minimal agar containing 1.0% w/v activated charcoal, colonies of the 'anaerobic' strain and the crystal violet-, barbitone- and acridine-resistant strains all showed some distinct tendency to form 'spikes' although in all cases this was less pronounced than in the colonies of the normal strain. The methylene blue- and Janus black-resistant strains on the other hand formed simple 'floral' type patterns and showed no tendency to form 'spikes' at all. It was observed, however, that colonies of the methylene blue-resistant strain produced a few distinct 'spikes' on agar containing 5.0% w/v charcoal. This may correlate, to some extent, with its lower metabolic rate.

Chapter 8.

Measurement and Simulation of Colony Development.

When the morphological development of large colonies of Aerobacter aerogenes was first considered, (Chapter 4), it was pointed out that in comparing two such colonies it was difficult to decide which was the more developed. Thus, in the ensuing investigation of this phenomenon, experiments were employed which obviated the need for such comparisons. Nevertheless, a function to describe the degree of development of a colony would be useful in the further study of this effect and also in any applications of this behaviour. The 'measurement of colony development' is considered below, while later in this Chapter attempts to simulate the shape of a developing colony are described.

Weiss, (1955), in discussing development in higher animals said that it was not possible to measure the degree of development, but only to study developing systems. This statement reflects the difficulty of rationalising the complex interplay of the various changes which take place during the development of these higher forms, which also applies albeit to a much lesser extent, to large single colonies of Aerobacter aerogenes and has been mentioned earlier. For example, in the development of a colony of the normal strain of the organism, (Chapter 4), it was reported that, in addition to distinct changes in the shape of the perimeter, complex changes in the internal appearance of the colony also took place. Also, when the morphologies of large colonies

of the various modified strains of Aerobacter aerogenes were compared, (Chapter 6), it was concluded that in addition to distinct differences in the shapes of the perimeters, the internal appearance of the colonies also varied considerably. The overall development of large colonies is thus sufficiently complex to defy simple numerical representation. However, enough is known about the mechanism of this development to justify measuring it in the first instance, in terms of the shape of the perimeter only. A practical limitation of this idea which has been mentioned previously, (Chapter 6), is that in a very irregular colony, the perimeter is not absolutely defined due to the rather indeterminant length of the many radial inlets. The above has limited the accuracy of such a procedure, but not prevented its successful application.

There are various criteria which the function which is chosen to describe the irregularity of the colony perimeter should satisfy. First, the function should be continuous and increase uniformly during the development of a typical colony. Secondly, it should be a dimensionless quantity and thus a measure of shape and independent of the size of the colony.

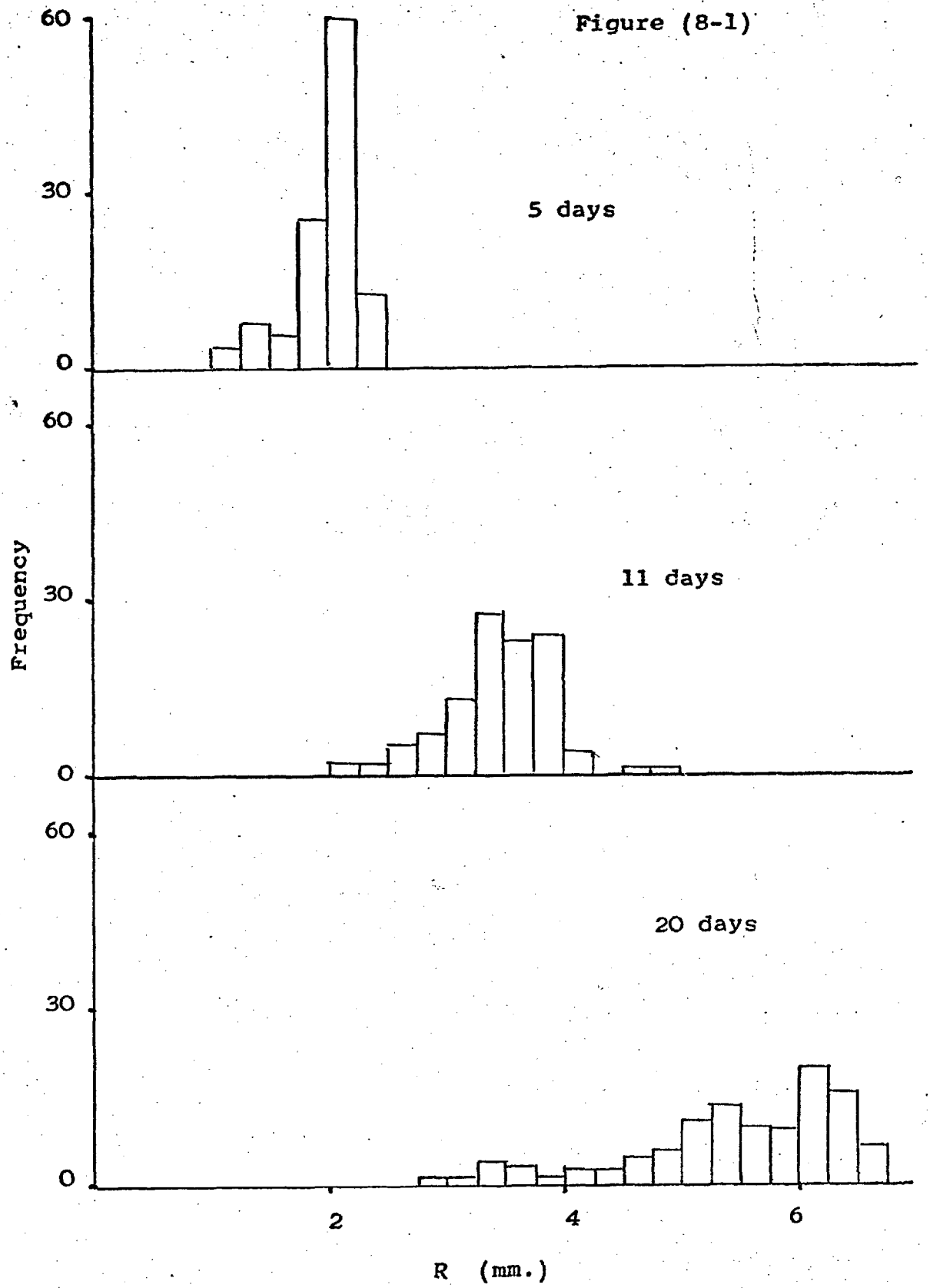
Various ideas were tested, using colonies which had been photographed against a scale and then drawings of known magnification were made with the aid of the negatives and an enlarger. In view of the previous discussions of colony development, it would seem that some function based on the variation of the colony radius would be the most direct

measure of peripheral irregularity. Drawings were made, as described above, from a colony of the normal strain of Aerobacter aerogenes at 5, 11 and 20 days after inoculation on normal minimal agar. The perimeter of the colony was marked off with a large number of points equally spaced at a distance of approximately 1% of the total length of the perimeter. Two arbitrary cartesian axes were set up beside the drawing of the colony and the coordinates of all the above points recorded.

For colonies formed by inoculation with a platinum wire, it is sometimes possible to see the original 'centre' of the colony. However, this is not always true and it is generally easier to define the colony centre as having the coordinates formed by separately averaging the abscissae and ordinates of all the peripheral points for a given drawing. The distances, (R), of each of the points on the perimeter from the colony 'centre' were then calculated. This data is shown in the form of histograms of common area at the above three times during the development of this colony, (Figure (8-1)). These histograms show clearly the expected increase in spread as the colony develops; Figure (8-4), page 192 shows the form of the perimeter of this colony at 5, 11 and 20 days after inoculation.

Since the histograms were not suitable for quantitative measurement, the average deviations from the mean, irrespective of sign, were calculated directly from the individual R values

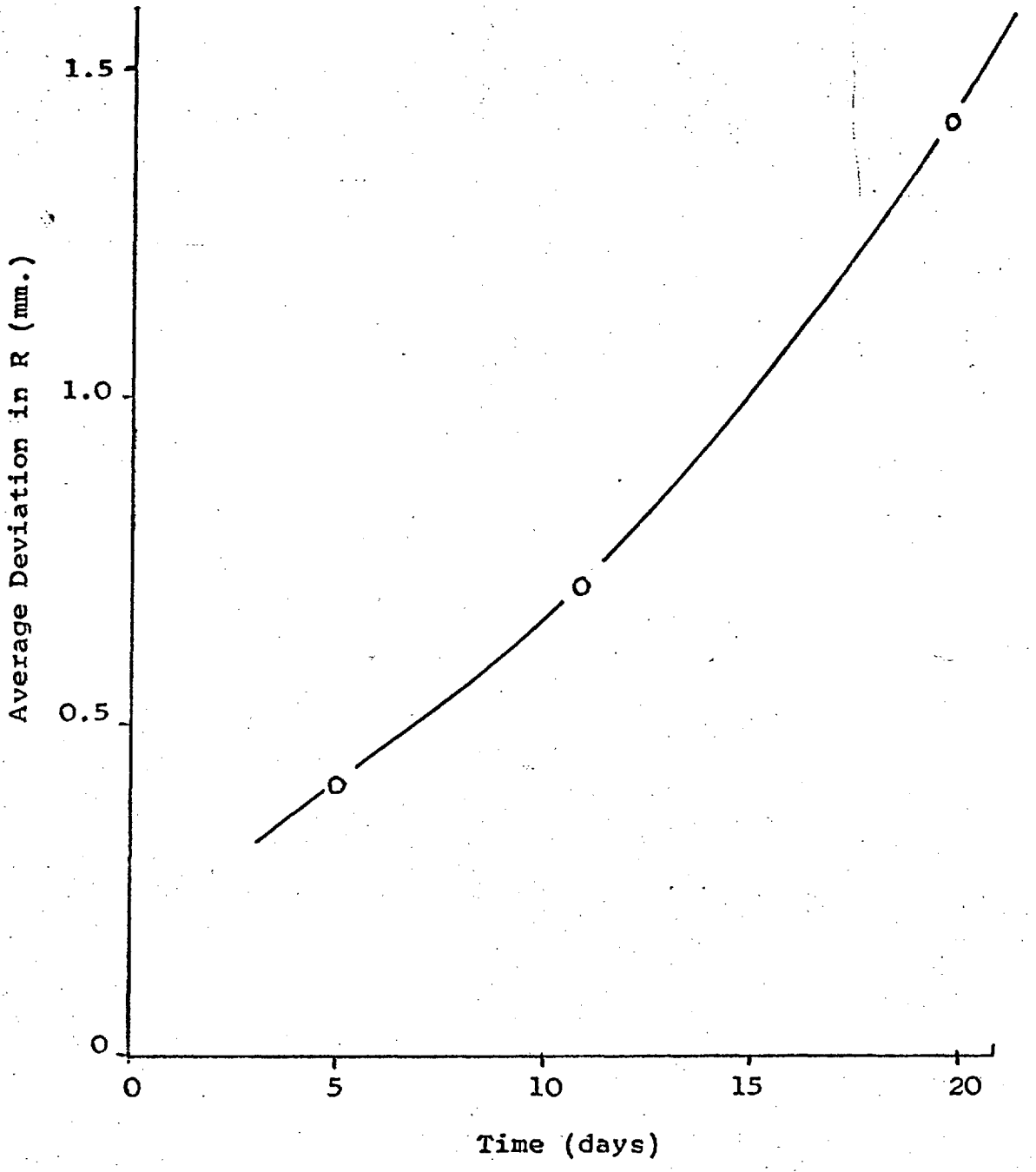
Figure (8-1)



and they are plotted against time in Figure (8-2). It is apparent from this Figure that the average deviation from the mean of the colony 'radius', as defined above, satisfies the criteria advanced earlier for a suitable function to describe the development of a colony perimeter. However, this function has the disadvantage that its evaluation is rather tedious.

It is possible to devise functions as measures of colony development which are based on the length of the perimeter. It has been mentioned, (Chapter 4), that one way of regarding colony development is to take the amount of nutrients consumed and by-products produced as proportional to the colony area and the cross-sectional area through which these materials can diffuse to and from the colony as proportional to the length of the colony perimeter. Thus, when a colony of basically circular form is increasing in area linearly with time, as is observed in practice, the rates of consumption of nutrients and production of by-products are also increasing linearly with time. However, the increase in the rates of supply and removal of these substances are proportional to the square root of the time. As already pointed out, the smooth circular perimeter of the colony eventually becomes unstable and the development of irregularities is regarded, in this approach, as a result of a tendency to maintain the ratio of the above two factors constant. This has already been acknowledged as a somewhat arbitrary approach to the phenomenon, but

Figure (8-2)



nevertheless it does mean that functions based on either the length of the perimeter or on perimeter to area ratios as measures of colony development have a certain degree of theoretical significance in addition to the purely geometrical viewpoint.

Scale drawings of colonies, (often 10 x actual size), were prepared as described earlier in this Chapter. The lengths of the colony perimeters were measured with a piece of cotton thread and the areas using a planimeter. The results described below have all been verified as applicable to duplicate colonies, but for simplicity, the diagrams are based on measurements of one colony only.

The length of the perimeter of a developing colony of the normal strain of Aerobacter aerogenes grown on normal minimal agar is plotted against its area in Figure (8-3a). The perimeter of a circle of common area is also shown; this represents the minimum perimeter of any shape surrounding a given area. This figure shows clearly the divergence of these two patterns with respect to the lengths of their perimeters. The lengths of the perimeters of the above colony and of the circle of equal area have also been plotted against the age of the colony (Figure (8-3b)). Since the colony area increases approximately linearly with time in the region of growth studied (Chapter 3), Figures (8-3a) and (8-3b) are very similar, but as the variation with respect

Figure (8-3a)

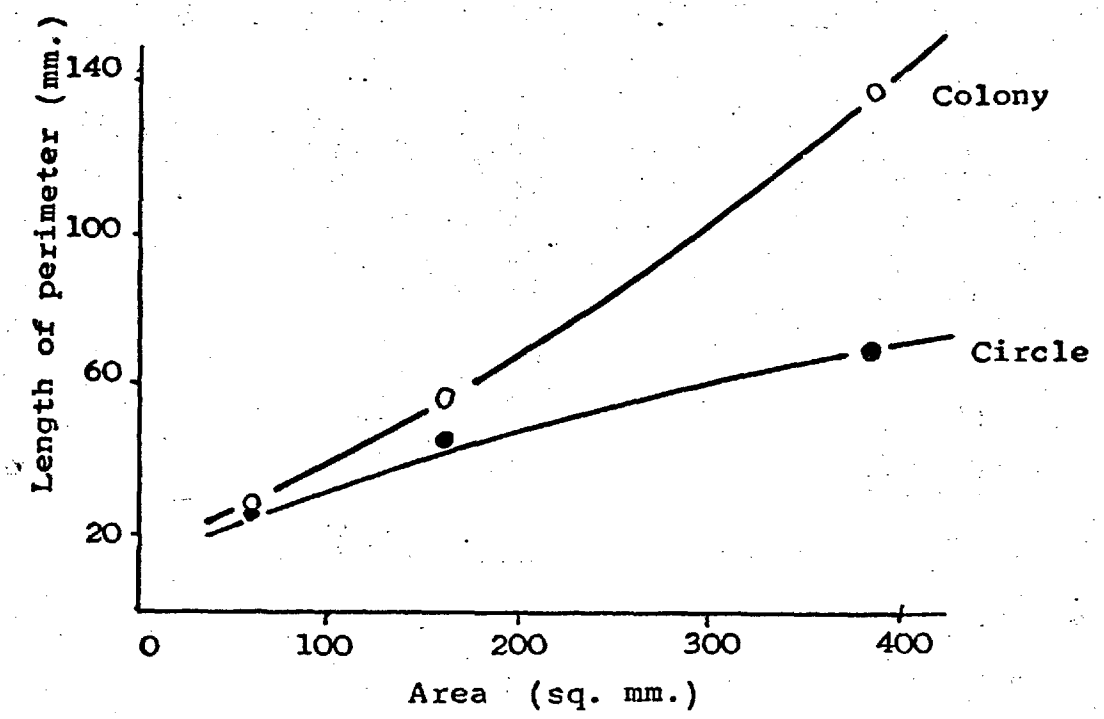
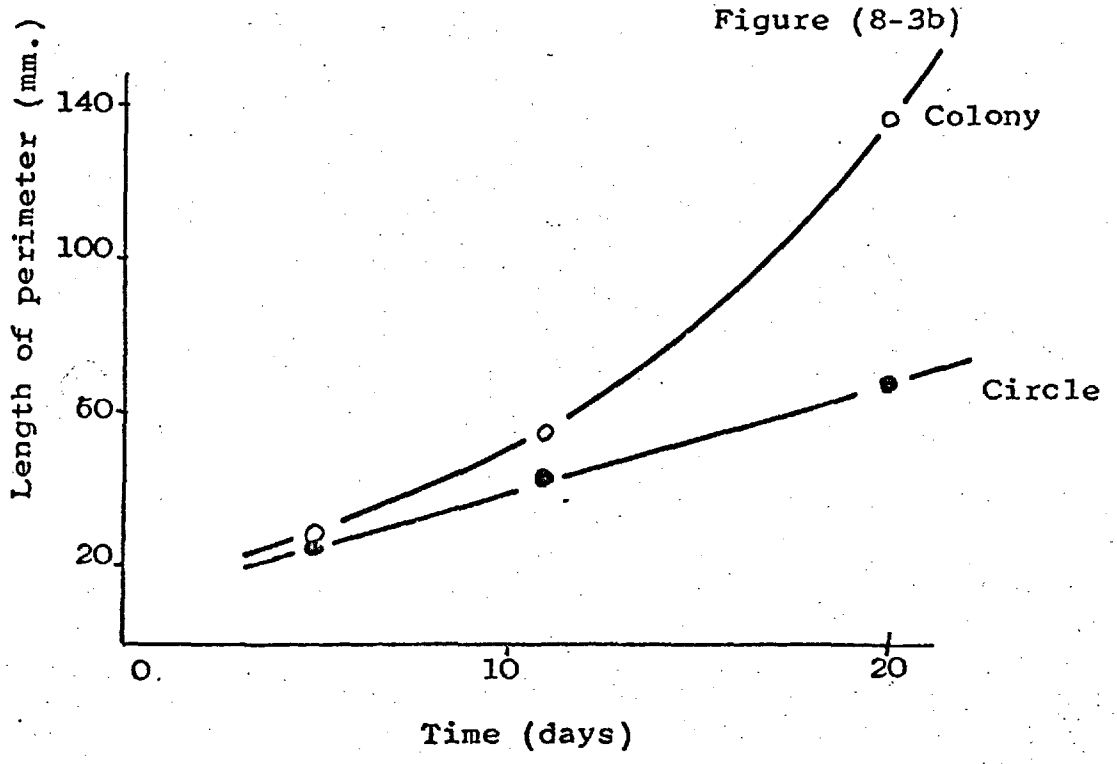


Figure (8-3b)

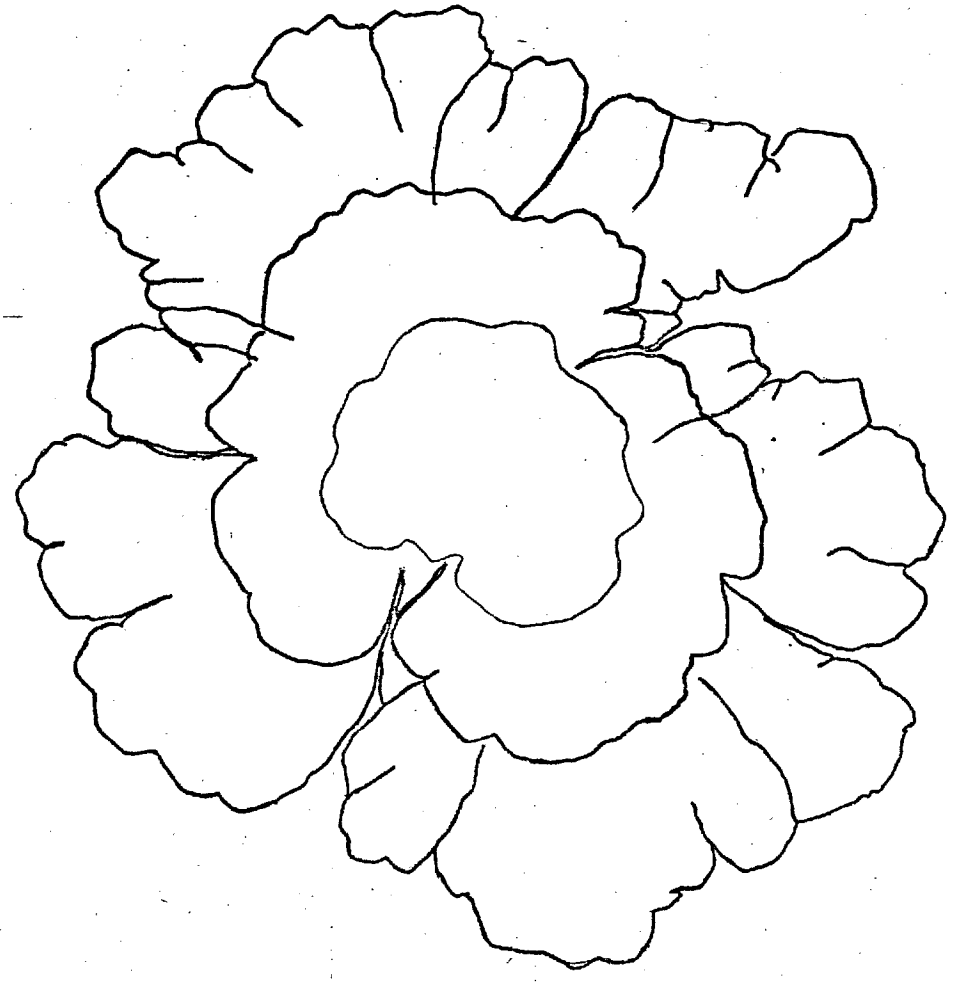


to time is more relevant in connection with colony development this will be used henceforth. It can be seen from Figure (8-3b) that while there is very little difference between the two curves at 5 days after inoculation, from this time on the curves diverge, (see Figure (8-4)) which shows the colony perimeters). This is in agreement with the conclusions reached earlier from diffusion calculations and morphological studies that peripheral irregularity starts at about this time.

It is also of interest, in connection with the approach to colony development described earlier in this section, to enquire how the perimeter to area ratio changes during the development of the colony. The variation with time of this ratio for the colony and for a circle of equal area is shown in Figure (8-5). A distinct difference is apparent, the colony development tending to maintain this ratio at a higher value than that observed with the circle.

Nevertheless, neither the colony perimeter nor the perimeter to area ratio are ideally suitable functions to describe the degree of development of a colony as both have dimensions and are dependent upon the size of a colony. However, a suitable function can be obtained by dividing the length of the colony perimeter by the perimeter of a circle of equal area. The variation of this function with time for a colony of the normal strain of Aerobacter aerogenes growing on normal minimal agar is shown in Figure (8-6). This functio

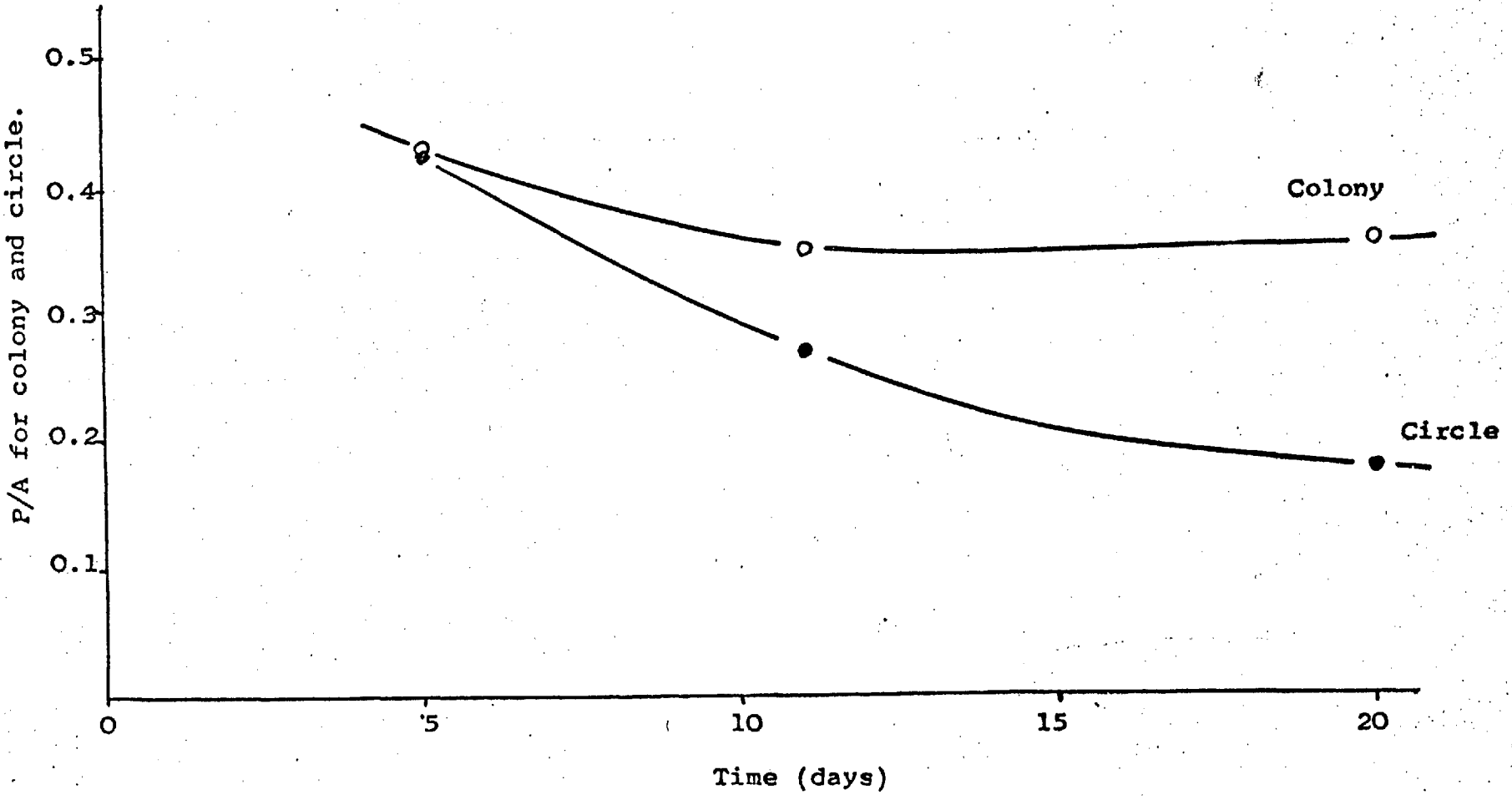
Figure (8-4)



Perimeters of 'Normal' colony at 5, 11 and 20 days.

(5 x Actual Size).

Figure (8-5)


P/A for colony and circle.
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
Time (days)
Colony
Circle

is dimensionless and it can be seen from Figure (8-6) that it increases in magnitude uniformly during the development of the colony. In addition, the function is quite easy to evaluate and is, therefore, a good measure of the degree of development of a colony.

Our knowledge of the mode of development of large single colonies of Aerobacter aerogenes is sufficiently complete from the work described in previous chapters to enable suggestions to be made as to the effect upon colony development of changing certain parameters. For example, on shallower agar, the cross-sectional area through which nutrients and by-products can diffuse to and from a growing colony is less and the tendency for the colony to develop into an irregular pattern is consequently greater. Previously, such theories could not be tested because it was not generally possible to compare the degree of development of two colonies. The effectiveness of the above function, namely the length of the colony perimeter divided by the perimeter of a circle of common area, will now be investigated in such a role.

This function, (hereafter 'P colony/P circle'), was evaluated for colonies of the normal strain of Aerobacter aerogenes growing on different depths of normal minimal agar and the results are given in Figure (8-7). It can be seen that the degree of development, as measured by this function, increases as the depth of the gel is decreased and this is in agreement with the conclusions reached in Chapter 4.

Figure (8-6)

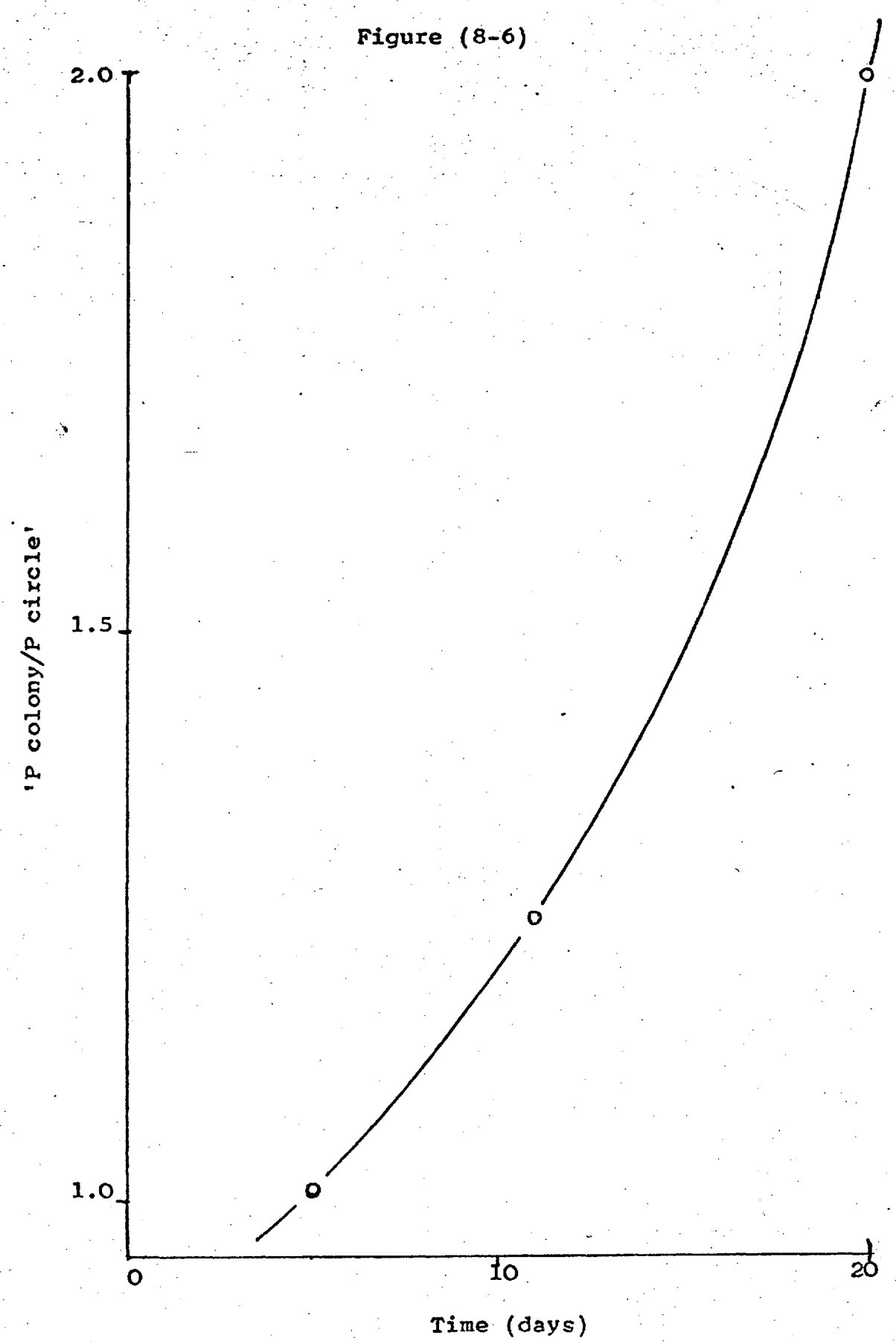
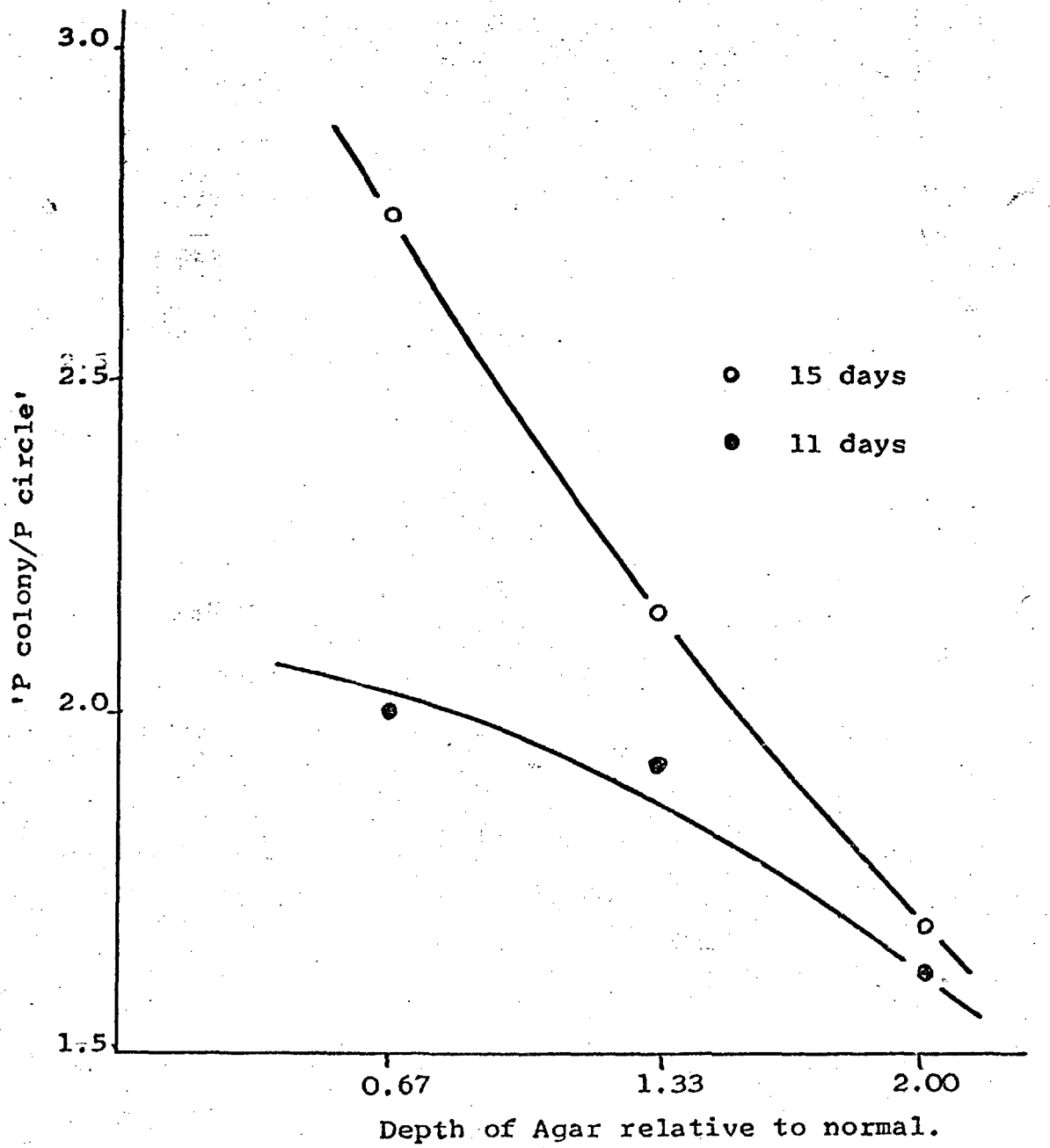


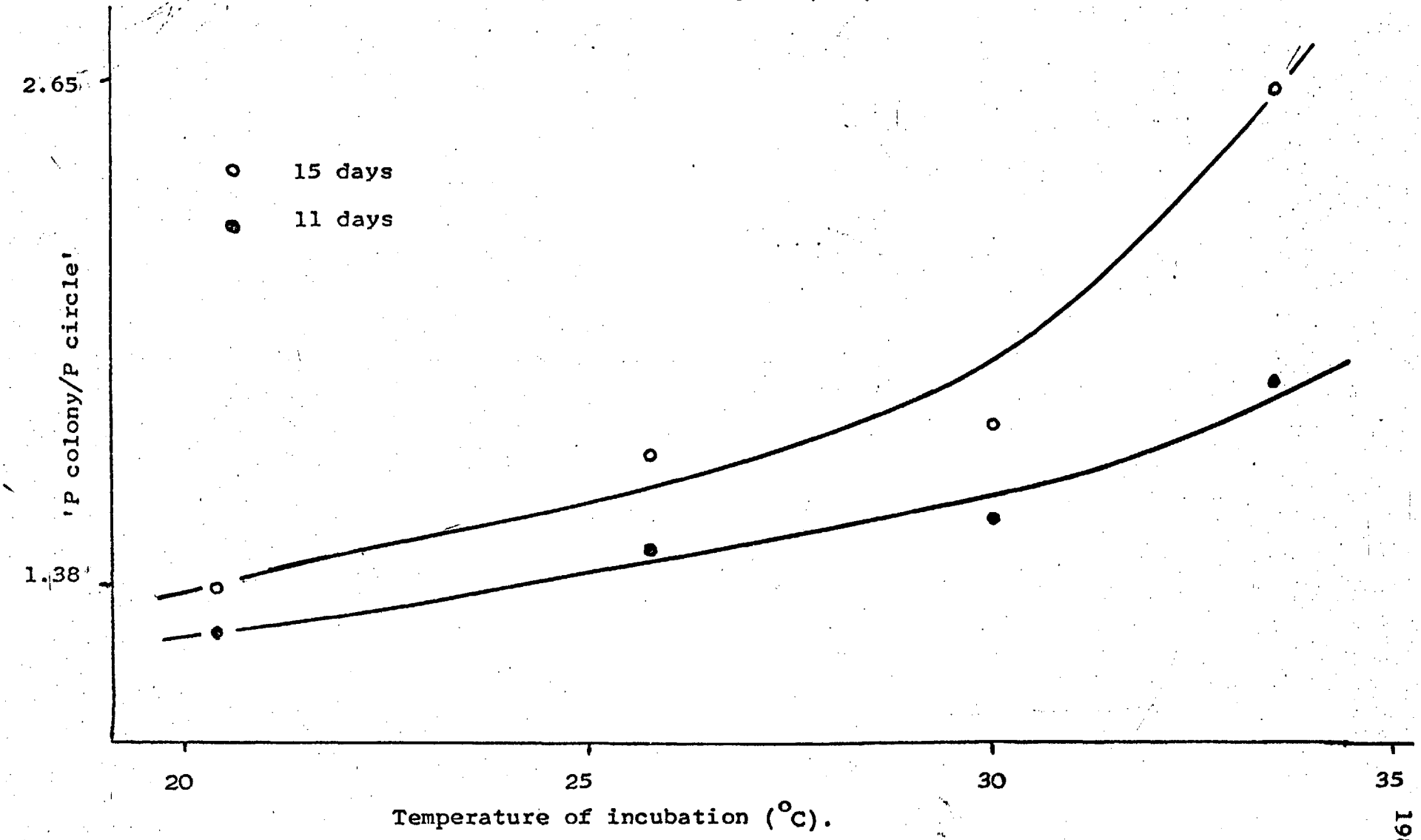
Figure (8-7)


3.0
2.5
2.0
1.5
'P colony/P circle'
15 days
11 days
0.67
1.33
2.00
Depth of Agar relative to normal.

The effect upon colony development of varying the temperature of incubation has also been investigated. It would be expected, that since decreasing the temperature of incubation below 35°C reduces the growth rate of the organism, the colonies formed by it at these lower temperatures should develop irregular perimeters more slowly much as colonies of the slow-growing methylene blue-and janus black-resistant strains do at normal temperatures, (Chapter 6). The concomitant decrease in the diffusion coefficient at lower temperatures will tend to oppose this trend, but the change in it is of a much smaller magnitude than that in the growth rate of the cells and hence the effect should still be observed, ($\approx$ 5% change in diffusion coefficient vs. 200-300% change in intrinsic cell growth rate). That this is indeed the case is shown in Figure (8-8) where the variation of 'P colony/P circle' with the temperature of incubation for colonies of the normal strain of Aerobacter aerogenes on normal minimal agar is plotted.

The growth and development of colonies of the normal strain of Aerobacter aerogenes on Millipore filters resting on normal minimal agar was described in Chapter 4. By using this method of culture it is possible to transfer a growing colony to a fresh agar plate without disturnbing the colony pattern. Transferring colonies in this way would be expected to retard the development of irregularities. 'P colony/ P circle' for colonies growing on Millipore filters resting on normal minimal

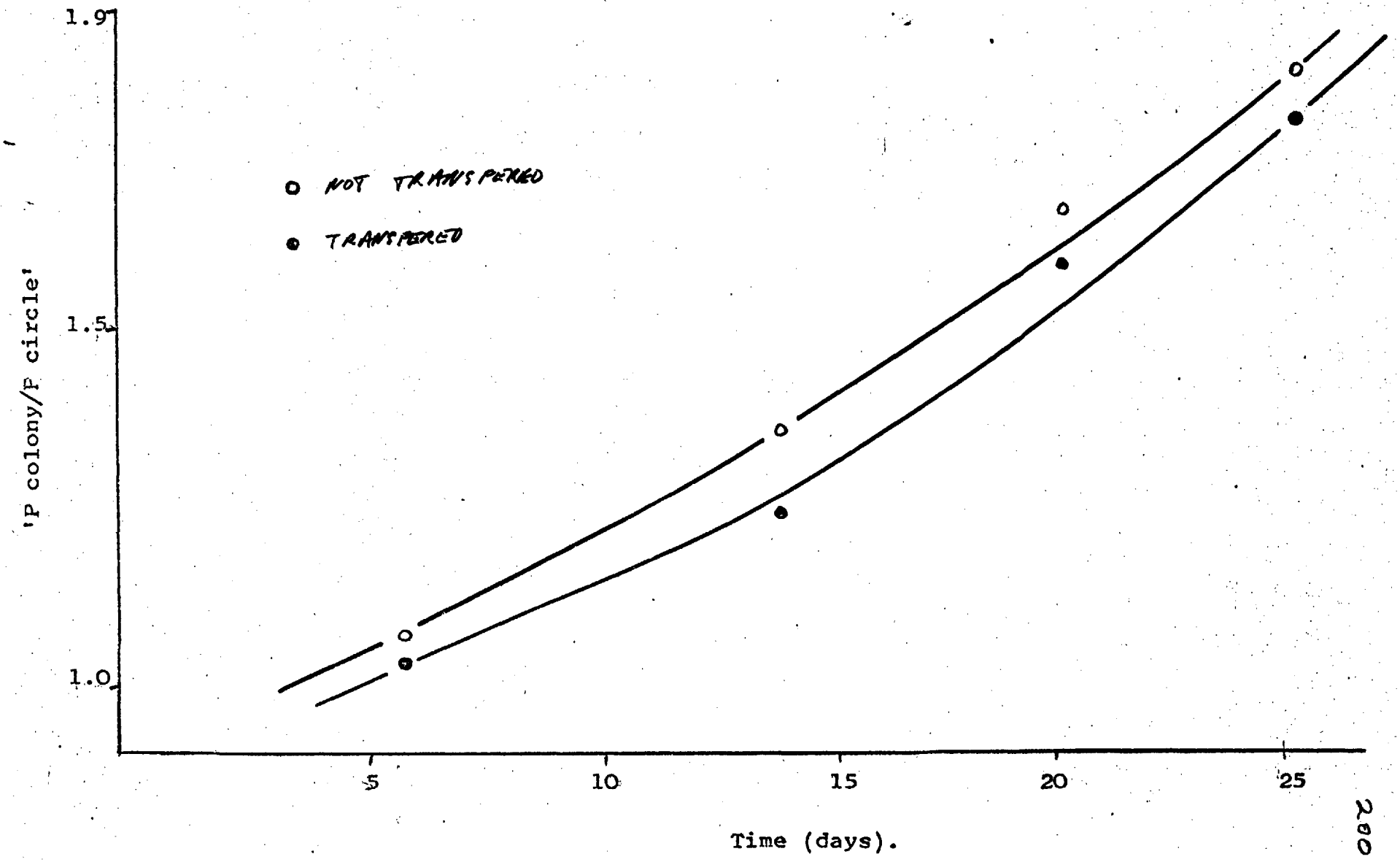
Figure (8-8)


2.65
1.38
'P colony/P circle'
15 days
11 days
20
25
30
35
Temperature of incubation (°C).

agar are shown in Figure (8-9). Each point is the average of readings for two colonies; the pair which were transferred were moved at intervals of six days. Although the transferred colonies were less developed at all times than the controls, the difference was not as great as might be expected. This may be due to the retention of growth by-products in the filter and in a thin adhering layer of gel or to the periods between transfer being too long.

It was mentioned in Chapter 4 that colonies of the methylene blue-resistant and normal strains of Aerobacter aerogenes were observed to develop more rapidly as the concentration of glucose in the gel was reduced. The type of criteria of development employed in this case were the formation of gentle undulations in the perimeter and the transition from a uniform to a patterned internal appearance by the colonies. Relatively low concentrations of glucose were used and the changes took place at quite an early stage. However, when 'P colony/P circle' was calculated from photographs of the above colonies, the results showed no such general trend. This illustrates two limitations to the use of such a function as a measure of colony development. First, the function is not sensitive enough to distinguish between the degree of development of colonies which have only slightly irregular perimeters. Second, as already pointed out, colony development also entails complex changes in internal appearance which were not considered in the formulation of the

Figure (8-9).


1.9
1.5
1.0
'P colony/P circle'
NOT TRANSFERED
TRANSFERED
5
10
15
20
25
Time (days).

function 'P colony/P circle'.

Simulation of Colony Development:

It was felt that the simulation of the perimeter of a developing colony using a mathematical model would be of fundamental significance in the study of this phenomenon and would also be interesting in its own right.

One such possible approach would involve drawing a circle to represent the initial colony perimeter and marking this off into a large number of small elements. These could then be advanced radially at a rate similar to the observed experimenta value for a small colony. To simulate the development of peripheral irregularities certain elements or groups of elements would need to be advanced at a faster rate for a short time. Some allowance for the advantage which these regions would then possess due to the various diffusion gradients would also be made. Since in a model of this kind a large number of elements are considered, the use of a computer seems inevitable, preferably one with graphical output.

Preliminary studies were carried out but were not proceeded with for the following reasons:-

First, it was necessary to make various assumptions regarding, for example, the method of choosing the elements to advance at an extra rate, how much faster to advance them and the way in which to incorporate the 'diffusion advantage effect', quantities which are difficult to estimate

experimentally. Often the choice was at best an arbitrary one, based on a value which would give rise to the observed experimental observation. There was, therefore, a very real danger that the computer model would become divorced from the system that it was supposed to represent and hence even if similar patterns to colonies could be produced, this need not be significant.

Furthermore, such a project would require a considerable amount of computer time. However, such an approach would be very interesting, but it was decided inappropriate to proceed with it in the present investigation.

Nevertheless, it was possible to devise a less time consuming procedure to represent the development of a part of the colony such as a single peninsula.

The probable mechanism of the development of small 'bumps' on the perimeter must first be considered. It seems reasonable to assume that cells of Aerobacter aerogenes are not able to respond individually to gradients of growth limitin substances and, therefore, that the expansion of a mass of such cells can only take place in a direction normal to the existing perimeter. When, however, a gradient of the growth regulating substance is present in the gel beneath the expanding mass of cells, the rate of outward growth will vary from point to point along the perimeter and in consequence the shape of the perimeter will change. This effect is

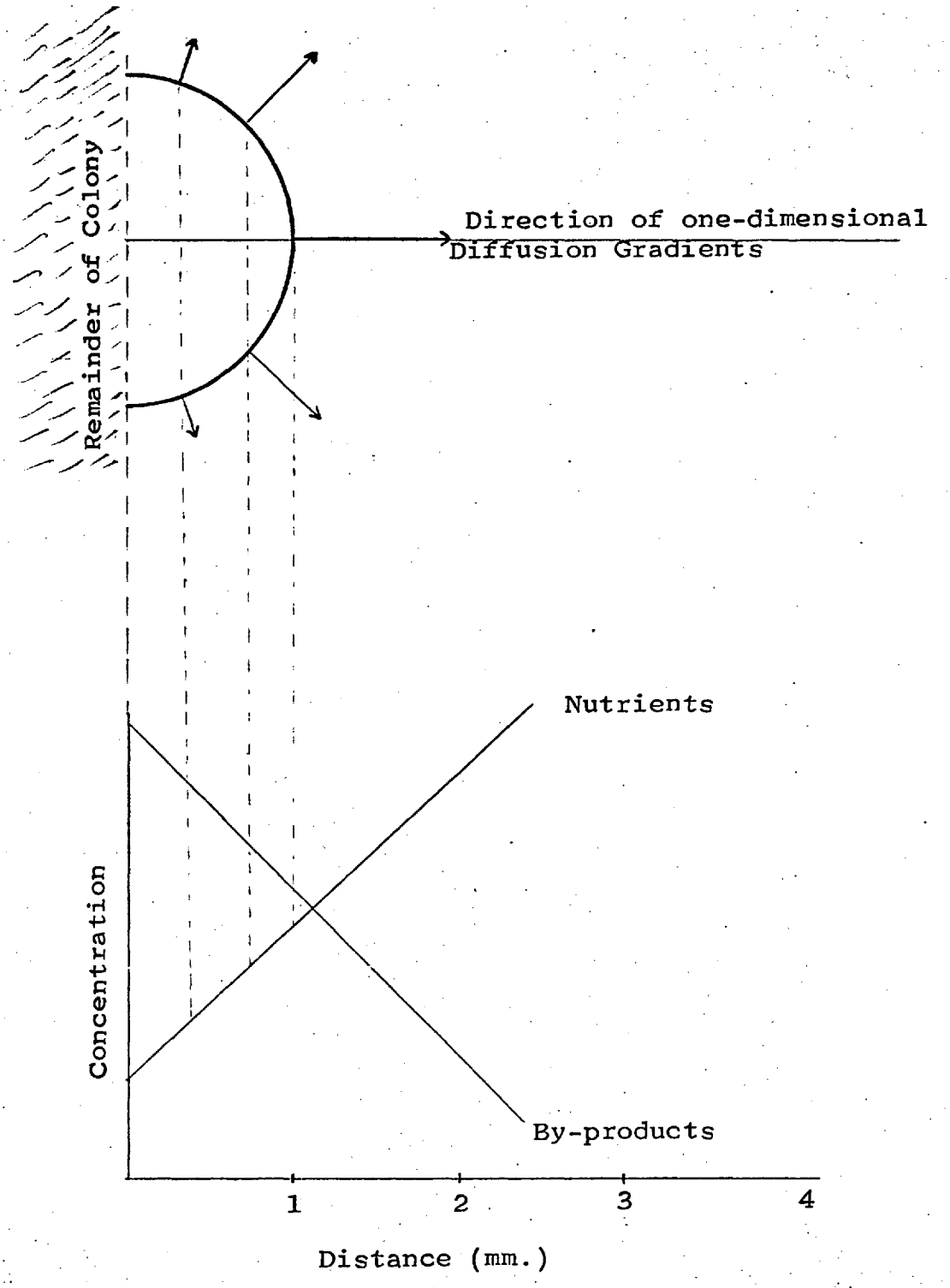
illustrated in Figure (8-10) for a semicircular protrusion of 1 mm. radius. The points on the perimeter expand faster at the higher nutrient concentrations or at the lower concentration of some toxic by-product, depending upon which is growth limiting. It can be seen that these two conditions will produce basically the same effect. This relatively simple mechanism lends itself to a stepwise graphical procedure.

To implement the above procedure it is necessary to be able to estimate the relevant diffusion gradients. In Chapter 3 it was assumed that the concentration of some growth limiting nutrient was maintained at zero in the gel immediately beneath the colony. This procedure gave the maximum rate at which material could diffuse to a colony and was useful in estimating when the diffusion of nutrients would become limiting. In a similar form it was also applied to the outward diffusion of by-products and in addition has proved satisfactory in other situations, (page 68). For the present purpose, however, it is of no use since we have postulated that gradients of growth regulating substances must exist beneath the developing regions of the colony's perimeter.

One way of overcoming this difficulty is to assume that the origin of the diffusion gradient is situated at some point between the 'centre' of the colony and its perimeter.

Another limitation to this method of simulating peripheral development is that, in practice small protrusions develop as such for a short time, but later they subdivide

Figure (8-10).


Remainder of Colony
Direction of one-dimensional Diffusion Gradients
Nutrients
By-products
Concentration
1
2
3
4
Distance (mm.)

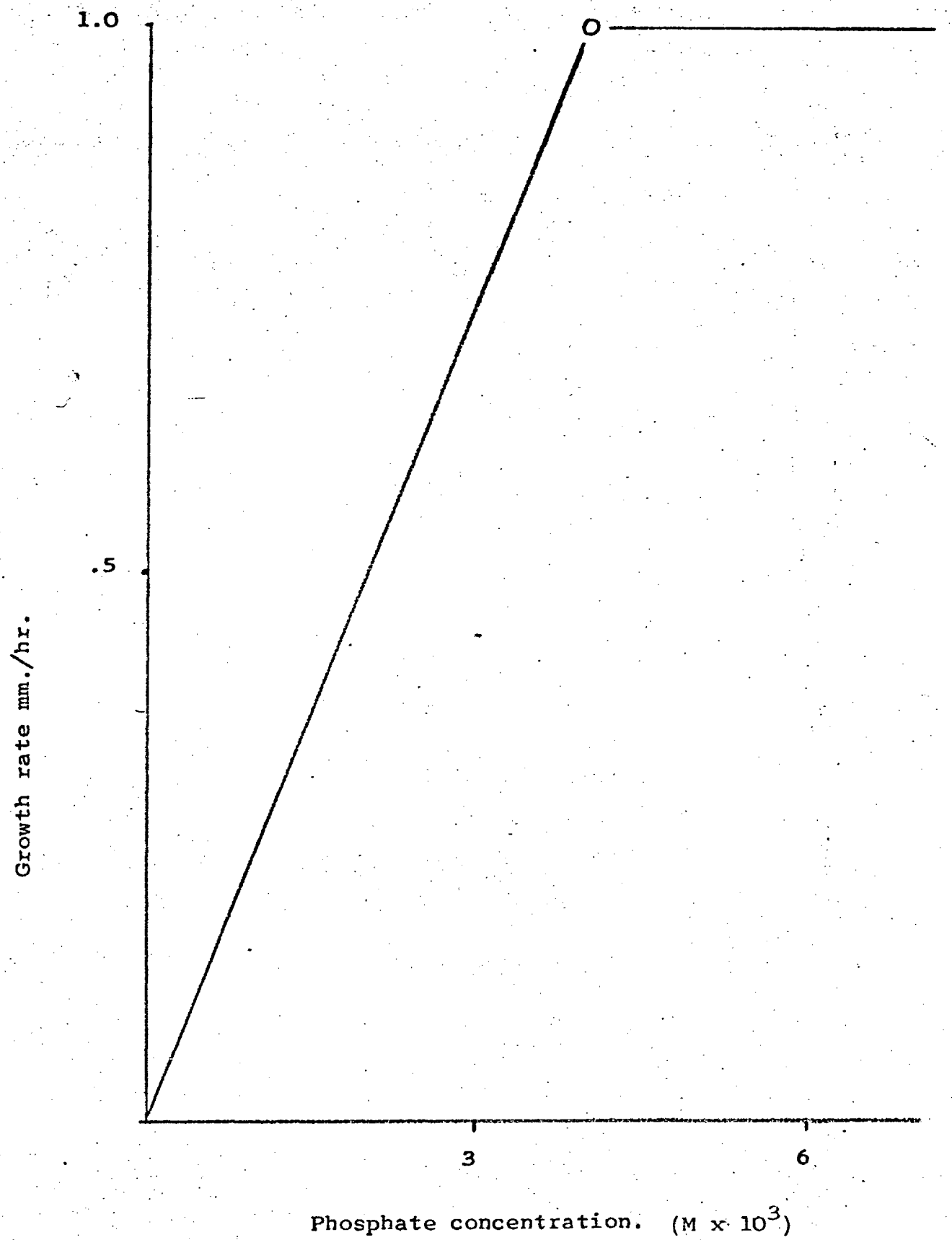
as they too become unstable. This effect is rather difficult to copy using the present simple model.

The treatment also neglects the modification of the diffusion gradients by the growth of the peninsula itself and apart from being complicated in this manner, the diffusion will in practice almost certainly be of a 3-dimensional nature.

In view of the operation of such factors as the above, it was felt best to adopt a fairly arbitrary approach to the simulation of the development of small peninsulae rather than attempt to represent growth in specific cases.

In the following calculations a small initial protrusion on the perimeter of a colony was represented as a semicircle of 1 mm. radius which was drawn on a much larger scale. A one-dimensional diffusion gradient (see diffusion programme, Appendix) of the growth regulating substance was then assumed to exist in a direction parallel to the axis of the semicircle, (see Figure (8-10)). The assumption was also made that when the concentrations of nutrients and by-products were not limiting, the perimeter of this semicircle would expand at the same rate as that found experimentally for a small colony of Aerobacter aerogenes, (0.05 mm/hr., Chapter 3). Figure (8-11.), which is adapted from some results obtained with this organism in liquid culture, (Dean and Hinshelwood 1966), shows the colony perimeter growth rate as a function of phosphate concentration. It appears from the work of

Figure (8-11).



Monod (1942), that the curve relating growth rate to glucose concentration is of a similar form.

A colony starts to develop irregularities at about 100 hours after inoculation and the concentrations fed into the model at the beginning were those produced by diffusion for this same period. At successive 20 hr. intervals, points on the perimeter of the semicircle were advanced outward in a direction normal to the perimeter by an amount calculated from the averaged concentration of growth regulating substance during this time increment.

Figure (8-12a) shows the development of a semicircle of 1 mm. radius for a period of 120 hr. starting at 100 hr. after diffusion had begun. The inward diffusion of phosphate was assumed to be growth limiting and the diffusion 'sink' to be 4 mm. behind the diameter of the semicircle. It was found that the concentration of both phosphate and glucose given by the conditions of this model were low enough to be growth limiting.

The effect of moving the diffusion sink to 2 mm. behind the diameter of the semicircle is shown in Figure (8-12b) in which all other parameters were kept the same as in Figure (8-12a). The pattern is now reduced in size due to the lower nutrient concentrations and also the shape is modified.

Figure (8-12a).

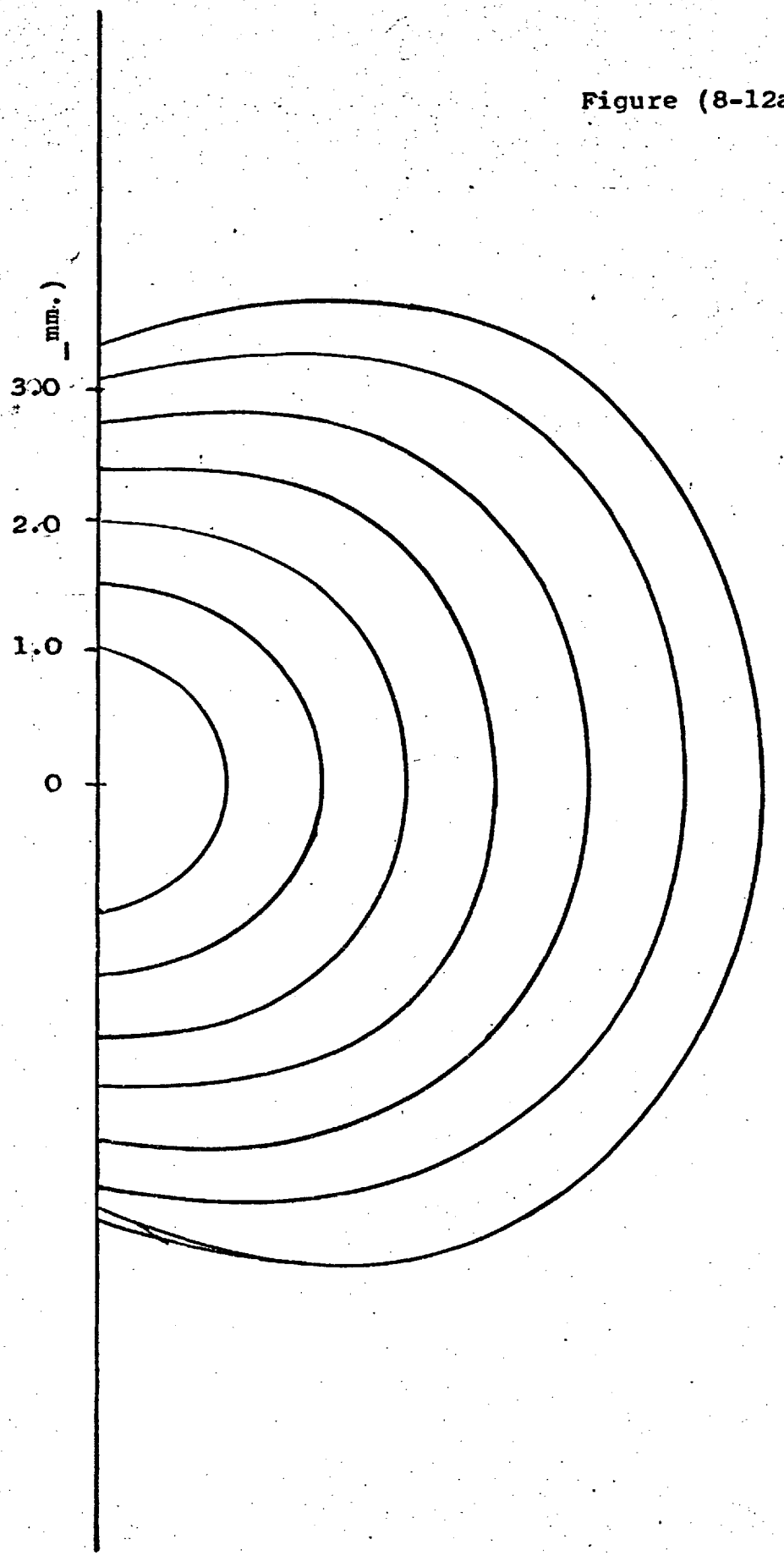
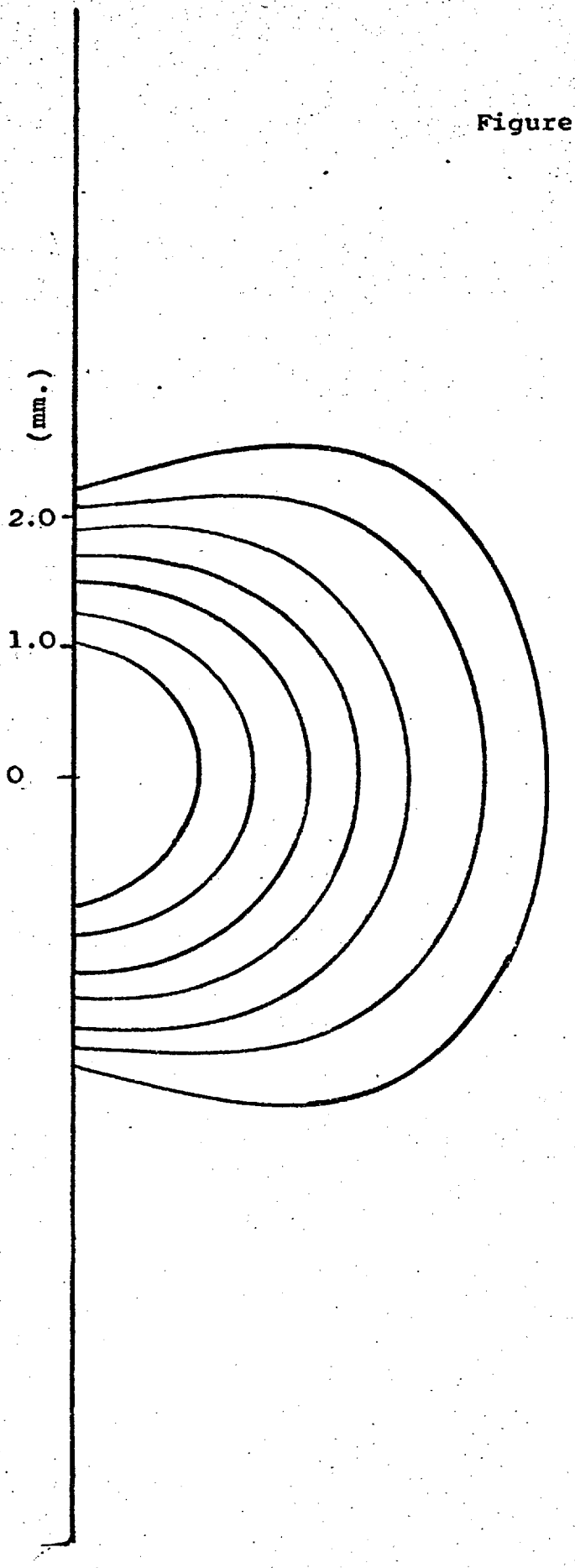


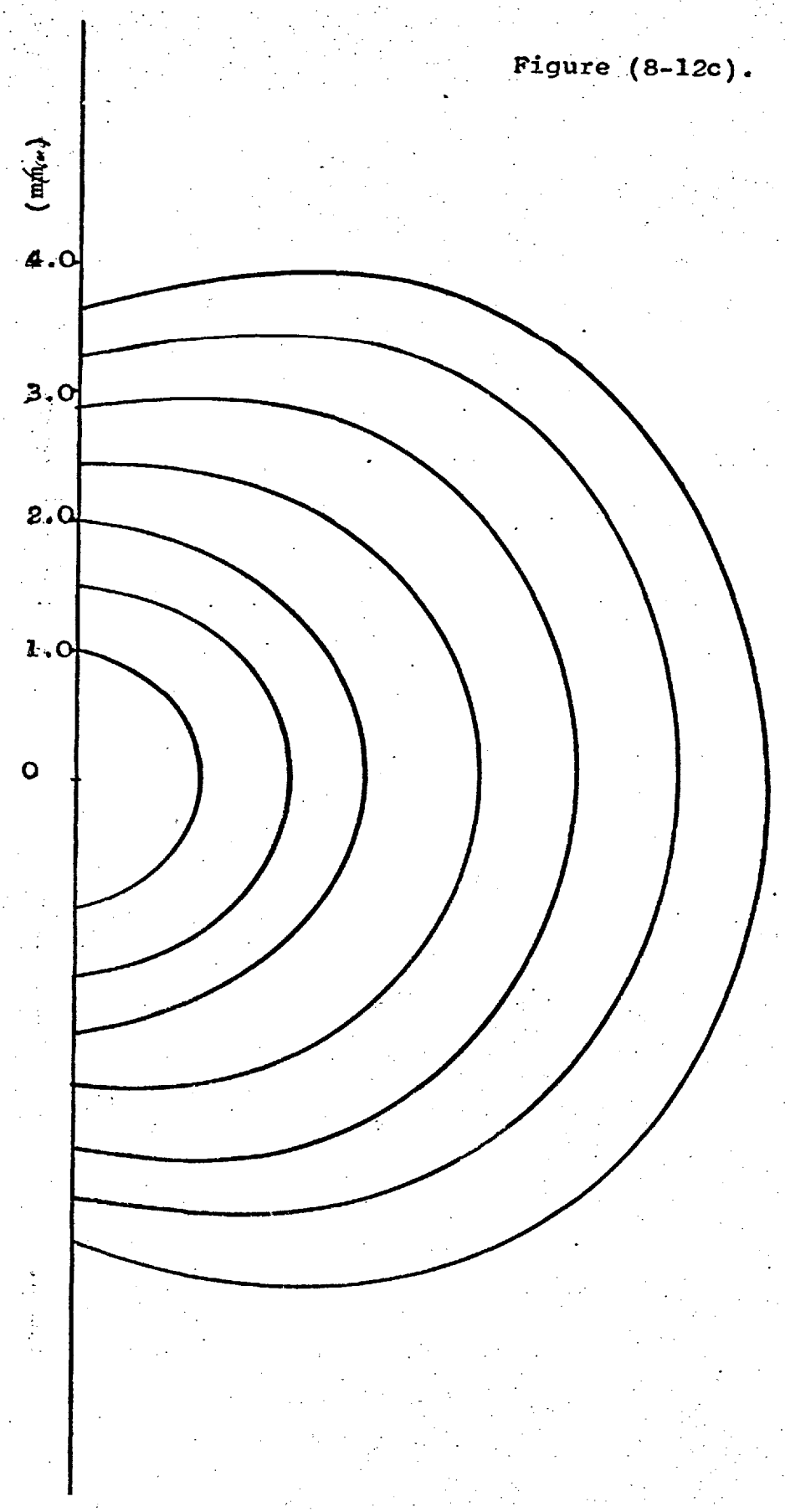
Figure (8-12b).



The simulated effect of adding charcoal to the agar, (Chapter 7), is illustrated in Figure (8-12c), in which the concentrations employed were those calculated with a computer programme, (Appendix), in which the mass of adsorbed material per litre of gel was 50 gm. This case is similar to that of Figure (8-12a) in all other respects except for the time at which it was postulated that the semicircle started to develop. It was also assumed that development would begin when the concentration of diffusing material at the diameter of the semicircle was the same as in the absence of charcoal. Thus, the growth represented in Figure (8-12c) began at 200 hrs. and finished at 320 hrs. after the start of diffusion. From a comparison of Figures (8-12a) and (8-12c) it can be seen that the presence of charcoal in the gel causes the semicircle to grow further from the parent colony radially, in a given period of time. The shapes of the two formations are not very different, that produced in the presence of charcoal having, however, a very slightly greater length to breadth ratio. This contrasts with what is observed in practice, (Chapter 7).

It will be apparent that some of the patterns which are produced in this way do bear a close resemblance to small regions of the perimeter of a developed colony, (c.f.page 76). However, it must be remembered that we have simulated the development of only a single outgrowth, while the developmentof a colony involves the complex interaction of many such events.

Figure (8-12c).



Chapter 9.

Discussion.

The growth of the colonies of Aerobacter aerogenes has been studied in this thesis and classified into several phases. There is good evidence that many other organisms may be similarly treated.

Immediately after its inoculation onto an agar plate, the bacterial cell suffers a lag, the length of which is governed by the same factors as those pertaining to liquid culture, (Chapter 2 and Dean and Hinshelwood 1966). This phase is terminated by the division of the inoculated cell from whence follows a phase in which the numbers of cells increase approximately exponentially with time. So far, the growth has been very similar to that in liquid media, but soon, due to physical or chemical factors, the growth of the 'colony' slows down. This transition, which has not been investigated in the present study, is complete by the time the colony has reached a diameter of about 0.3 mm.

The colony retains the shape of a flat circular disc and increases its diameter linearly with time in the range 0.3 - 4.0 mm. These kinetics have previously been reported by others for several different organisms, for example, Chaetomium globosum, (Plomley 1959), Escherichia coli and Streptococcus faecalis, (Pirt, 1967). It seems that the most likely explanation for this type of growth is that the actively dividing cells are confined to a peripheral annulus

of fixed width. Experiments described in Chapter 3 lend some support to this.

Later this phase gives way to a slower one in which the colony area increases approximately linearly with time. This type of growth is only clearly distinguishable when single colonies are grown on agar plates and their growth followed for the period from about 5 to 30 days after inoculation. It has been shown, (Chapter 3), that growth under these conditions is regulated by the diffusion of nutrients or by-products to and from the colony. This phase of growth is accompanied by complex morphological changes in the colony which have in themselves, formed a large part of the work described in this thesis.

Mathematically, the linear increase of colony area with time may be explained in terms of a decrease in the width of the actively growing annulus inversely proportional to the colony radius. On spread plates, however, any operation of this area-linear growth is generally short-lived since it is soon terminated by the general decrease in growth rate as the agar becomes depleted of some essential nutrient, (Chapter 3). The progressive depletion of the agar by randomly spaced circular colonies presents a somewhat formidable diffusion problem for rigorous solution, even when certain assumptions are made. Instead, various semi-empirical functions have been devised as measures of the interaction of colonies in this way. The operation of competition among individual

colonies of Aerobacter aerogenes on spread plates has been demonstrated and so also has its effect upon the distribution of colony sizes, (Chapter 3). The fact that competition was not evident on spread plates of the slower growing organism, Saccharomyces cerevisiae provides an indication of the delicate balance that exists between the rates of supply of nutrients, (or removal of by-products), by diffusion and their rates of consumption, (or production), by the cells in the colonies.

The morphological changes which are observed on the prolonged growth of single colonies have been extensively studied, (Chapter 4). These changes involve the appearance of regions of different thickness in the colony and the progressive development of the smooth circular perimeter of the latter into a complex 'floral' type of pattern, (Plate 4-1) page 75). Experiments have shown that the production and selection of mutant cells is not important in this phenomenon and similarly specific cell-cell interactions (c.f. Bacillus mycoides, Chapter 4), and the effect of gel structure have been ruled out. It was proposed that as the diffusion of a nutrient or a by-product became growth limiting for a colony, the smooth perimeter of the latter became unstable. Thus, under these diffusion limited conditions, any small protrusions in the perimeter would be at an advantage over the remainder by virtue of being at a higher concentration of some growth limiting nutrient or at a lower concentration of a rate

limiting by-product. The small protrusions would thus grow faster than the intervening regions and reinforce their advantage. With time, these large peninsula-like regions of growth would themselves develop unstable perimeters and the above procedure would repeat itself leading to the eventual formation of the complex pattern observed in practice. The analogy between this proposed mechanism and that of dendritic crystal growth was pointed out.

Experimental support for this theory includes the fact that for a modified strain of Aerobacter aerogenes the age and area of a colony growing on a gel of relatively low buffer concentration when peripheral irregularity developed were both found to be proportional to the concentration of buffer in the gel, (page 97). Also, it was found that for colonies of the same strain on a series of plates of different low glucose concentrations, peripheral irregularity and internal inhomogeneity appeared after a period of growth which was proportional to the concentration of glucose in the particular plate. These, together with other experiments, showed that the development of colonies of Aerobacter aerogenes into complex patterns could be induced by a shortage of an essential nutrient or by by-products. That gradients of these materials are required for this process was illustrated by the retention of a smooth perimeter in a colony growing on a Millipore filter in contact with the surface of stirred minimal medium, (page 88). Less direct experiments have

shown that the rate of development of peripheral irregularity in colonies of the normal strain increases with the temperature of incubation and decreases with the depth of the gel, (Chapter 8). Both of these observations find ready explanation in the proposed 'dendritic' type of growth.

When 1% w/v of activated charcoal is present in the norma minimal agar, a colony which resembles a dendritic crystal in geometric form is produced, (page 172). Apart from this striking similarity, such a colony pattern shows that new growth takes place normal to the existing perimeter and hence in the same direction as the various gradients of nutrients and by-products.

The brand of agar powder used to form the gel was found to have a considerable effect upon the colony growth rate and morphology. It seems that the most probable reason for this is chemical in origin (Chapter 5).

It has also been shown that replacing the supply of air to a colony by nitrogen caused marked changes in the growth rate (reduced) and the morphology. This may be due directly to the reduced efficiency of glucose utilisation under anaerobic conditions or to the relatively greater production of toxic by-products. However, due to the similarity in the appearance of such a colony to one grown on agar containing twice the normal glucose concentration, it would seem that toxic by-products are the growth regulating

factors.

The fact that aerating a plate did not modify the morphology of a colony appreciably suggests that the exhaustio of oxygen is not important in the normal method of incubation.

The effect of varying the glucose concentration has been investigated, (Chapter 5), and it was found that on agar of glucose concentrations greater than 4 g.p.l., the growth of colonies of Aerobacter aerogenes was considerably inhibited. This has also been reported by Pirt (1967) for spread plates of this organism and it seems most likely that it is due to the production of excessive quantities of toxic by-products; direct measurement of the pH in such colonies supports this idea. This result also implies that the glucose concentration in the centre of a large colony on normal minimal agar is quite low.

The variation of the growth rate of large colonies as the buffer concentration was altered has been investigated and found to pass through a maximum. At low concentrations, the growth was regulated by the diffusion of buffer to the colony, while at higher concentrations the decrease in growth rate was attributed to the degree of toxic by-product production which the increasing buffer tide permitted at the centre of the colony.

These results illustrate that a colony, (or any expanding mass of cells), will not necessarily grow faster

simply by increasing the nutrient supply to it.

The reduction in the concentration of ammonium sulphate in the gel lowered the colony growth rate proportionally, but no inhibition of this rate was observed at higher concentrations, presumably because excess of this substance does not lead to the production of toxic by-products as in the case of glucose. At higher concentrations of ammonium sulphate the colony growth rate is regulated by factors other than the inward diffusion of this substance to the colony.

The changes in the pH in a growing colony have been investigated, (Chapter 5), and good correlation with the behaviour of Aerobacter aerogenes in liquid culture was obtained. On spread plates, with glucose as the carbon source, the pH initially fell to a level governed by the concentration of buffer in the gel. Between one and two days later this level rose somewhat, due probably to the breakdown of the initially produced acid by-products. This behaviour was observed in spread plates and single colonies; the pH in the centre of a single large colony was generally slightly higher than that at its perimeter for the same reason.

Attempts have been made to correlate certain characteristics of the colony formation of various modified strains of Aerobacter aerogenes with their biochemical properties. A rough dependence of the total area of colonies on spread plates on the maximum populations reached in liquid

minimal medium containing 10 g.p.l. glucose has been demonstrated for some of the strains. It was proposed that, as with the normal strain, toxic by-product accumulation rather than glucose exhaustion limits growth on such plates. The growth rates, however, of large single colonies of the modified strains remained difficult to correlate with other parameters. This may have been partly due to the fact that the varying properties of the modified strains caused different factors to be growth-limiting in each case. It was found, that by reducing the concentration of buffer in the gel to 1/10 of the normal, the diffusion of this became the rate controlling factor and the growth rates of several strains were observed to be similar.

All of the modified strains were found, on prolonged culture, to develop in the same general way as the normal strain, but at very different rates. Although it seems most probable that the colonies of the modified strains developed by the same general mechanism as the normal strain, the colonies produced however, were characteristic of a particular strain and generally distinguishable from those of other strai Colonies of the methylene blue- and Janus black-resistant strains developed irregularities relatively slowly compared with the other strains and this fact has found good correlation with the biochemical properties of these strains, (Chapter 6). The majority of the modified strains exhibited reduced

efficiencies of glucose utilisation which could be explained in terms of a certain degree of their metabolism proceeding by anaerobic routes, but only the acridine-resistant strain reflected this trend appreciably in its colony morphology, (c.f. Plate (6-1) page 160).

The prolonged culture of Aerobacter aerogenes in minimal medium under anaerobic conditions did not seem to produce a strain with markedly altered properties and this contrasts with the report of Warburg (1956) regarding the culture of yeasts.

In the light of the proposed mechanism of colony development the effect of adding adsorbents to the gel has proved interesting. Activated charcoal at 1% w/v in normal minimal agar produced most striking changes in the colony morphology of the normal strain of Aerobacter aerogenes. During the first 10 days of growth, a colony retained a uniform internal appearance and a smooth perimeter relative to one on normal agar; in the presence of the adsorbent, colonies of significantly greater thickness were produced. It appears that the adsorption of toxic growth by-products produced these effects.

After this initial period of growth, spike-like protrusions appeared around the perimeter of the colony. Experimental evidence showed that although these outgrowths differed considerably in appearance from the floral-type of patterns produced in the absence of the adsorbent they

were nevertheless a manifestation of the same type of growth and resulted from an instability of the original smooth perimeter of the colony. The particular mechanism for the formation of these spikes has been considered. While it is true that the diffusion gradients of all growth regulating substances will be steeper in the presence of charcoal than in its absence,(assuming that such substances are adsorbed), the modification to the development of any outgrowths which would take place by this means does not seem to be as extensive as that which is observed in practice. It was proposed, therefore, that just as the presence of charcoal produced colonies initially of much smaller area and greater thickness than those on normal agar, probably by adsorbing toxic by-products, so too the form of the peninsula regions is modified in a similar way.

The very close similarity in pattern, (though not in size which old colonies on 1% charcoal-agar bear to dendritic snow crystals has been pointed out and is very interesting in the light of the proposed mechanism of development of large colonies of Aerobacter aerogenes. It was shown that the addition of 1% charcoal-agar of appreciable amounts of certain fatty acids, (which were known to be produced by the cells), caused a colony to develop into a basically normal pattern, (Chapter 7). This result may be taken as indicating that while such acids may be the growth regulating substances in this case, as other workers have suggested, this has not been proved.

Several of the modified strains of Aerobacter aerogenes were found to yield colonies which produced 'spikes' on 1% charcoal-agar, but to a lesser extent than the normal strain. However, the methylene blue-resistant strain did not produce such 'spikes' until the charcoal concentration in the gel was raised to 5% w/v, which correlates well with its much lower metabolic rate, (Chapter 7).

The comparison of the degree of development of large colonies of Aerobacter aerogenes by visual inspection has proved difficult. A mathematical function, which increased uniformly during the development of a normal colony and was based on measurements of the perimeter, was devised. This function, 'P colony/P circle', has been used to show that colonies develop irregular patterns more rapidly on shallower agar and also at higher incubation temperatures, results which are both in keeping with the proposed mechanism of colony development. However, this same function was not able to differentiate between colonies with only slight irregularities in their perimeters and also made no allowance for the complex internal changes in the appearance of a colony which takes place during the development of peripheral irregularity, (for example, Plate (6-1), Barbitone-resistant strain).

The way in which small peripheral bumps modify in shape by interacting with a diffusion gradient of a growth regulating substance has been demonstrated. The successful simulation of the shape of the entire colony perimeter would require a computer model. [illegible]

However, rather more must be known about certain quantitative aspects of this phenomenon in order to avoid the introduction of several arbitrary factors to enable any representation to be meaningful.

The analogies between a colony and a mammalian tissue, which it was suggested in Chapter 1 might exist, will now be considered.

It has already been reported that small colonies of many microorganisms exhibit a phase of growth in which the colony diameter increases linearly with time. The colony at this stage exhibits little tendency to modify its morphology and a tumour might be considered to represent a similar mass of mammalian cells. It is, therefore, of interest to note that certain tumours exhibit a linear increase in diameter with time, (Maynard 1932; Schrek 1935, 1936). The most probable explanation for this behaviour is that growth in each case is confined to a thin peripheral region, an annulus in the case of a colony and a shell for a spherical tumour. The thickness of this growing layer is determined by the penetration of some nutrient diffusing into the mass of cells which are consuming it, (c.f. Hill 1965). Alternatively, an explanation could also be provided in terms of some toxic growth by-product.

The current method of culturing colonies of mammalian cells is on glass slides immersed in a suitable liquid nutrient medium, (Puck and Marcus 1955), and hence comparison with

bacterial colonies is rather difficult. Fisher and Yeh (1967) observed an exponential increase in the diameter of small colonies of the Chinese-hamster cell line CHL-1 cultured in this way. On the other hand they reported that the mammalian cell line 3T3 showed only a linear increase in colony diameter with time because these cells suffered 'contact inhibition of replication' and hence only the peripheral cells of the colony were able to divide.

As the techniques for culturing macroscopic colonies of mammalian cells are improved they may well provide a very interesting way of studying such cells under defined tissue-like conditions.

Embryonic development in higher animals is extremely complex and our understanding of it is still rather fragmentary A possible classification involves a progressive reaction between the coding built into the nuclei of the cells and a physical type of effect produced by the environment of the individual cells, (Weiss 1955). This 'in-built' type of behaviour has been demonstrated by the ability of individual cells or small groups of cells which have been removed from an embryo and artificially cultured, to form a mass of characteristic shape, perhaps similar to that produced in the actual embryo. The possible operation of environmental factors on the other hand, has been shown by the orientation of cells growing in a gel along lines of strain, (Weiss 1929, 1933).

It seems reasonable, however, that bacterial cells, which only form colonies by virtue of being placed on a solid surface, possess no predetermined coding with regard to the development of the ensuing colony. Some microorganisms do tend to form aggregates in liquid culture and in such cases this conclusion may not be so clearly justified. Thus, any morphological modifications exhibited by colonies of Aerobacte aerogenes must be produced by mechanisms of the so-called environmental type and although this represents a limitation of the possible similarity to mammalian tissues, it does mean that such colonies are unique in illustrating the sole operation of these factors.

One very direct analogy has already been drawn between mammalian tumours and small bacterial colonies, but the most interesting aspect of colony growth is observed on prolonged culture. The morphology of large colonies of the normal strain of Aerobacter aerogenes is very sensitive to such factors as the brand of agar powder, depth of the gel, temperature of incubation, concentration and nature of nutrients and air supply. Responses of this type would be expected more from developing tissues than from mere expanding masses of cells.

Under a given set of conditions, colonies of the normal strain of Aerobacter aerogenes form patterns which are recognisably similar, but not superimposable. This again is

characteristic of a given region of cells in different specimens of the same type of multicellular organism.

Similarly, the morphology of large colonies of the various modified strains exhibited great sensitivity to the composition of the agar, often reacting in completely different ways to a given change. Each strain also produced characteristic, but not superimposable patterns, under a given set of conditions.

Weiss (1955) has already been reported as stating that embryonic development is such a complex phenomenon that it cannot be classified in terms of a one-dimensional scale. This difficulty has been experienced to some extent in connection with the internal patterns of colonies, but nevertheless the principle morphological change in such systems is the development of the perimeter and this has proved amenable to simple mensuration.

In the present investigation, it has generally been satisfactory to postulate either nutrients or by-products as the growth regulating substances, and this appears to contrast with mammalian tissues in which quite specific substances may control a region of growth or initiate some development. Nevertheless, colonies of Aerobacter aerogenes do show considerable specificity in their morphology with respect to the substance which is regulating the development of peripheral irregularity. For example, the type of morphology which is exhibited by a colony growing on '1/10 buffer-agar', in which the rate of diffusion of buffer to the colony is the

rate controlling factor is completely different from that observed when glucose is the rate controlling factor. To a certain extent, modified strains of Aerobacter aerogenes produce different colony patterns when the same substance is growth regulating.

Colonies of the normal strain of Aerobacter aerogenes on '1/10 buffer-agar' were observed occasionally to exhibit a stepwise type of growth in which periods of arrest of 2-3 days duration were observed. Probably the low flux of buffer reaching the colony allowed the pH there to fall to a value of about 4, which is growth inhibiting. The cells stopped growing, concomitant with this the pH rose due to the reduced acid production and the cells exhibited a lag due to their exposure to acid conditions, (this is well known from work with liquid cultures). A satisfactory explanation can thus be given for the observed periods of arrest and growth. (The similarity of this type of behaviour to the phenomenon of 'hunting' in certain control systems will be apparent). Likewise, during the development of tissues in higher animals, periods of complete arrest are sometimes observed. This may be a prerequisite for the formation of certain complex structures.

In principle, there is no reason why the type of 'dendritic' growth already shown to occur in bacterial colonies should not take place in certain regions of developing embryos. Embryonic cells can divide as rapidly as bacterial

cells, (Burrows 1955), and so suitable gradients of growth limiting substances could be produced, and by this means the transition from a smooth interface of cells into an irregular form is possible.

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Appendix.

Computer Programmes for one-dimensional Diffusion.

The basic programme uses a method of successive approximations, (Barrow et. al. 1947), in which Fick's first law is applied to small sequential distance elements for short periods of time. Alternative methods for the numerical solution of diffusion equations are given in Carslaw and Jaeger, (1959); Crank (1956).

A 5.0 cm. length was treated as 50 cells of 0.1 cm. and 1.0 min. was found to be a suitable time increment. The programme, which was written in Fortran IV language and ran on an IBM 7090 digital computer, consisted of 2 concentric 'DO' loops. The inner one of these loops applied Ficks first law to calculate the amount of material diffusing from the n^{th} to the $(n+1)^{th}$ distance element over the range of n from 1 to 49, during one time increment. The outer 'DO' loop increased the total time by 1.0 min. at the end of each distance scan.

One commonly used way of testing the validity of the results obtained by such procedures involves varying the size of the time and distance increments. The computed concentrations of diffusing material should be independent of such changes. In the present case, however, calculated concentrations were compared directly with those obtained from the rigorous solution of the one-dimensional diffusion equation (Chapter 3) at a time before the diffusing substance

had made contact with the end wall. The results at 10 hr. after the start of diffusion were found to be virtually identical by both methods. The test corresponds to 600 cycles of the computer programme and it seems, therefore, that the procedure is reasonably accurate.

9 min. of computer time were required to simulate 500 hr. of diffusion, using the programme which is given on page 236.

A modified form of this programme which takes into account the adsorption of the diffusing material within the gel is given on page 240.

These computer programmes can be modified quite easily to study the diffusion from a source where material is being produced at a constant rate rather than from a reservoir of constant concentration. The programme could also be modified to allow for the advance of the colony perimeter. Moreover, also using an extended form of this procedure, it might be possible to study cases of two-dimensional diffusion.

Key to Symbols used in Computer Programmes.

TFLUX	=	Total flux of diffusing material.
DC	=	Amount diffusing (gm.) in each small increment.
AVC	=	Time average concentration in the n^{th} cell during small diffusion step.
DT	=	Time increment (sec.).
DX	=	Distance increment (cm.).
D	=	Diffusion coefficient ($cm.^{2}$ $sec.^{-1}$).
A	=	Cross sectional area through which diffusion is taking place (sq. cm.).
ADS	=	Mass of diffusing material (as a percentage of C_o) adsorbed per cc. of gel.

```
$IBFTC MANPROG
MANPRO    -  EFN   SOURCE STATEMENT  -  IFN(S)  -
C     THIS PROGRAM STUDIES A 5.0 CM. LENGTH AS 50 CELLS OF 0.1 CM.
C      AND USES 1 MIN TIME INCREMENTS
C
C     ROUTINE TO INITIALISE VARIABLES AND CONTROL EXECUTION OF PROGRAM
C
      DIMENSION CELL(50)
      COMMON CELL,D,A,N,DC,AVC,DT,FLUX,TIME,TFLUX
      FLUX=0.0
      TIME=0.0
      DC=0.0
      AVC=0.0
      DX=0.1
      D=0.00001
      A=10.0
      DT=60.0
C
C     ZERO ARRAYS
      DO 4000 N=1,50
      CELL(N)=0.0
 4000 CONTINUE
C
C     WRITE TITLE
      CALL OUTP(1)
C
C     START TIME INCREMENT LOOP                                          9
      DO 2000 I=1,28800
C
C     START DISTANCE INCREMENT LOOP
      CELL(1)=100.0
      AVC=100.0
      DO 1000 N=1,49
```

```
C
C     CALCULATE DC, THE MASS TO FLOW FROM CELL(N) TO CELL(N+1)
      DC=D*A*((AVC-CELL(N+1))/DX)*DT
      IF (DC.LT.0.0000001) GO TO 1900
C
C     SUBTRACT THIS MASS FROM CELL(N)
      CELL(N)=CELL(N)-DC
C
C     CALCULATE TIME AVERAGED CONCENTRATION IN CELL (N+1)
      AVC=CELL(N+1)+DC/2.0
C
C     SET CONCENTRATION IN CELL(N+1) AT ITS NEW VALUE
      CELL(N+1)=CELL(N+1)+DC
C
 1000 CONTINUE
 1900 CALL RFLUX
C                                                                      28


      TIME=I
      TIME=TIME/300.0
      TIC=1/300
      IF(TIME.EQ.TIC) CALL OUTP(2)
C                                                                      31
 2000 CONTINUE
 2001 STOP
      END
```

```
$IBFTC OUTPDK

OUTPDK     - EFN   SOURCE STATEMENT  - IFN(S) -

      SUBROUTINE OUTP(IJK)
      DIMENSION CELL(50)
      COMMON CELL,D,A,N,DC,AVC,DI,FLUX,TIME,TFLUX
C     IF IJK=1 OUTP TITLE
C     IF IJK=2 OUTP RESULTS
      GO TO (10,20),IJK
C
C     1 OUTPUT TITLE
 10   WRITE(6,1010)                                                    3
1010  FORMAT(1H1,20X,33HONE DIMENSIONAL DIFFUSION MARK 2 )
C
C     OUTPUT RESULTS
 20   WRITE(6,1020) TIME                                               4
1020  FORMAT(1X/6X,28HCONCENTRATIONS WHEN TIME IS ,F8.3)
C
      WRITE(6,1030)(CELL(N),N=1,50)                                    5
1030  FORMAT(1X,5(6X,10(F7.3,2X)/))
      WRITE(6,1040) TIME,TFLUX                                         12
1040  FORMAT(1X,6X,19HTOTAL FLUX AT TIME ,F8.3,1X,3HIS ,F8.3)
      RETURN
      END
C
```

```
$IBFTC DFLUX

 DFLUX      -  EFN   SOURCE STATEMENT  -  IFN(S)  -

      SUBROUTINE RFLUX
      DIMENSION CELL(50)
      COMMON CELL,D,A,N,DC,AVC,DT,FLUX,TIME,TFLUX
      TFLUX=0.0
      DO 3000 N=2,50
      IF(CELL(N).LT.0.0000001) GO TO 3001
      TFLUX=TFLUX+CELL(N)
 3000 CONTINUE
 3001 RETURN
      END
```

```
£IBFTC MAWPROG

MAWPRO      -  EFN   SOURCE STATEMENT  -  IFN(S)  -

C       THIS PROGRAM STUDIES A 5.0 CM. LENGTH AS 50 CELLS OF 0.1 CM.
C        AND USES 1 MIN TIME INCREMENTS
C                    DIFFUSING MATERIAL IS ASSUMED TO BE ADSORBED
C
C       ROUTINE TO INITIALISE VARIABLES AND CONTROL EXECUTION OF PROGRAM
        DIMENSION CELL(50),STORE(50)
        COMMON CELL,STORE,D,A,N,DC,AVC,DT,FLUX,TIME,TIC,TFLUX,ADS
        FLUX=0.0
        TIME=0.0
        CC=0.
        AVC=0.0
        DT=60.0
        DX=0.1
        D=0.00001
        A=10.0
        ADS=50.0
C       ZERO ARRAYS
        DO 4000 N=1,50
        CELL(N)=0.0
        STORE(N)=0.0
 4000   CONTINUE
C
C       WRITE TITLE
        CALL OUTP(1)
C
C       START TIME INCREMENT LOOP                                        10
        DO 2000 I=1,28800
C
C       START DISTANCE INCREMENT LOOP
        CELL(1)=100.0
        AVC=100.0
        DO 1000 N=1,49
```

```
C
CC      CALCULATE DC THE FLOW FROM CELL(N) TO CELL(N+1)
        DC=D*A*((AVC-CELL(N+1))/DX)*DT
        IF (DC.LT.0.0000001) GO TO 1900
C
        IF(STORE(N+1).EQ.ADS) GO TO 1800
        IF(STORE(N+1)+DC.EQ.ADS) CALL ADSB(1)                          27
        IF (STORE(N+1)+DC.EQ.ADS) GO TO 1900
        IF(STORE(N+1)+DC.LT.ADS) CALL ADSB(3)                          35
        IF(STORE(N+1)+DC.LT.ADS) GO TO 1900
        CALL ADSB(2)                                                   41
        GO TO 1000
C
C       SUBTRACT THIS MASS FROM CELL(N)
 1800   CELL(N)=CELL(N)-DC
C



C       CALCULATE TIME AVERAGED CONCENTRATION IN CELL (N+1)
        AVC=CELL(N+1)+DC/2.0
C
C       SET CONCENTRATION IN CELL(N+1) AT ITS NEW VALUE
        CELL(N+1)=CELL(N+1)+DC
 1000   CONTINUE
 1900   CALL RFLUX
C
C
C       SUM TIME                                                       52
        TIME=I
        TIME=TIME/300.0
        TIC=I/300
        IF(TIME.EQ.TIC) CALL OUTP(2)                                   55
 2000   CONTINUE
 2001   STOP
        END
```

```
£IBFTC OUTPDK

OUTPDK      -   EFN    SOURCE STATEMENT   -   IFN(S)   -

      SUBROUTINE OUTP(IJK)
      DIMENSION CELL(50),STORE(50)
      COMMON CELL,STORE,D,A,N,DC,AVC,DT,FLUX,TIME,TIC,TFLUX,ADS
C     IF IJK=1 OUTP TITLE
C     IF IJK=2 OUTP RESULTS
      GO TO (10,20),IJK
C
C     1 OUTPUT TITLE
 10   WRITE(6,1010)                                                    3
 1010 FORMAT(1H1,10X,42HONE DIMENSIONAL DIFFUSION PLUS ADSORPTION )
C
C     OUTPUT RESULTS
C
 20   WRITE(6,1020) TIME                                               4
 1020 FORMAT(1X/6X,28HCONCENTRATIONS WHEN TIME IS ,F8.3)
C
      WRITE(6,1030)(CELL(N),N=1,50)                                    5
      WRITE(6,1030)(STORE(N),N=1,50)                                   12
 1030 FORMAT(1X,5(6X,10(F7.3,2X)/))
C
      WRITE(6,1040) TIME,TFLUX                                         19
 1040 FORMAT(1X,6X,19HTOTAL FLUX AT TIME ,F8.3,1X,3HIS ,F8.3)
      RETURN
      END
```

```
£IBFTC DADSB

DADSB      -  EFN   SOURCE STATEMENT  -  IFN(S)  -

      SUBROUTINE ADSB(IJK)
      DIMENSION CELL(50),STORE(50)
      COMMON CELL,STORE,D,A,N,DC,AVC,DT,FLUX,TIME,TIC,TFLUX,ADS
      GO TO (30,40,50),IJK
C     HERE (ADS-STORE(N+1)) IS GREATER THAN DC
   30 STORE(N+1)=ADS
      CELL(N)=CELL(N)-DC
      RETURN
C     HERE (ADS-STORE(N+1)) IS LESS THAN DC
   40 CELL(N+1)=CELL(N+1)+DC+STORE(N+1)-ADS
C     SUBTRACT DC FROM CELL(N)
      CELL(N)=CELL(N)-DC
C     CALCULATE TIME AVERAGED CONCENTRATION IN CELL(N+1)
      AVC=CELL(N+1)-0.5*(DC+STORE(N+1)-ADS)
      STORE(N+1)=ADS
      RETURN
   50 STORE(N+1)=STORE(N+1)+DC
      CELL(N)=CELL(N)-DC
      RETURN
      END
```

```
£IBFTC DFLUX

DFLUX       -  EFN    SOURCE STATEMENT  -  IFN(S)  -

      SUBROUTINE RFLUX
      DIMENSION CELL(50),STORE(50)
      COMMON CELL,STORE,D,A,N,DC,AVC,DT,FLUX,TIME,TIC,TFLUX,ADS
      TFLUX=0.0
      DO 3000 N=2,50
      IF(CELL(N).LT.0.0000001) GO TO 3001
      TFLUX=TFLUX+CELL(N)+STORE(N)
 3000 CONTINUE
 3001 RETURN
      END
```