DIVISION AND GROWTH RELATIONSHIPS

IN SINGLE CELLS

by

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I INTRODUCTION.

The growth of a culture of cells or of a multicellular structure is the statistical result of the growth of the cells of which it is composed. This overall growth of the community is due mainly to the multiplication of the constituent cells, the actual size of these latter being generally unrelated to the size of the whole. In fact, during the period of such overall growth, cells of any one type remain, within limits, a constant size (Adolph, 1929; Loefer 1952). In order that this situation can arise the rate of division of the cells clearly must be balanced by the rate at which they increase in size. The individual cells must undergo a repetitive cycle of division, growth and division, the growth between divisions being such as to double the size of the cell.

When cells are growing under conditions which permit cellular functions to proceed at their optimal rate, growth will have the appearance of a more or less continuous process periodically interrupted by the division of the cell. As the size of the cell must double between divisions, the occurrence of division cannot be arbitrarily determined, and the question arises as to whether the form or extent of growth between divisions in some way dictates the timing of division.

In 1903 Hertwig introduced the idea of a 'karyoplasmic' ratio being responsible for the induction of division. This concept suggested that there exists in any one cell type a definite relationship between the volume of the nucleus and of the cell, and that the immediate cause of cell division was a state of cytoplasmic and nuclear 'tension' induced by an overgrowth of the nucleus. This work induced others to investigate the nucleo-cytoplasmic relation. Boveri showed in 1905 that nuclear size in sea urchin embryos was proportional to the degree of ploidy and Tessier (1939) showed that cell size and ploidy are proportional in many organisms. More recently, Fautrez et/

et al.(1955) have found a more or less constant relation between DNA content, ploidy and nuclear volume in rat liver cells, and it has been shown for a polyploid series of yeasts that cell volumes (Mortimer, 1953), dry weight per cell, and DNA per cell (Ogur et al.1952) are related to the degree of ploidy. However, although such relationships may in general be true, exceptions have been reported (Swift, 1950) and interesting aberrations have been found. In the case of the Desmid alga, Micrasterias, for instance, Kallio (1951) has found a surprising situation. Micrasterias consists of two "semi-cells" joined by a narrow isthmus which usually contains the nucleus. Normally the nucleus divides and a cell plate forms in the isthmus, the two semicells separate and each grows another half. By centrifuging cells which are either undergoing nuclear division or are near it, it is possible to produce multinucleate cells and cells containing a single polyploid nucleus. Kallio found that the size of the polyploid uninucleate cells was related to the degree of ploidy, but that the size of the multinucleate cells differed little from the normal uninucleates. Polynucleates of other organisms (eg. Chalkley's amoebae, 1931) seem to behave like polyploids and a satisfactory explanation of Kallio's findings is still lacking.

The idea that the relationship between the quantity of DNA and the cell size acts as a stimulus to division might be more convincing if the increased size of polyploid cells was due to an increased generation time as a result of the extra amount of DNA to be synthesised. This does not generally seem to be the case. Burns (1956) has shown that diploid, triploid and tetraploid yeast cells of the same series have similar generation times which must mean that it is the growth rate that is proportional to the ploidy. Furthermore, the constancy of the DNA content of a cell, expected on the basis of its genetic function, would lead one to expect the size of the cell at division to be rigidly determined. However, Prescott (1956) has shown for Amoebae that/

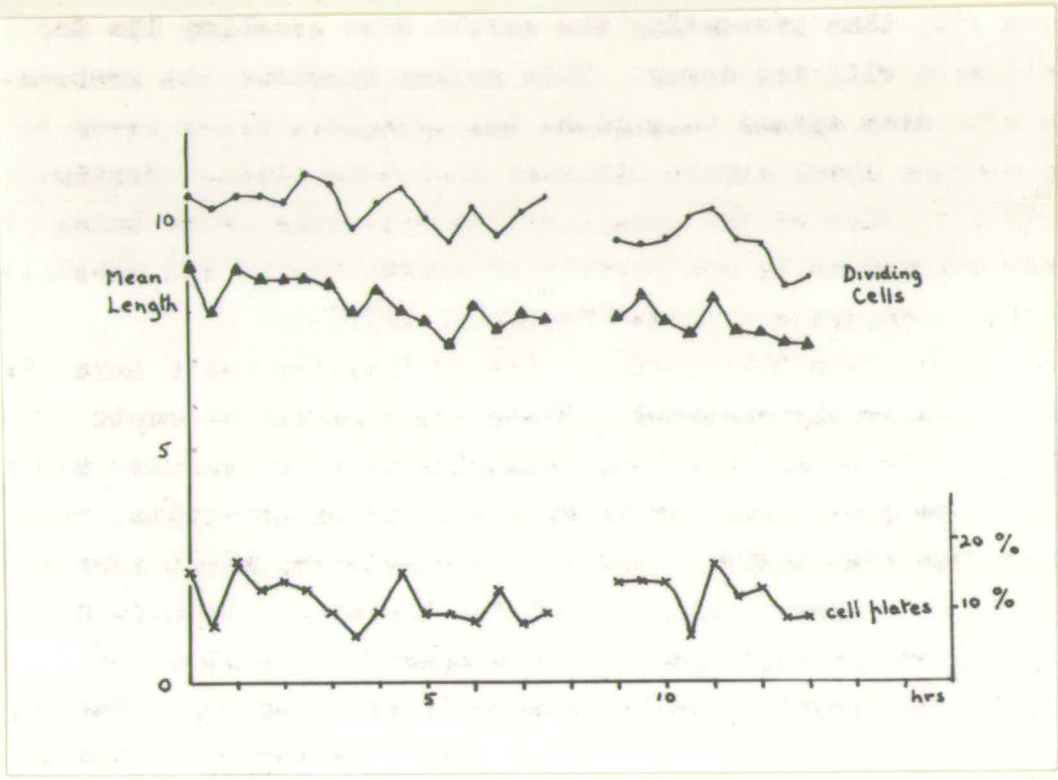

Figure 1. Mean length of the cells of S.pombe during logarithmic growth in culture. Percentage of cell plates in sample shows slight waves of synchronous division. First sample taken twelve hours after inoculation. One unit of length equal 1·5μ.

that starved cells will eventually divide although they never reach their normal fully grown size. On the other hand both Prescott and Hartmann (1928) have shown that when pieces of cytoplasm are continually cut off, thus preventing the amoeba ever reaching its full size, division will not occur. This rather suggests the presence of some substance spread throughout the cytoplasm which needs to reach a certain level before division will take place. Continual removal of a portion of the cytoplasm prevents this level being attained but starved cells are capable of manufacturing the substance slowly from endogenous sources (Prescott, 1956).

Deviations from the 'normal' size of dividing cells have, in fact, been frequently reported. There are numerous accounts of the size of dividing cells in culture changing in a progressive manner when cells are grown from low to high population densities, and in most cases the size becomes less as the stationary phase approaches (Fogg, 1953 for algae; Henrici, 1928 for bacteria; Richards for protozoa, 1941, and yeast, 1928). I have myself shown that the fission yeast Schizosaccharomyces pombe behaves in the same way. (See Fig. I). It is evident from all this work that cell size per se is not the 'trigger' for division and that the processes leading to division are separable from those responsible for growth, though the extent to which they are independent is not altogether clear. It thus becomes necessary to investigate more fully the conditions which will induce division to occur and to see to what extent they are related to the mechanisms controlling growth.

The dissociation of division from growth which occurs during the life of a culture has already been mentioned. Unfortunately, the changing conditions of such cultures are hard to define and it is not possible to decide whether the changing size of the cells is due to one or other process having a priority for materials required by both, or to the disappearance from the medium of some substance necessary for one and not the other. The possibility of priorities for/

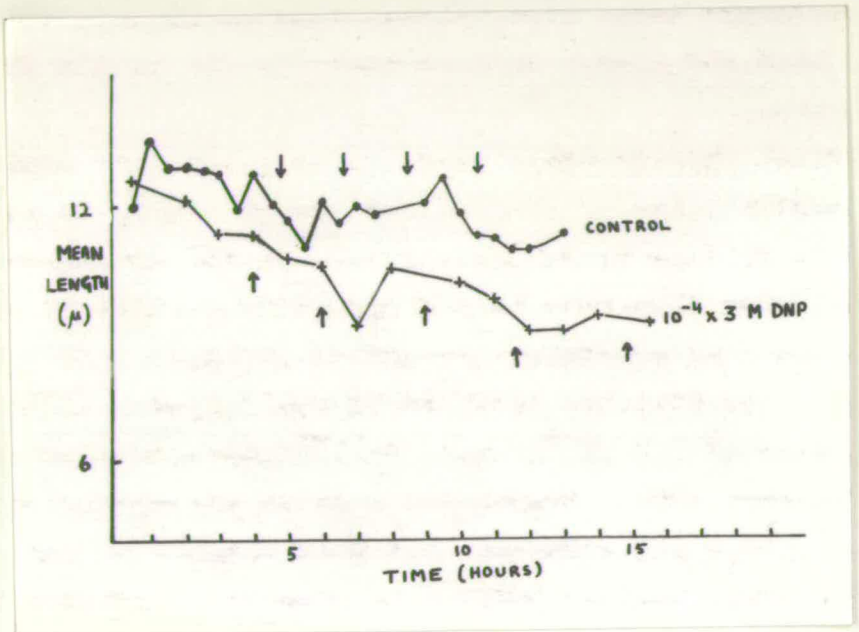

Figure 2. Effect of 3 x 10^{-4} DNP on the mean length of cells of S.pombe. Population doubling times indicated by arrows.

for the energy supply has been discussed in greater detail by Swann (1954). The fact that cells may either decrease or increase in size during the growth of the culture (Adolph, 1931) would suggest that the change in size is due to a different balance between those materials required specifically for division and those required for growth in the different media used, though this in no ways denies the possibility that the change in some cases is due to the action of a priority system.

During my final undergraduate year, in an attempt to decide between these possibilities, I limited the energy supply to cells of a fission yeast in culture by subjecting them to the influence of 2:4 dinitrophenol when they were in the logarithmic phase of growth. Progressively increasing concentrations of dinitrophenol slowed the growth rate before the division rate and it would appear that between concentrations of 3×10^{-4}M and 6×10^{-4}M there was no direct effect on the division rate. Eventually a point was reached at which the division rate was affected, but this appears to have been due to the cells having been so reduced in size that division could not take place at its previous rate, and was thereafter controlled by the rate at which the cell could grow up to the minimum size for division. (See Fig. 2). At a concentration of 10^{-3}M the rate of division was also reduced. This suggests that division exercises a priority for the available energy. However, the effects of dinitrophenol, like many other inhibitors, are not sufficiently well understood to decide whether this result is due to a restriction of the energy available to the cell caused by uncoupling phosphorylation from oxidation (Loomis and Lipmann, 1948; Lipmann, 1949) or due to some specific interference with the growth mechanism. Pace and Ireland (1945) found that raising the oxygen tension increased the oxygen consumption of Tetrahymena and induced the formation of smaller cells. This suggests that the additional energy acquired is used for division.

In/

In spite of the difficulties of interpretation of the action of chemicals many 'inhibitory' substances have been applied to cultures, and some have been found to have differential effects on division and growth. Loveless et al. (1954) tested a large number of compounds for this effect although the range of concentrations used appears in many cases to be inadequate (e.g.for Urethane, see Harnden, 1957). They concluded that none of the inhibitors used, with the exception of radiomimetic compounds, succeeded in affecting division without also affecting growth, although many did reduce the growth rate without comparable effect on division. It would thus appear that division is less sensitive to inhibitors than is growth and a priority system might well explain this.

The effect of high energy radiation and of radiomimetic substances is to inhibit division and not growth so presumably must act solely on the division mechanism. The mode of action is far from clearly understood but Swann (1957), who has discussed the problem at some length, concludes that it seems likely that the radiosensitive mechanism is to be found in the nucleus, probably in a number of discrete centres which may be located on the chromosomes. The effect may well be concerned with DNA synthesis as suggested by Mitchell (1942) but the evidence is contradictory and the question must be left open for the time being. There do, however, seem to be some significant similarities with the effect of respiratory inhibitors which we will return to later.

Filament formation has been induced by chemical means in a number of micro-organisms but it seems unlikely that the entire division, consisting of nuclear and cytoplasmic cleavage and cell separation, is suppressed. Webb (1949), for instance, finding that deficiency or excess of magnesium induced filament formation, noticed that there was no appreciable interference with the division of the chromatinic bodies. Similarly, Robinow (1944) has shown the presence of intrafilamentous partitions in some bacteria and reported that chromatinic/

chromatinic bodies are scattered along the length of such filaments. Nevertheless it is noticeable that many of the substances found effective in inducing filament formation might be expected to act on sulphydryl groups. (Rapkine, 1937; Nickerson and Van Rij, 1949; Scherr, 1957). Nickerson (1954) found that both a filamentous mutant and induced filamentous forms of the yeast Candida albicans, which grew at the same rate as the normal budding cells, accumulated and reduced tetrazolium dyes. He has suggested that this is due to impairment of a cellular oxidation mechanism at a flavoprotein locus, and that the reactions at this site are essential to cellular division. He has further suggested that the block to cellular division is due to impairment of a dissociable metal chelating mechanism which normally couples a reaction essential to cellular division to flavoprotein oxidation. The question of the importance of sulphydryl groups to cell division is a large one and will be considered again later.

It has long been known that temperature has a differential effect on division and growth, and it generally appears that extremes of temperature induce the formation of longer cells. In other words the division mechanisms are more sensitive to temperature extremes than are the growth mechanisms. Mucibabic (1956) has found that although cell size in Chilomonas paramecium is maximal at extremes of temperature, over the greater part of the range there is a progressive change in the relative rates of division and growth resulting in smaller cells at the higher temperatures. It seems probable that this increasing rate of division relative to growth is a general effect of temperature. Cells like Histoplasma capsulatum which change from yeast like forms to mycelial forms do so less readily at higher temperatures (Scherr, 1957).

Observations on randomly dividing cell cultures, useful as they are in telling us something of the general relationships which exist and the factors affecting the relative rates of division and growth,/

growth, tell us little of the relationships of the various possible controlling influences within the cell cycle. If, on the other hand, cells which are dividing simultaneously can be used, analysis on the whole culture can be equated with the situation in the individual cells, providing any other cyclic cellular processes are similarly in step.

When fertilized simultaneously, synchronous divisions occur in the cleavage stages of the eggs of several organisms and those of the sea urchin have been widely used for studies on the mechanism of division. Up to the stage of the blastula of the sea urchin there are good reasons for thinking that little or no growth occurs. The cells certainly do not increase in volume although it might be argued that there is conversion of inactive foodstuffs into living material. This however, does not seem to be the case, as Zeuthen (1953) has shown that the rate of oxygen consumption increases only slightly inspite of great increases in cell number. Swann (1954) has argued that in spite of the fact that no marked synthesis occurs between divisions there is, none the less, a time lag between the end of one division and the onset of the next, presumably indicating that time is required to bring the cell to a state in which division is possible. He has found that when respiration is inhibited for a given period of time the succeeding division is delayed by the same amount of time, provided that the cell has not already reached a certain critical point just before prophase. Inhibition of respiration after this critical point has no effect on that division although the next one is delayed. Furthermore, blockage of energy pathways by dinitrophenol or glycolytic inhibitors after this critical point does not stop the division, which indicates that all the energy required for the division processes must be present in the cell by prophase. Swann suggests that respiratory inhibition before prophase halts the filling of this 'energy reservoir' and thus delays division. If such an energy reservoir exists one would expect to be able to find/

find evidence of the presence of an energy carrying substance, the level of which rises during interphase and falls during division. Rapkine (1931) found that in sea urchin eggs the quantity of free -SH groups fell during interphase but rose throughout mitosis. This is suggestive of the build up of an energy mechanism envolving fixed -SH groups. Swann (1957) has recently found evidence of fluctuations of an activated acyl compound, probably a thiol ester. The fluctuations follow the mitotic cycle in exactly the reverse manner to the fluctuations in free -SH. Thiol esters bonds, moreover, are energy rich and the bond is exchangeable with the high energy phosphate bond. Suggestions concerning the fluctuations in free -SH bonds are numerous in the literature, however, and are more fully reviewed by Swann (1957), Barron (1951) and Needham (1942).

Whether or not this energy build-up is the system that limits the rate of division in all cells is another matter. Even in the case of cleaving eggs there are various other processes which must be completed before a normal division can occur. There appears to be an excess of DNA in the egg (Brachet,1946 ; Vendrely, 1955; Mc. Master, 1956) and even if this can be used without modification, the duplication of the chromosomes might well take a measureable time to achieve. Moore's experiments (1933) on cross-fertilizing eggs of Strongylocentrotus and Dendraster would suggest that the rate of cleavage is cytoplasmically determined, though it may well be that the cytoplasm exerts its effect by altering the rate of nuclear activities. It has also been shown (Briggs et al.1951) that enucleate frog's eggs are capable of forming asters and cleaving. These cleavages are slow and irregular but it would appear that chromosome reduplication is not absolutely essential to division. Such eggs cannot, however, proceed beyond the blastula. Thus a cell which needs to grow and differentiate between divisions requires the presence of a nucleus and a different rate-limiting system may operate. Whatever decision one might come to as to the final limiting factor for/

for cleavage in the egg its applicability to the growing cell is doubtful. On the other hand it is not unreasonable to expect those processes which do occur in the cleaving egg also to be found in the growing cell and it has been shown that growing cells like eggs, will complete a division which has started inspite of violent environmental changes (Bullough, 1952) and so might be expected to have a similar energy reservoir system. The equivalent of Swann's experiments on eggs (1953, 1954a) has not been done for growing cells, however, so it is not known whether inhibition at all points of interphase is effective in delaying division or whether the energy reservoir is built up entirely in the period just before prophase. Such experiments as Swann's however, could not usefully be applied to growing cell unless it could be shown that the processes leading to division, and in particular to the filling of the energy reservoir, can be separated from other cellular activities. While specific examples of a relationship between cell division and certain energy pathways have been reported (e.g. O'Connor's work (1950) shows a connection between the number of dividing cells in the mid brain of a chick and aerobic glycolysis) it would seem that the division mechanism is fairly adaptable; the total energy entering the cell being more important. Bullough (1952) and Swann (1957) give further discussion of this problem.

Natural synchrony, unfortunately, does not occur to any marked extent in growing cells, although small peaks of division activity have been reported in cultures of some cells (Adolph and Bayne Jones, 1932; Hegarty and Weeks, 1940). This has been found to be the case in cultures of S.pombe and has been investigated by Harnden (1957). The effect, however, is too slight to be useful. Recently there have been a number of attempts to induce synchrony in cultures of growing cells. Scherbaum and Zeuthen (1954) have succeeded in synchronizing the division of Tetrahymena pyriformis by subjecting them to repeated shifts of temperature. Scherbaum and Zeuthen shifted the/

the temperature repeatedly between the optimum temperature of 29°C and a sub-lethal temperature of 34°C. At the sub-lethal temperature neither growth nor division occurs. On return to the optimum temperature of 29°C the cells again grow and for a while do not divide. Before any division does occur the temperature is returned to the sub-lethal level. Once again on return to the optimum temperature growth resumes but no division occurs. When this process has been repeated a number of times all the cells have grown considerably larger than their normal division size. When they are finally returned to their optimum temperature eighty-five per cent of them divide together after a time interval corresponding to about two-thirds of their normal generation time at that temperature. Two further synchronous divisions occur, again with a reduced generation time. The growth rate while these divisions are occurring is greatly reduced but it regains its normal rate when the divisions have reduced the cells to their normal sizes. The synchrony rapidly disappears after the first three divisions. These workers have suggested that the effect of the sub-lethal temperature is to set back the preparations for division so that on return to the optimum temperature the cells have to prepare for division again. Meanwhile growth is taking place and a series of such set backs produce a population of cells all of which are capable of division in all respects except the temperature sensitive one, and all cells are comparable with respect to this. While this is another interesting manifestation of the possibility of separating growth from division it seems unlikely that the behaviour of these cells between divisions can profitably be compared with the normal cell cycle, except with respect to the temperature sensitive mechanism. The fact that division occurs at a shorter interval than usual could be taken to mean that this is not usually the mechanism limiting the rate of division, but it might also mean that a certain amount of growth is necessary before the mechanism starts to operate, or it might be that in this system energy normally directed/

directed to growth, which is much reduced during the period of the divisions, is utilised by this mechanism alone which can thus proceed faster. Similar effects have been found by other workers using other organisms (e.g.Lark and Maaloe, 1954; Szybalska et al. 1955) but for studying the cell cycle such induced synchrony seems to offer little advantage over cleaving eggs which have a rather similar behaviour.

Barner and Cohen (1956) have induced synchrony in a thymine requiring mutant of E.coli by starving it of thymine. So far this method is limited to organisms with a thymine requirement although Cohen and Barner (1955) claim to be able to induce a thymine deficiency in normal cells. As is the case in the heat shocked cells this system unbalances the synthetic machinery and comparisons with normal growth must be undertaken with care.

Physical methods of separating cells in similar stages of development such as the filtration and differential centrifugation methods of Maruyama and Yanagita (1956) seem more likely to be able to provide synchronous cells showing the normal form of growth but they are probably limited to certain types of cell. The information so far reported from the use of these methods will be discussed at some later point.

In lieu of a satisfactory method of obtaining synchronous cells it is necessary to make direct observations on single cells and to follow their progress from one division to the next. This is something which has been relatively rarely done. There are good reasons for this. Any parameter of growth, such as volume, is usually difficult to measure; animal cells in particular tend to have irregular and changing outlines and the basic variability of biological material is such that a tediously large number of cells must be measured.

Inspite of these difficulties, a number of attempts have been made to estimate the form of the growth curve of individual cells. Initially the parameters which can be measured on single cells are limited/

limited to those which can be obtained from observations of the living cell. Until a short while ago the only method available was to follow the growth in volume. Possibly for the reasons mentioned above, reports of this kind have given very variable results (Adolph, 1931), and it is difficult to decide whether the differences found are genuine or not. For instance, while Gerassimoff (1901) found the growth of Spirogyra to be logarithmic, Chalkley's results (1931) for Amoeba proteus show that the percentage rate of volume increase is a decreasing function of time, which means that the volume increase is not exponential, but his curves for the mean volume of twenty uninucleate amoebae plotted directly against time show a slight acceleration, although this effect may be due to the fact that when a division occurred the volumes of the two daughters were summed and entered on the curve as a single cell. This, obviously, makes it impossible to determine the true shape of the curve between divisions, as the cells were by no means synchronous, and probably accounts for the apparent discrepancy between Chalkley's results and those of Prescott (1955) using the same organism. Prescott found that the rate of volume increase was greatest after division and steadily became slower until it ceased altogether, the cell then remained a constant volume for some time before division occurred. Both Chalkley's and Prescott's results are, however, open to the criticism that the experimental organism has to catch the bulk of its food and it is well known that its feeding behaviour varies at different points in the growth cycle.

Mitchison's measurements (1957) of the volume of the fission yeast Schizosaccharomyces pombe do not suffer from this disadvantage. The cell is cylindrical and easy to measure and grows on sterile nutrient media. The volume growth in this organism shows a slight acceleration for the first three quarters of the growth cycle, the remaining quarter consisting of constant volume stage. It is noticeable, however, that the combined rates of growth of the two daughter cells/

cells is seldom, if ever, equal to twice the rate of the parent. This seems likely to be due to deteriorating conditions around the cells, as the quantity of nutrients available was limited by the method of mounting necessary for determination of dry mass which was measured at the same time. For the same reason it is also very difficult to subject the cells to controlled changes of environment (other than temperature).

Changes in volume undoubtedly give useful information on many of the changes which occur during the cell cycle but interpretation of them must be made with care. This is particularly true if one is endeavouring to trace the course of synthesis as the degree of hydration may well alter. Until recently it has not been possible to measure any other parameter on single cells. In 1955 Prescott measured the reduced weight of single amoebae and found that the curve for weight increase closely followed that for volume. These determinations, however, suffer from the same disadvantage as have already been discussed for his volume determinations. The method used, moreover, is limited to cells of fairly large dimensions. Mitchison (1957) has succeeded in measuring the dry mass of considerably smaller cells by means of the interference microscope and has found that the dry mass of S.pombe increases in a linear manner between divisions. In view of the curve found for volume growth this must mean that the concentration of the cell falls during the first three-quarters of the cell cycle and rises rapidly over the remaining quarter.

With accurate information on the course of volume growth over the cell cycle (or of any other progressively changing visible characteristic of the cell) it is possible to follow the synthesis of a number of cellular materials by analysis of single cells. Thus if any one cell can be 'aged' by some visible characteristic such as size it is possible to relate determinations on dead or living single cells to their position in the growth cycle. Direct chemical anal-

yses/

yses on single cells are clearly impossible but the absorption characteristics of many important cellular materials make it possible to obtain, in combination with suitable extraction procedures, fairly accurate estimations of the quantities present. Mitchison and Walker (in press) have effectively used this technique to follow the course of RNA synthesis over the cell cycle.

Accurate information on the course of volume growth, apart from its own intrinsic interest, is thus important for other determinations. Estimations of dry weight, though certainly giving a better indication of the course of synthesis, are beset by considerable technical difficulties and the conditions under which the cell must be grown are not ideal, nor can they be appreciably altered.

I therefore considered it worth while to develop a system which permitted direct observation of individual cells grown under conditions which could be kept stable or varied at will, and by this means to follow the cell's response in terms of volume increase and division rate to constant conditions or deliberately altered ones. In this way I hoped to discover something of the rate controlling mechanisms and the inter-relations which exist between them.

II THE EXPERIMENTAL ORGANISM

A. THE CELL.

The fission yeast, Schizosaccharomyces pombe Linder N.C.Y.C. 132 was the cell used throughout this work. The characteristics of this cell have a distinct advantage over those of most other available organisms for a study of this type.

It will grow satisfactorily in simple media (see page 17) and unlike many animal cells it is non-motile and shows no characteristic feeding behaviour which might be expected to vary with the stage in the division cycle.

The cell is cylindrical in shape with hemispherical ends, divides centrally by means of a cell plate, and grows terminally, thus making volume measurements a simple function of length. It is 3.5μ in breadth and varies between 5μ and 20μ in length which makes it considerably larger than any bacterium.

Under the conditions used in this work multiplication is purely vegetative. The generation time in wort broth at 27.5°C is rather less than three hours. Sexual reproduction, by conjugation, does occur however, four ascospores being formed. These ascospores, and the vegetative cells which develop from them appear to be haploid. The diploid condition only occurs in the zygote (Leupold 1956).

Cytological information about the nucleus has, until fairly recently been rather doubtful, but Mitchison (1957), using phase contrast microscopy to observe the living cell, has reported seeing a central vacuolar region which divides equally shortly before the formation of the cell plate. Rustad (1958) has managed to stain this central body with the fluorescent dye, acridine orange, which would indicate the presence of deoxyribonucleic acid and has found that only the largest cells, which are/

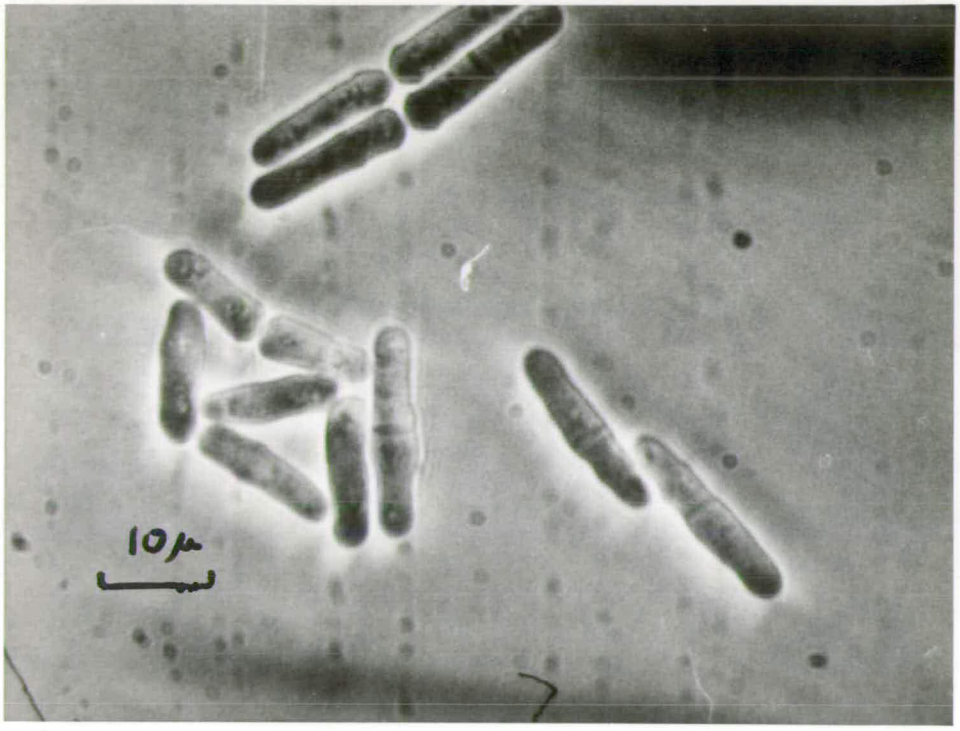

Figure 3. 'Normal' cells of S.pombe in various stages of growth and division. Field typical of those used for measurement.

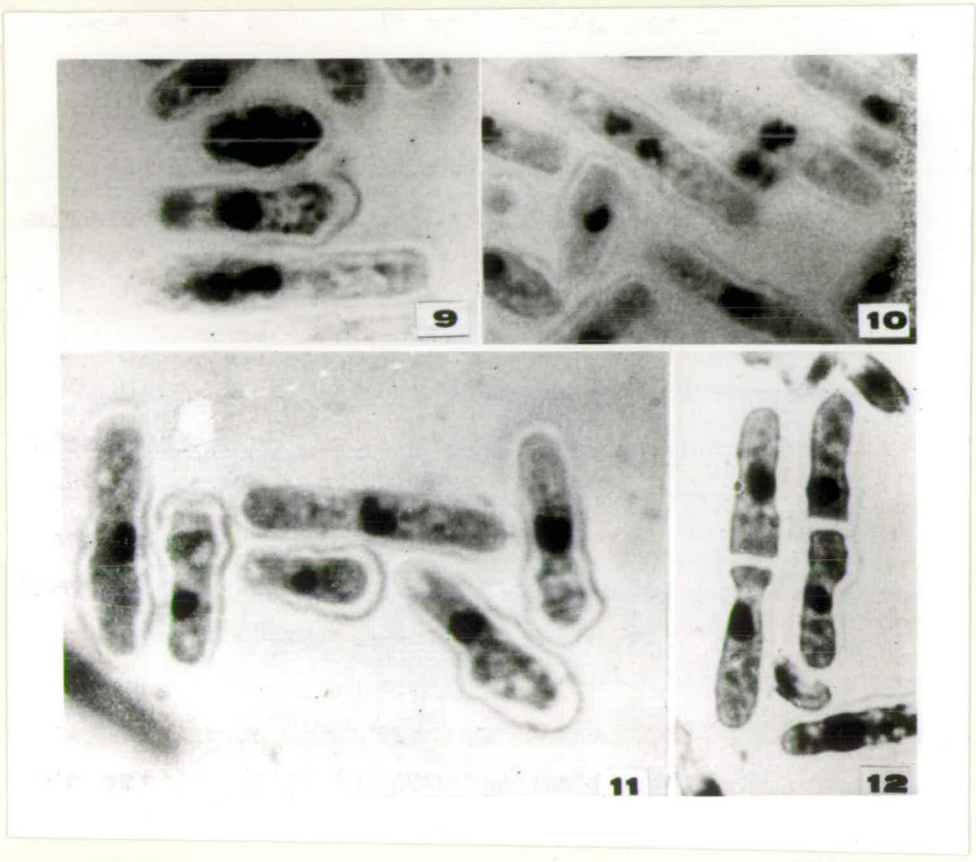

Figure 4. Cells of S.pombe stained with Feulgen. Nucleus clearly visible. Ganesan and Swaminathan, 1958.

are presumably near division, contain more than one of these bodies. Gamesan and Swaminathan (1958) have also recently reported staining techniques which show that this body contains chromatin, that the cells are uninucleate, and that various stages of what appear to be more or less orthodox mitosis can be seen. In this respect then, the cell seems more akin to a normal animal or plant cell than to a bacterium.

B. MEDIA and CONDITIONS of GROWTH.

(i) Stock cultures.

At first stock cultures were kept in test tubes with metal caps, each tube containing 10 mls. of 2% w/v 'Oxoid' wort broth, but over long periods of frequent subculturing it was found that this had the unfortunate result of speeding up the growth rate making comparisons difficult, although the variability was also reduced (see Fig. 5)

Latterly, however, stock cultures were kept on wort agar slopes in McCartney bottles which has served to stabilize this trend. Cells were subcultured from slopes to liquid media at least a week before being used for a series of experiments.

Normal sterile precautions were observed.

(ii) Experimental cultures.

Cultures required for experimental purposes were inoculated from a five day old culture, the effective dilution of the inoculum on inoculation being 1 in 500. This gave an initial cell population of about 75,000 per ml.

Inocula for defined or dilute media were washed in distilled water.

The yeast was found to grow satisfactorily in the defined medium described below and this medium was the one used in section/

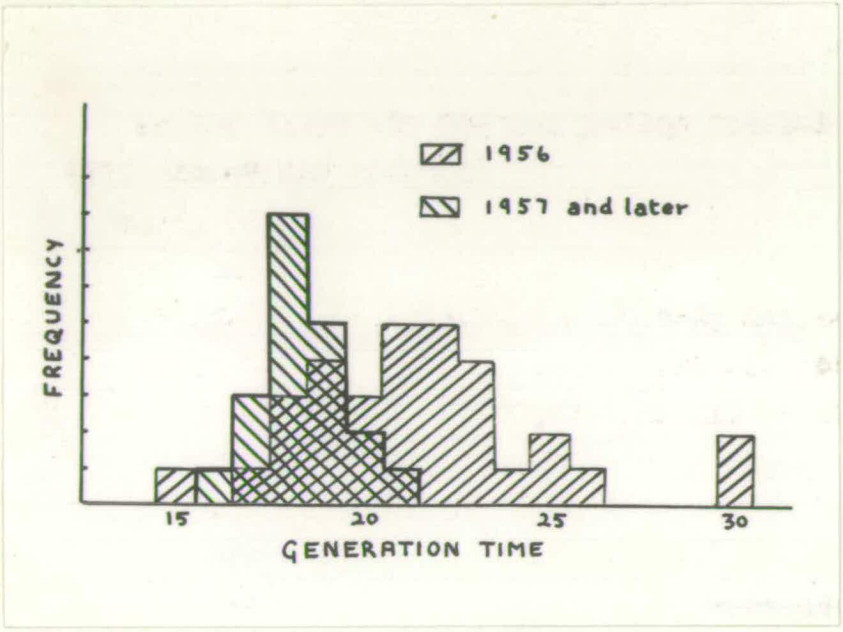

Figure 5. Histogram to show the reduced and less variable generation times of cells after several months subculturing in liquid media. Generation time in nine minute units.

tion IV D. The same medium was used in section IV C but all nitrogenous substances were omitted.

All tube cultures were incubated in a water bath kept at 27.5°C (0.3°C).

Composition of defined medium per 500 ml. final volume (Northam and Norris 1951)

Glucose	10 grams
L-asparagine	1.0 "
Potassium dihydrogen phosphate (KH_2PO_4)	0.75 "
Calcium chloride ($Ca\ Cl_2,\ 6H_2O$)	0.25 "
Magnesium sulphate ($Mg\ SO_4,\ 7H_2O$)	0.25 "
Ammonium sulphate	1.0 "
Aneurin H Cl	250 "
Pyridoxin H Cl	250 "
Calcium D-pantothenate	250 "
Nicotinic acid	250 "
d-Biotin	0.1 "
Potassium iodide (K I)	50 "
Trace element solution	0.5 mls.
Inositol	6.7

The pH was adjusted to 5.2 by addition of 2.5 mls of N/10 Sodium hydroxide.

Trace element solution (Emery, McLeod and Robinson 1946)

Boric acid	0.1 grams
Manganese Sulphate ($Mn\ SO_4,\ 4H_2O$)	0.04 "
Zinc sulphate ($Zn\ SO_4,\ 7H_2O$)	0.04 "
Copper sulphate ($Cu\ SO_4,\ 5H_2O$)	0.045 "
Ammonium molybdate	0.02 "
Ferrous sulphate ($Fe\ SO_4,\ 7H_2O$)	0.25 "
Distilled water	1000 mls.

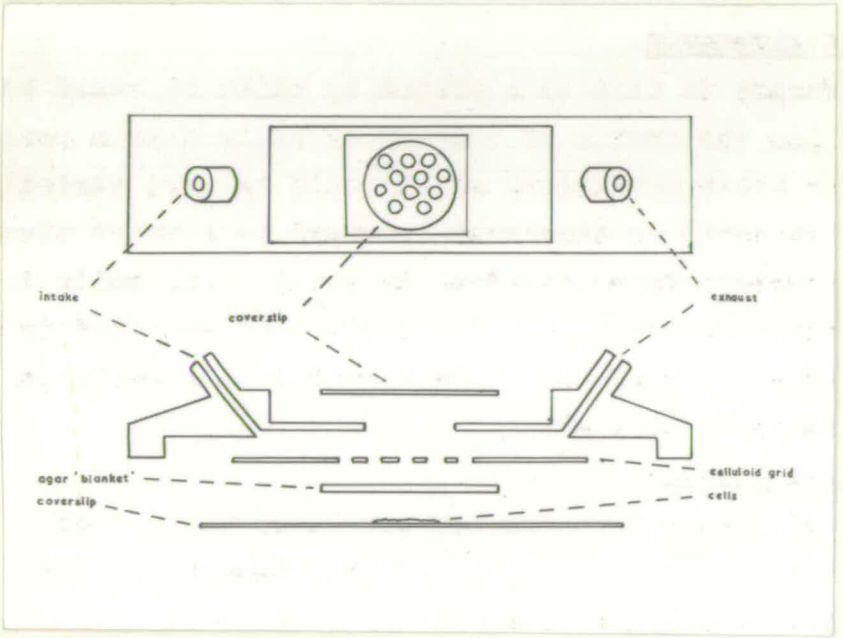

Figure 6. Diagram to show the structure of the culture chamber used and the method of mounting the cells for observations on the growth of individual cells.

III METHOD

A. OBSERVATIONS ON INDIVIDUAL CELLS.

(i) General requirements.

It was necessary to develop a system by which it would be possible to follow the growth of a group of cells over a period of several hours under conditions which could be kept strictly constant or which could be completely changed in a short time.

These requirements were satisfied by growing the cells in a perfusion chamber on the stage of a microscope enclosed in a thermostatically controlled box. The growth of the cells was recorded by time-lapse photography.

(ii) The Culture Chamber.

The design of the culture chamber was based on that of Christiansen et al.(1953). The body of the chamber is quickly and easily made from acrylic if a milling machine is available. However, this chamber had originally been designed for use with tissue culture cells, which, apart from being somewhat sticky, could also be held against the upper coverslip in a clot. Such a technique is not applicable to yeast cells.

Several methods of mounting were attempted. The eventual method used is illustrated in Fig.6. This was the only method which held the cells sufficiently still for photographic purposes, readily permitted exchange of nutrients and waste products by all cells, encouraged growth to take place in the plane of focus, and did not in any way damage the cells.

The procedure was as follows:-

A drop of the culture of cells was placed on the centre of a $2\frac{1}{2}$" by $\frac{7}{8}$" No.1 coverslip and the cells allowed to settle. Excess liquid was withdrawn with filter paper. An agar blanket was then made by dipping a $\frac{3}{4}$" square coverslip into molten $1\frac{1}{2}\%$ agar/

agar at 65°C., withdrawing it rapidly, and holding it horizontal until the agar had solidified. A thin strip of agar was then cut away from the edge and a drop of distilled water used to separate the agar from the coverslip. It was then possible to slide the agar off onto the cells on the other coverslip. Excess moisture was withdrawn by filter paper and then the edges of the 'blanket' were painted with liquid agar which to some extent sealed it to the coverslip. This coverslip, the celluloid grid (made from film strip), the main body of the chamber and the upper coverslip were then stuck together with vaseline and the edges of the coverslip sealed onto the body of the chamber with a molten mixture of paraffin wax and vaseline.

The assembled chamber was connected to the perfusion system as soon as possible.

(iii) The Perfusion System.

There were two nutrient tanks which could hold 100 mls. of fluid each. These fed the culture chamber by gravity. The system could be changed from one tank to the other by means of a tap at a T-junction near the chamber. The rate of flow was controlled by a screw clip between the T-junction and the chamber. There were no restrictions on the exhaust end of the system thus preventing a pressure build up in the chamber. The rate of flow was estimated by drop count at the exhaust end. The rate was generally kept at ½ drop/min.(.3ml./hr.) The exhaust was only permitted to form drops when the flow rate was being estimated as the pressure changes occurring upset the focus.

The agar blanket might be expected to delay the effect of a change of medium on the cells beneath it. As the diffusion rate is proportional to the square of the thickness, ten agar blankets were made in the normal way and their thickness measured by means of the fine adjustment of the microscope. The mean of these estimations was 229μ with a standard deviation of 28μ

All/

All the components of the system were kept sterile but once an experiment had started no further attempt was made to maintain sterility. No infection was ever apparent during the period of an experiment.

(iv) Temperature control.

The entire system, culture chamber, nutrient tanks and microscope, was enclosed in a thermostatically controlled 'hot box'. Provided the room temperature did not fluctuate excessively the temperature inside the 'hot box' could be controlled to within $\pm 0.3^{\circ}C$.

In the mounting procedure temperature shocks were avoided as far as possible by keeping the component parts of the culture chamber in an oven at $30^{\circ}C$. until required, and by having the 'hot box' turned on at least half an hour before mounting the cells. Furthermore, no recordings were made for at least two hours after setting up the system thus permitting the cells to equilibrate.

(v) Optical system.

As the cells were on the bottom of the chamber it was necessary to use a Prior inverting microscope.

A Baker phase contrast set was used, with an oil immersion 1mm. objective for maximum resolution in conjunction with a X4 eye-piece. However, even with such a low power eye-piece there were generally too few cells in the field. An additional convex lens of +4.5 dioptres was interposed between the objective and the eye-piece. The aberration in the field of the camera was negligible.

A Beck Tenslite was used as a light source and the beam was passed through heat absorbing glass and a Chance O Gr 1 green filter, which gives light of a suitable wave length for work with phase contrast. The intensity of the light was largely dictated by the requirements of the film being used for recording, but/

but, within these limits, it was kept as low as possible.

The agar used for mounting was twice filtered through Whatman No.1 filters. Albumen was not used for clearing as it appeared that the cells might obtain nitrogen from this source.

(vi) Recording and analysis.

Photographs were taken at intervals of three minutes on 16 millimetre film. The film was projected by means of a modified 32 millimetre Leitz Valoy enlarger fitted with a frame mover worked by a solenoid. Cell dimensions were measured on millimetre squared graph paper and the magnification was such that 1mm = .5μ

From these measurements it is possible to calculate volume and surface area. Thus Volume (V)

$$= \frac{\pi D^2}{12}(3L - D)$$

and Surface area (S)

$$= \pi DL$$

where D is the cell's diameter and L its length, and the shape of the cell is taken to be a cylinder with hemispherical ends.

B. OBSERVATIONS ON CELL POPULATIONS.

(i) Conditions of growth.

In a number of cases, it was necessary to conduct experiments on whole populations grown in test tubes. During such an experiment and for at least an hour before it, the cells were kept in suspension by frequent shaking.

The tubes were incubated in a water bath at 27.5°C. and only removed for very short periods for sampling.

(ii) Sampling.

The culture was very thoroughly shaken before sampling. When the same sample was required for more than one analysis, a measured quantity, usually .5ml., was withdrawn and added to an/

an equal quantity of 8% formalin. Wherever possible, however, this treatment was avoided as the formalin did not seem able to prevent many cells with cell plates completing division although growth was stopped altogether.

(iii) Counts.

A Burker double chambered, .1mm. deep, haemacytometer was used for population counts. Each chamber was filled from a separate sample. Distribution was good and about four hundred cells were counted. According to Student (1907) this should give an expected error of five per cent.

(iv) Measurements.

Measurements of length were made under oil immersion with a X 10 eyepiece containing a micrometer grid. Measurements were made to the nearest whole unit of the grid. At this magnification 1 grid unit = 1.25μ. At least fifty cells were measured. A tendency to select cells is inevitable in a crowded field and so samples for measurement were diluted sufficiently to permit measurement of all cells in the field.

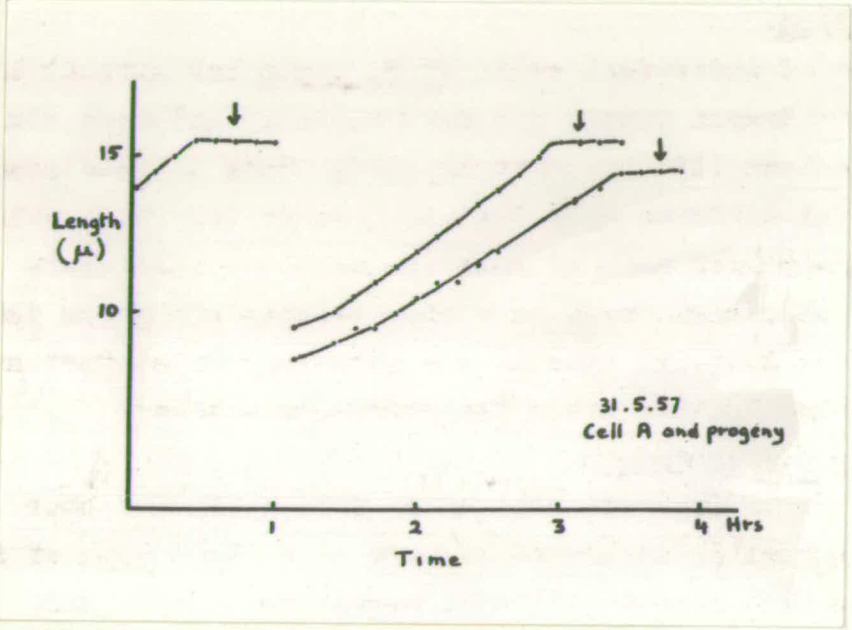

Figure 7. 'Normal' growth in volume of two sister cells of unequal sizes. First appearance of the cell plate indicated by arrows.

IV EXPERIMENTAL

A. NORMAL GROWTH.

(i) Introduction.

The growth of individual cells of S. pombe has already been investigated by Knaysi (1940) and more recently and more thoroughly by Mitchison (1957). However, both these authors used techniques which differed from the one used in this work notably in that no attempt was made to keep the nutrient conditions constant. As this might well have considerable effect on the growth curve and division rate it was necessary to conduct an independent investigation using the perfusion chamber.

(ii) Experimental procedure.

The cells to be observed were taken from a sixteen hour culture and mounted as described on page 18 . Perfusion of full strength medium (2% w/v "Oxoid" wort broth) was immediately started but the cells were then left for a further two hours to equilibrate before filming started.

(iii) Results.

(a) The Cell Cycle. The general form of the volume curve can be seen from Fig. 7 which shows the growth of a typical pair of sister cells.

It can be seen that the young cell starting at about 8.5μ in length grows in a continuous and more or less linear manner for the first four fifths of its life. At about 15μ it enters a 'plateau' stage at the end of which it divides. The cell plate first becomes visible about half way through the 'plateau' phase.

Volume increase stage. Although the period of volume increase superficially appears to be linear it is noticeable that at the beginning of growth, and sometimes at the end, there is/

is an acceleration. The growth curve over the period of volume increase was tested for linearity. Twenty-five curves were tested and only five of these were significantly linear (P = .05). The remainder, with one exception, were all significantly, or highly significantly, positively curved. The exception was just significantly negatively curved. Unfortunately it is not possible to obtain readings sufficiently precise to determine whether the curvature is a characteristic of the ends of the phase or whether there is constant acceleration throughout the phase.

The central vacuole can generally be seen during the growth phase but the optical system used for these experiments was not sufficiently good to detect the moment of its division. Mitchison (1957), however, has reported that it occurs shortly before the plate appears and it seems probable that the formation of the cell plate follows its completion.

Plateau stage and division. Although the cell plate does not become readily visible until sometime after the initiation of the plateau stage, it is reasonable to expect that its formation starts earlier and that fission occurs when it is complete.

If the cell wall thickness is assumed to be constant, the rate of growth of cell wall can be taken to be equivalent to the rate of growth of surface area. Calculating from the mean values shown in Table 1 the rate of increase of surface area during the growth phase is 4.87μ^2 per nine minute period. If it continued at this rate during the plateau period the area of wall formed would be 17.14μ^2. Two new end walls formed by a flat cell plate would have a surface area of 19.24μ^2. The mean observed difference in surface area between a parent cell and its offspring is 20.89μ^2. It would thus appear that no significant stretching of the cell wall occurs at division and that the rounding/

TABLE 1 .

Data from cells grown in 2% wort broth.

	Units	Mean	S.D.	N.
Initial length	μ	8.44	0.76	41
Final length	μ	15.09	0.95	42
Breadth	μ	3.5	-	42
Growth rate (length)+	μ/9mins	0.51	0.05	41
Plateau time	9 mins	3.52	0.87	21
Generation time	9 mins	18.58	1.81	24

+ This is the rate during the growth phase and is measured on the apparently linear portion of the curve.

TABLE 2 .

Calculated rates of increase from data in Table 1 .

	Rates of increase /9min period (growth phase)	
Length	0;443	μ
Volume	4.27	μ^3
Surface area	4.87	μ^2

rounding up of the new ends is not due to expansion but merely deformation of the existing wall. Furthermore, it should be noted that the rate of cell wall formation during the plateau phase is not significantly greater than the rate during the growth phase.

The change of shape at fission from two cylinders each with one hemispherical end, to two cylinders each with two hemispherical ends allows a small volume increase, which is, in fact, found. (Table 3)

In Table 3 the observed volume increase has been compared with the volume increase expected if growth in volume 'lost' during the plateau phase is made up at fission, and with the volume increase expected when cell wall synthesis continues at the rate characteristic of the single cell. The observed value is not significantly different from the value calculated when cell wall synthesis remains constant.

When a cell divides the two daughters spring apart. The shape and volume of the cells changes immediately on fission which suggests a high internal pressure.

(b) Rates of growth and division. The combined data from about forty cells is shown in Table 1 . The derived rates of increase are shown in Table 2 . The rate of increase in length in Table 2 is calculated as the difference between the initial and final lengths divided by the time spent in the growth phase. The figure for growth rate shown in Table 1 is obtained by direct measurement of the slope of the linear portion of the growth curve. The lower value of the calculated figure would suggest that the curvature is more marked at the beginning of the growth phase than at the end.

Variation. On analysis of twenty cells it was found that the division length of the cell was positively correlated with the initial length. The correlation is not excessively good/

TABLE 3.

Table to show that the observed change in overall length at division is best explained by a change of shape envolving no stretching of the cell wall.

	Length increase	Volume increase
Observed. (Mean)	1.9 μ	6.5 μ^3
Estimated		
(i) Daughters each half volume of parent	1.17 μ	--
(ii) Volume 'lost' during plateau made up on fission	2.72 μ	14.95 μ^3
(iii) Increase due to change of shape on division	1.75 μ	5.62 μ^3

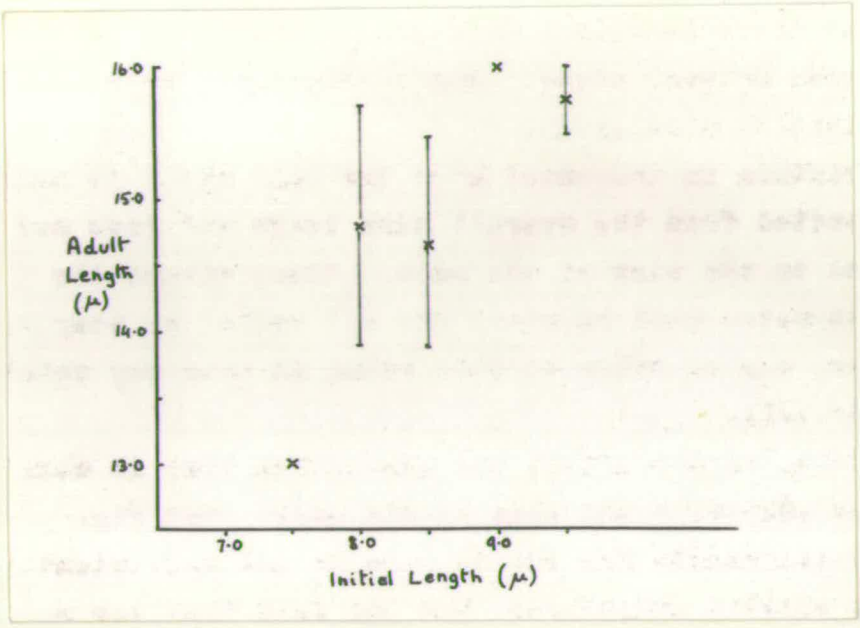

Figure 8. The relationship of the length of the cell at division to the initial cell length. Means of adult length shown with Standard deviations. Correlation coefficient = +0·45 (N = 20)

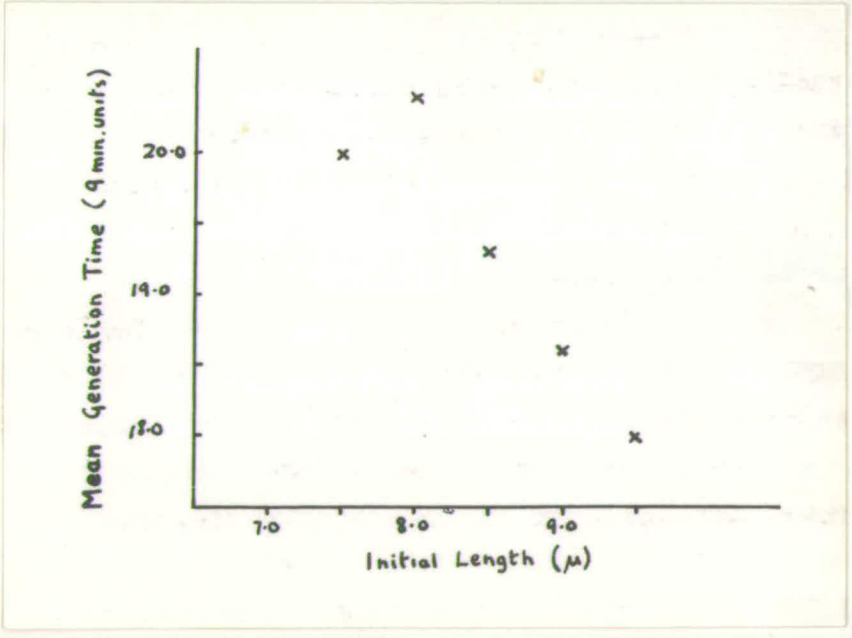

Figure 9. The inverse relation between generation time and initial length. Correlation coefficient = −0·603 (N=14)

good (P = .05) but there is only a difference of 2μ between the smallest and largest initial sizes. Fig. 8 shows the mean adult length for each initial size group.

Thus the variation in increment over the cell cycle is less than might be expected from the overall size range and does not seem to be related to the size of the cell. Thus, either the division andgrowth rates must be equal for all cells, or they must fluctuate together, one or other or both being in some way related to the size of the cell.

It can, in fact, be shown that the generation time is negatively correlated (P = .02) with the size of the cell. See Fig. 9 The accuracy of the measurements for growth rate is not sufficiently great to permit a similar comparison, but the fact that the generation time is related to the initial size must mean that the growth rate is also, as the increment is constant.

Variation between sister cells. Of ten pairs of sister cells only two pairs contained cells of the same initial size. The mean difference between two sister cells was .5$\mu$and the correlation was not significant. (coefficient correlation = .295). The correlation between sisters was more marked for adult sizes and was just significant (P = .1) probably because of the greater size range permitting more accurate measurement. (coefficient correlation = .55); mean difference .35).
The above coefficients are calculated without reference to sign. However, in forty-one cases cells which had been followed from one cell through two divisions, thus giving four cells in a row, it was found that the cells on the outside were larger (mean length 8.7μ) than those on the inside (mean length 7.9μ).

Measurements were also made of the position of the cell plate in the cell.

Approximately the same degree of inequality occurred as between the daughter cells. It was, however, noticeable, that the inequality/

TABLE 4.

Comparison of the size of sister cells relative to their position in a chain of four cells.

Description of pair	Number of pairs
Cells of equal length	7
Outer cell larger	57
Inner cell larger	4
Total	68

TABLE 5.

The position of the cell plate in a dividing cell when the cell is in contact at one end with a sister cell.

Position of cell plate	Number of cells
Central	10
Displaced to end in contact	42
Displaced to free end	2
Total	54

inequality of length was counter-balanced by a slight difference in diameter in the two halves of the cell. The difference was too small to be satisfactorily measured but would be sufficient to make up the difference in volume between the two halves. However the cells do not get progressively thinner and thinner, so the diameter must be increased shortly after division.

Efficiency of system. The validity and significance of any data on the normal cell cycle is dependent on whether or not the conditions are maintained constant. In other words, the parameters measured for one generation should not be significantly different from those of the succeeding generation. Table shows that this is, in fact, true for this system.

Direction of growth. Although, for most purposes, the cells may be considered to have a constant diameter, the surface is, in fact, sufficiently uneven to give each cell a characteristic outline. This made it possible to trace the outline of a young cell, and by fitting it to the fully grown cell, identify that portion which is new. This determination was facilitated by the tendency of the original portion of a cell to remain anchored. All cells were found to grow at one end only for the majority of the growth period although a few showed slight growth at the other end shortly before the plateau stage. In three cases out of those examined it was not possible to tell at which end growth was taking place.

When growth in length started again after a division, it was invariably the old ends which showed the growth.

TABLE 6 .

Comparison of 1st. generation with 2nd. generation parameters.

		Units	Mean	S.D.	N.
1st. generation	(adult length	μ	15.00	.84	17
	(growth rate	μ/9min.	.52	.053	14
2nd. generation	(adult length	μ	15.16	1.0	25
	(growth rate	μ/9min.	.50	.037	25

TABLE 7 .

Observations on the region of growth.

Growing region	Number of Cells
One end	39
Both ends	2
Indeterminate	3
Number examined	44

B. GROWTH IN DILUTE MEDIA.

(i) Introduction.

In the previous section the growth of the cells under more or less optimal conditions has been reported. The next step is to see how these same cells behave when they are grown in media containing nutrients in the same proportions but in considerably lesser quantities. When the concentration of nutrients is kept at a level which prevents the cells from fulfilling their normal functions at their full rate are all the cells' activities curtailed or is one affected before another?

(ii) Procedure.

Pilot experiments were run in test tubes at various dilutions. Dilutions used were ½, 1/5th, 1/10th, 1/15th, and 1/20th of the normal concentration. The cells of the inoculum were washed in distilled water before inoculation and the same culture was used to inoculate all dilutions. Samples were taken at intervals of fifteen minutes starting fifteen hours after inoculation.

Single cell estimations were made in the same way as for normal cells. The cells were taken from the log phase of the appropriate dilution, mounted in the chamber and perfused with the dilute nutrient at the usual rate.

(iii) Results.

(a) Effect on populations. Dilutions down to a concentration of .2% w/v wort broth had virtually no effect on the generation time. Below this concentration the generation time increased rapidly though below the .1% level test tube conditions do not permit the cells to grow logarithmically sufficiently long to make a reliable estimate of generation time.

As the concentration is decreased the efficiency of conversion of/

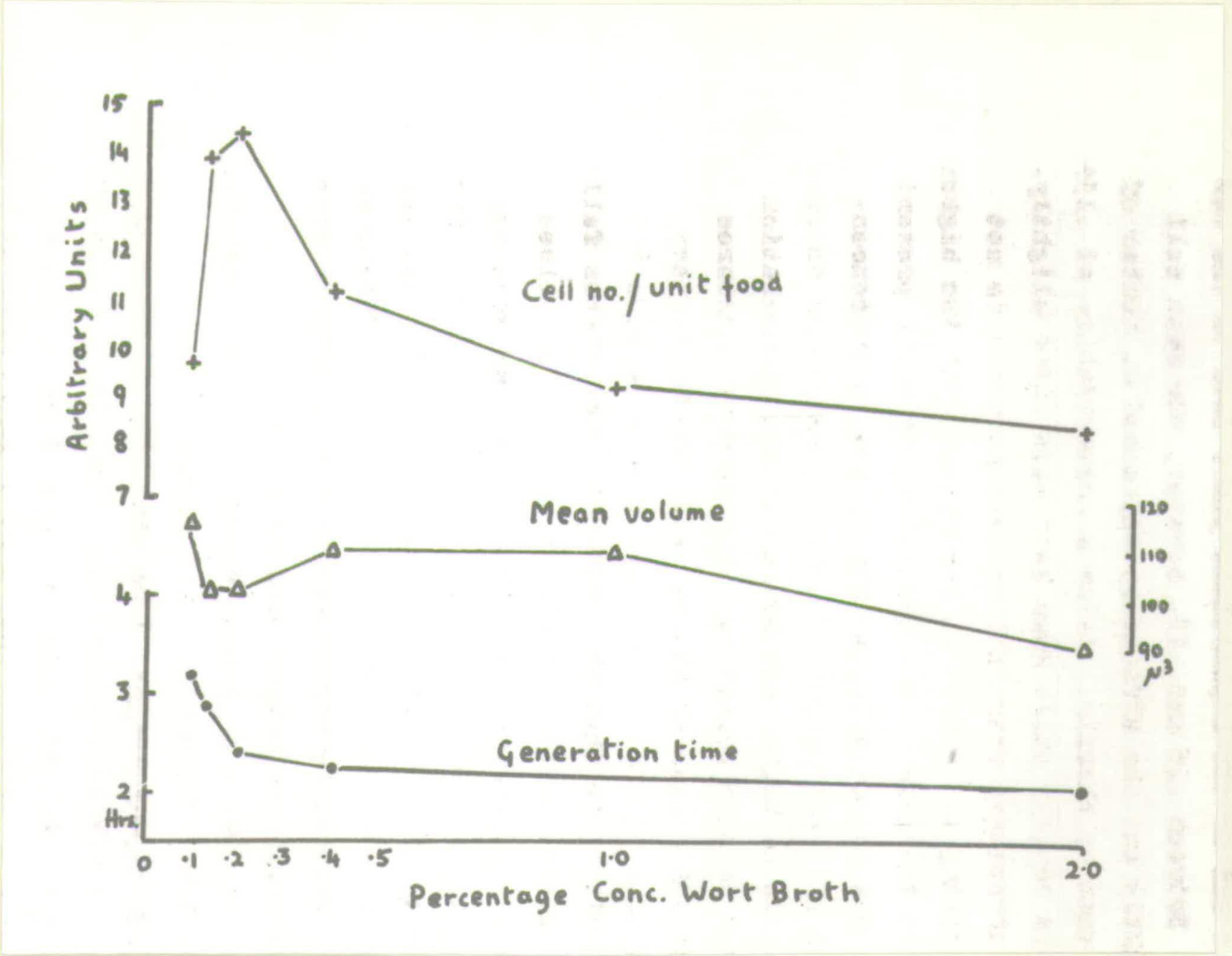

Figure 10. The effect of various concentrations of wort broth on the mean generation time of cells grown in culture. The effect on the mean volume of the cells at the end of the logarithmic phase of culture growth, and the efficiency of convertion of food in terms of cell number is also shown.

of food to living matter gradually increases until the concentration falls below .2%. This increase in efficiency is shown both as an increase in cell number and as an increase in mean cell size at the end of the logarithmic phase down to the .4% concentration. Between .4% and .2%, however, the mean cell size falls slightly but the efficiency expressed as number of cells produced goes up sharply. Below a concentration of .13% cell number falls rapidly while mean cell size rises slightly.

The rising efficiency with falling concentration is not altogether surprising as it must be remembered that the higher the concentration the greater are the number of cells present per unit volume and it may well be that at the lower concentrations lack of food acts alone in bringing about the cessation of growth, but at higher concentration the accumulation of waste products is also effective. Furthermore, cultures grown at low concentrations of medium reach the stationary phase first and thus have a smaller maintenance requirement.

It is also noticeable that the mean cell size starts falling before the generation time starts to increase but rises again at the lowest concentration. This latter increase may be due to the cells having insufficient time to 'recover' from the lag phase, but the fact that the mean size falls when the generation time is still constant and the over all efficiency is in fact still increasing would suggest that growth is more susceptible to adverse conditions than is division.

It appeared that the most useful concentration for use for single cells would be 1/15th normal dilution.

(b) Effect on single cells. A dilution of 1/15th normal concentration (.13% w/v wort broth) was used. The results of growing the cells at this dilution are summarized in Table 8 . The generation time is slightly less than twice as long as in full strength. The growth rate, on the other hand, is rather less/

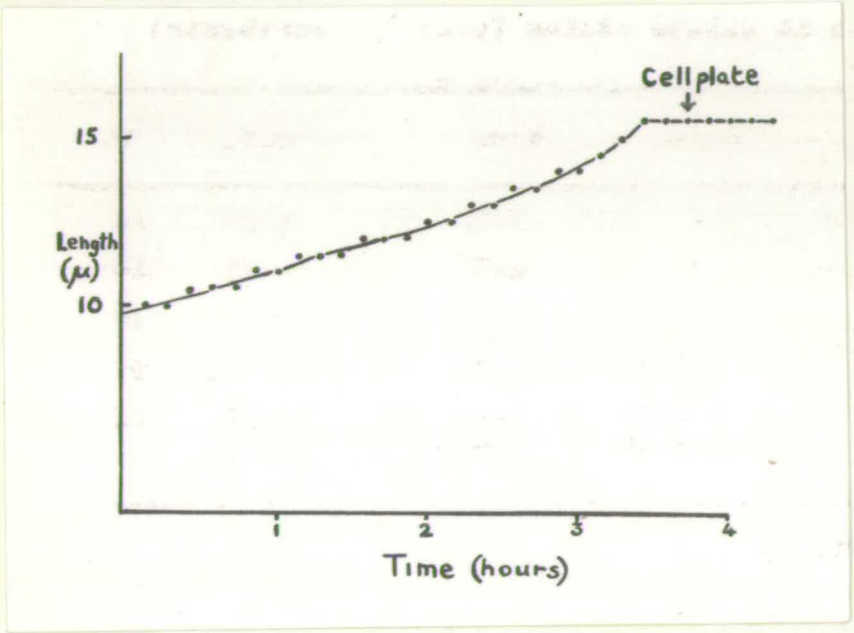

Figure 11. The growth in volume of a cell in 0·13% w/v wort broth.

TABLE 8.

Data from cells grown in dilute medium (0.13%w/v wortbroth)

	Units	Mean	S.D.	N.
Initial Length	μ	7.89	1.32	14
Final Length	μ	14.38	1.79	16
Breadth	μ	3.5	-	16
Growth rate (length)+	μ/9 mins	0.214	0.012	22
Plateau time	9 mins	5.83	0.92	12
Generation time	9 mins	32.00	4.12	11

+ See footnote for Table .

TABLE 9.

Percentage of Generation Time spent in plateau phase

	%	S.D.	N.
Cells in full strength medium	19.29	3.78	20
Cells in dilute media (0.13%w/v wort broth)	18.24	1.60	7

less than half that in full strength with the resulting slight drop in division size. The variability of all these parameters is much greater than for cells grown under 'normal' conditions.

The general characteristics of the curve do not alter; they are merely extended in time. Thus, the percentage of time spent in the plateau phase does not change significantly (See Table 9). This lends strength to the argument that the plateau phase is regulated by the same growth mechanisms as the growth phase.

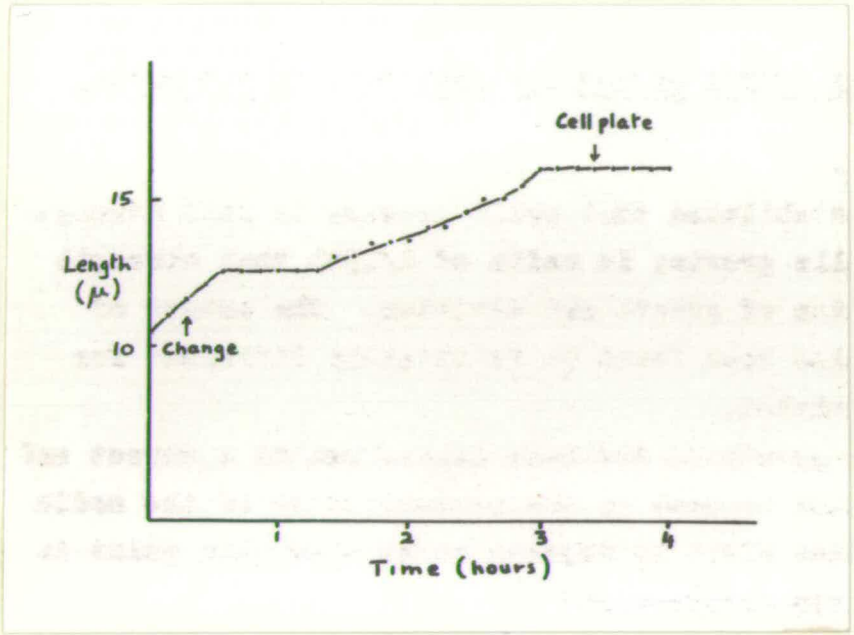

Figure 12. The effect of a change from 2·0% w/v wort broth to 0·13% w/v wort broth on the volume growth of the cell.

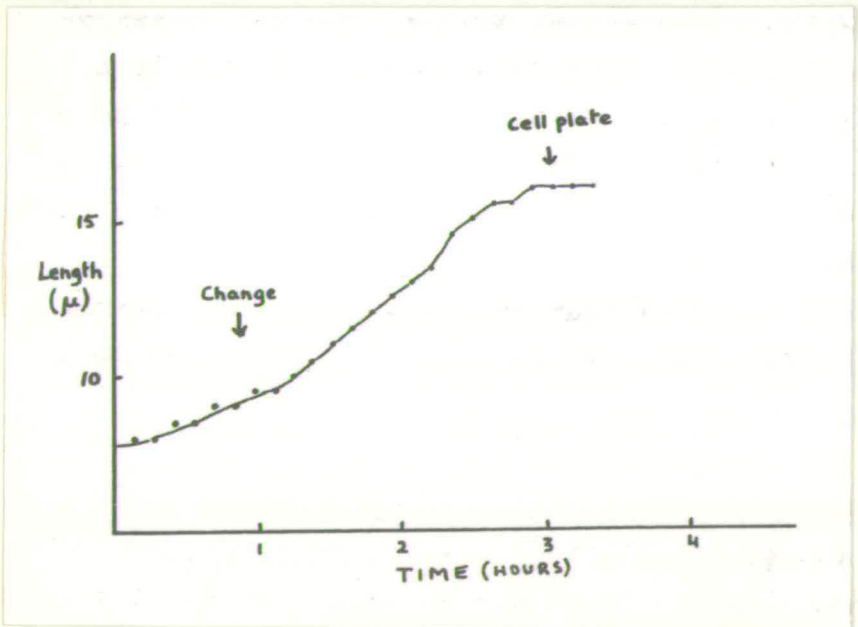

Figure 13. The effect of a change from 0·13% w/v wort broth to 2·0% w/v wort broth on the volume growth of the cell.

C. EFFECT OF A CHANGE IN THE CONCENTRATION OF THE MEDIUM.

(i) Introduction.

It has been established that cells growing in full strength wort broth and cells growing in media of 1/15th that strength show different rates of growth and division. The extent of this effect has also been found to be slightly different for growth and for division.

Is the slower growth in the more dilute medium a direct and immediate limitation imposed by the concentration of the medium, or does the organism adapt by degrees or at a certain point in the cycle to the new environment?

(ii) Procedure.

The cells were mounted in the usual way and allowed to equilibrate in the first medium. When it was required to change the medium the control tap was fully opened for one minute which was sufficient to clear out and replace the original medium. The normal rate of flow ($\frac{1}{2}$ drop per minute) was then re-established. When the change was from full strength medium to a lower concentration it was arranged to take place twenty hours after inoculation. In the reverse case cells were inoculated from a normal growing culture into the dilute medium and allowed to grow for ten hours before they were mounted, still in the dilute medium, in the culture chamber. They were then permitted the usual time to equilibrate before any further changes were made.

(iii) Results.

(a) Change from full strength to 1/15th normal concentration.

Any apparent change of behaviour due to the change of medium did not appear immediately. There was a lag of between 2 x 9 and 5 x 9 minutes. This can be accounted for as being the time for the medium to penetrate the agar blanket.

The/

TABLE 10

Data for cells changed from full strength medium (2%) to $^{1}/_{15}$ concentration (.13%).

A. Cells with separate induced and division plateaux.

	Units	Mean	S.D.	N.
Initial Growth Rate	μ/9min	0.50	.046	19
Time from Change to Effect	9 min	2.42	1.02	21
Induced plateau	9 min	6.5	2.0	16
Growth rate after plateau	μ/9min	0.26	0.09	16
Length at division	μ	15.48	0.75	7
Division plateau	9 min	6.85	1.83	7

B. Cells with one long plateau only.

Cell	Time from change to plateau (9min.)	Length at Division (μ)	Plateau time (9 min.)
$9.4.D_1$	3	16.0	12
$9.4.D_2$	2	15.0	12
$15.3.C_1$	4	14.0	12
$15.3.C_2$	5	12.5	9
$28.3.A_1$	2	17.0	10
$28.3.A_2$	3	15.5	12
$28.3.C_1$	1	15.0	15
$28.3.C_2$	1	13.5	14
$28.3.D_1$	0	17.0	11
Mean		15.05	

The variability of this time was less marked for any one experiment and is probably due to the variation of the thickness of the agar blanket from one experiment to another.

At the end of this lag period the growth in volume ceased altogether for a while. The length of this growth plateau is somewhat variable but there is no correlation with the size of the cell at which the plateau is induced, and so it is probably merely a reflection of the inherent variability of the cells.

At the end of this induced plateau growth resumed at a rate characteristic of growth in the new medium and division eventually occurred at the normal size. It would thus appear that both growth and the build up for division is inhibited over the period of the induced plateau. The length of the division plateau was similarly characteristic of the rate for the dilute medium. In a number of cases the induced plateau was followed by division without further growth. This, however, only occurred when the cells had reached a size at which they would be dividing in any case and the plateau was long enough to account for both the induced and the division plateaux.

(b) Change from full strength to 1/10th and 1/20th normal concentration. When the concentration of the new medium was .2% w/v wort broth (1/10th normal concentration) there was very little change in rate and the induced plateau, when it occurred, was very short.

At a concentration of .1% w/v wort broth (1/20th normal concentration) the plateau was longer than at 1/15th normal concentration and the new growth rate slower. The values for the growth rate (Table II) are, in fact, directly proportional to the concentration and the length of the induced plateau is more or less inversely proportional to the concentration.

(c) Change to 1/15th normal concentration with Xylose added. In/

TABLE 11

Comparison of the length of the induced plateau and the new rates of growth of cells changed from full strength medium (2%) to various dilutions.

	Conc.	Mean	S.D.	N.
Induced plateau (units of 9 mins.)	.20%	2.57	2.59	7
	.13%	6.50	2.00	16
	.10%	11.72	1.86	11
	.13% + xylose	6.42	1.82	8
New rate of growth (μ/9min)	.20%	0.40	0.04	8
	.13%	0.26	0.93	18
	.10%	0.19	0.63	11
	.13% + xylose	0.28	0.80	7

TABLE 12

Data for change from dilute medium (0.13%) to full strength medium (2.0%)

	Units	Mean	S.D.	N.
Initial Growth Rate	μ /9min	0.21	0.11	9
New Growth Rate	μ/9min	0.52	0.05	9
Length at Division	μ	16.1	1.20	9

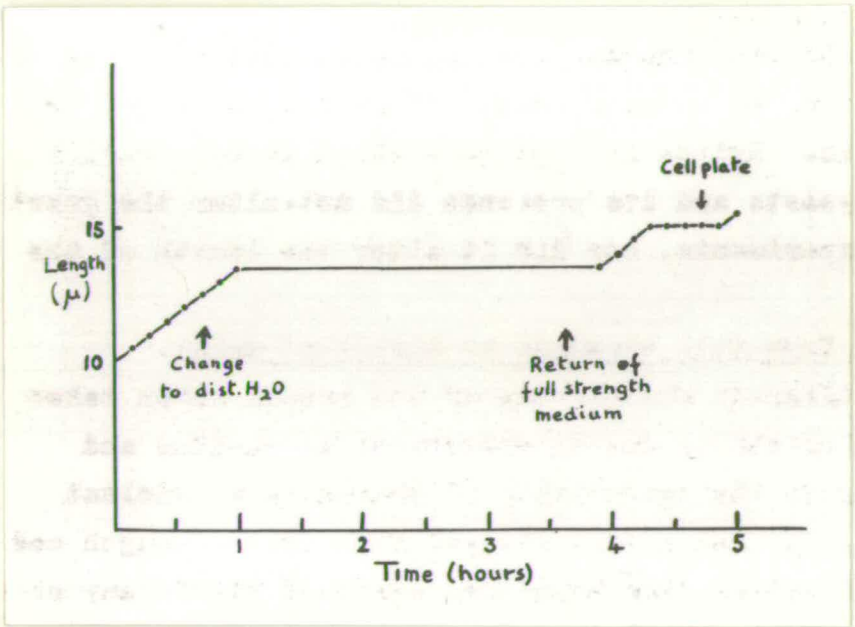

Figure 14. The effect of distilled water on the volume growth of the cell.

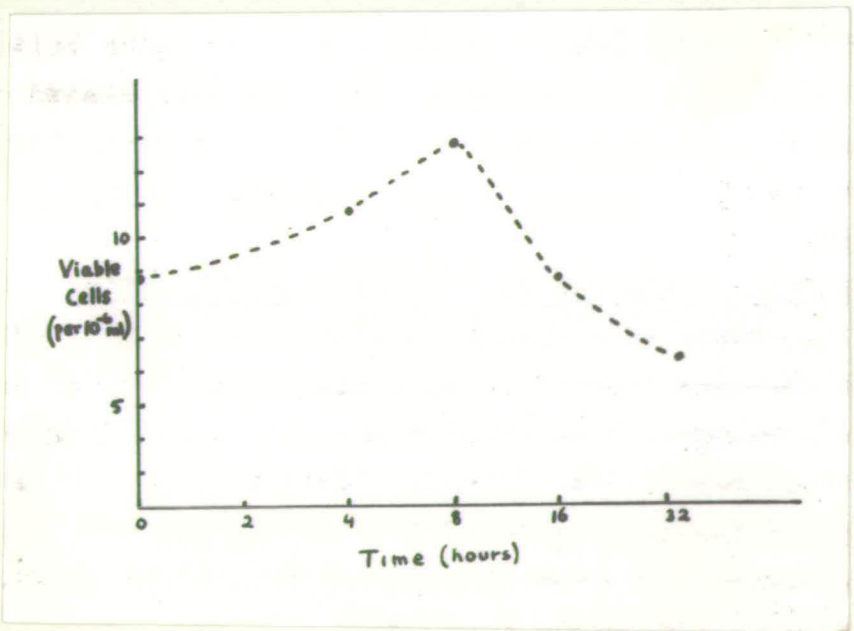

Figure 15. The effect of various periods in distilled water on the viability of the cells. Time is plotted logarithmically.

In order to discover whether the induced plateau was due to the cells receiving an osmotic shock, 2% xylose was added to the dilute medium. Xylose is a pentose which is not readily metabolized by yeasts and its presence did not alter the growth rate in these experiments, nor did it alter the length of the plateau period.

(d) Change from full strength to distilled water.

In order to discover whether any of the growth which takes place at low dilutions is due to endogenous mechanisms and also to investigate the sensitivity of the cells to violent osmotic changes, the cells were changed from full strength medium to distilled water. The usual lag occurred before any change became apparent, after which the cells ceased growing. There was no further growth and division only occurred in those cells which were in any case fully grown. If full strength medium was returned, even after a period of three hours in distilled water, growth resumed, after the usual short lag, at the full rate.

These results were confirmed by washing a twenty hour culture and transferring it to distilled water. Samples were plated out on nutrient agar at intervals thereafter. It can be seen from Fig.15 that the viability count does not start falling for at least eight hours.

(e) Change from dilute medium to full strength medium. If the plateau which occurs on a change from full strength to dilute medium is an adaptational period of some kind, one might expect a similar plateau to occur when the reverse change is effected.

In the event no such plateau occurs. Growth is resumed almost immediately at the full rate. This rate is uniformly high (.52μ per nine minutes), but does not differ significantly from the normal rate in full strength wort broth.

D. EFFECT OF A CHANGE IN THE BALANCE OF THE MEDIUM.

(i) Introduction. While it is true that energy can be obtained from nitrogenous sources, and, in the yeast cell at any rate, much structural carbohydrate is required for growth, it is none the less true that energy will mainly be provided by carbohydrate sources and nitrogen is essential for growth even if it is merely required for membrane and enzyme formation. Furthermore, although division will undoubetedly require nitrogen for the formation of DNA its demands on the energy supply may well be greater in proportion.

Nevertheless, if the total nitrogen available to the cell is divided in a constant proportion between division and growth, limitations imposed upon the nitrogen supplies should produce a slow growth and a slow division rate irrespective of the amount of energy provided. Similarly, if division is in some way dependent on growth the rate of division will remain proportional to the rate of growth.

There is an indication, however, from the effects of dilute media at critical levels (see page 29) that the two rates do not remain proportionally related. This might be due to a slight unbalance of nutrients in the medium which would be expected to manifest itself only at critical concentrations.

If, then, the cells are grown in a deliberately unbalanced medium any such tendency for the two rates to dissociate should become clearly apparent.

(ii) Results.

(a) Change to dilute media with glucose added. The cells were grown in full strength medium and changed over in the usual way to dilute medium (1/15th normal strength) enriched with 2% w/v glucose.

In the majority of cells, growth continued at the same rate as before until the division plateau was reached. The length of this plateau was rather variable but in all cases the growth of each of the two daughters after fission proceeded at about half the rate of the/

TABLE 13

A. Cells growing normally until division after change to dilute (.13% w/v wort broth) medium + glucose (N = 22).

Mean growth rate before division	0.518 μ/9 min
Range of increment before division plateau	1.5 - 6.0 μ
Mean rate of growth of daughter cells	0.240 μ/9 min

B. Cells changing growth rate before division after change to dilute (.13% w/v wort broth) medium + glucose (N = 9)

Mean growth rate before change occurs	0.505 μ/9 min
Range of increment before rate changes	3.5 - 6.0 μ
Mean rate of growth after change	0.300 μ/9 min

the parent. This rate does not differ significantly from that of cells grown in medium of 1/15th normal concentration (see page 29).

The remainder of the cells, some third of the total, showed a change of rate before division. These were cells which were young and small when the medium was changed and in nearly all cases had continued to grow normally for at least an hour after the change. The point of change of growth rate in these cells was not, however, so clearly marked as in the straightforward change from full strength to 1/15th normal concentration. The induced plateau appeared to be short or non-existant. In two cases the change to the new rate was gradual.

Four cells showed no change of growth rate during the period of the experiment.

It seems probable that the division plateau is masking the point at which the change of rate occurs in those cells which grow normally until division, as the period of time spent by those cells in the plateau spans the point in time at which the other cells from the same experiment show a change of rate.

(b) Change to Nitrogen free medium.

Effect on single cells. As the results presented in the previous section were somewhat confused and thus difficult to interpret, it was decided to remove all the nitrogen from the medium and to observe the cells over a longer period. The medium used consisted of glucose and salts made up in the proportions shown on page 17 but with all nitrogenous materials omitted.

The effect of this change showed in two phases. During the first phase growth continued more or less as before but eventually the cells ceased to increase in length, and the second phase consisted of a period of division without growth. Finally, division also ceased.

The length of time for which a cell continued to grow varied from experiment to experiment and also from cell to cell within one experiment. Variation within any one experiment was considerably less than the/

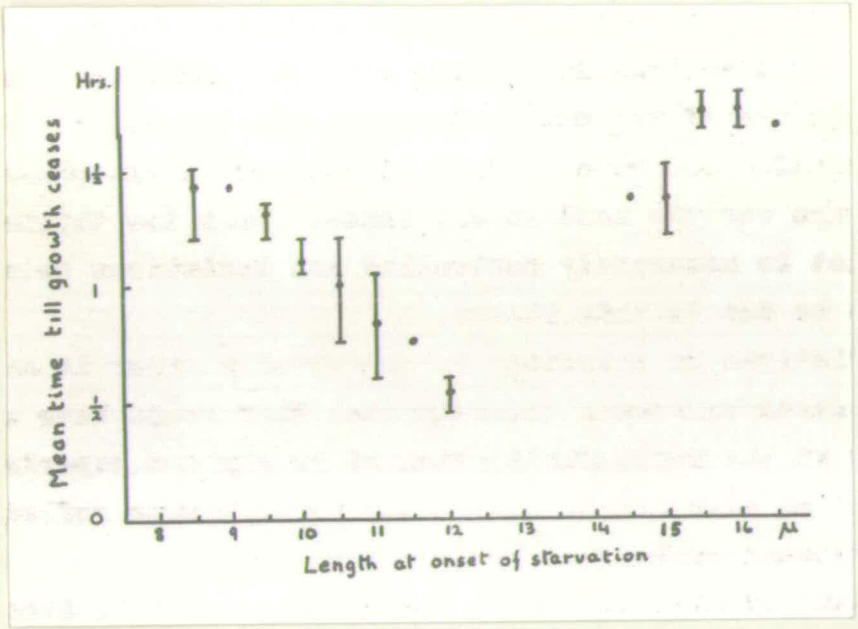

Figure 16. The effect of the size of the cell when Nitrogen starvation commences on the time for which volume growth continues. The range of times for each size is indicated.

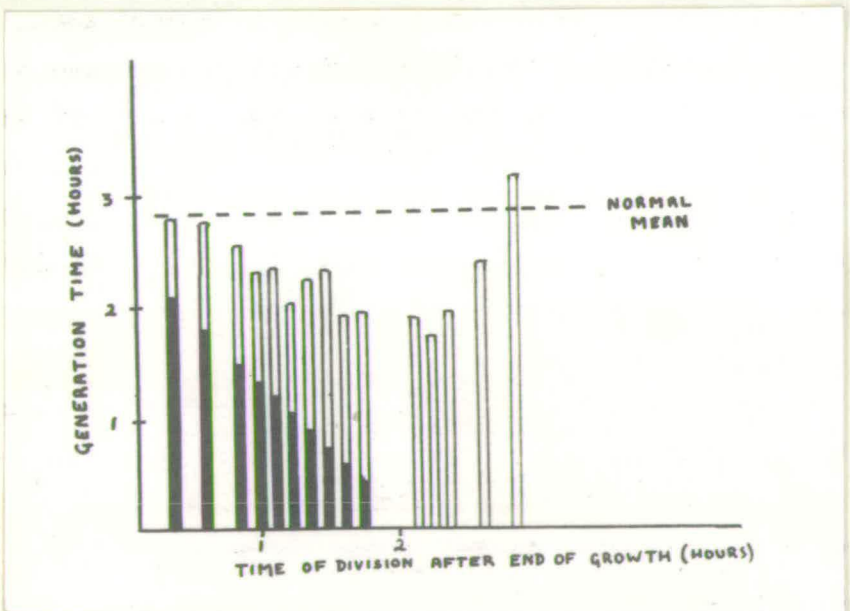

Figure 17. The generation time of cells after growth in volume ceases. The shaded portions of the generation time represents that part of the generation time spent in the period of continued growth.

the overall variation. Thus it must be assumed that the environmental conditions were not identical in all experiments. Care had been taken to ensure that the age of the cells since inoculation was constant, that the concentration and rate of flow of the medium was constant, and the temperature was the same in all cases. Only the thickness of the agar could not be accurately controlled and variations between experiments must be due to this factor.

Although variations in thickness do undoubtedly occur in any one agar blanket it seems extremely unlikely that this would have any effect on the cells in the small field observed in any one experiment. Variations within an experiment, then, must be a genuine reflection of differences between cells.

Cells of a similar size at the time when the medium is changed are found to continue growing for similar lengths of time. In figure 16 the experiment with the shortest lag before growth ceased is taken as a standard and the other experiments were compared with it by finding a cell in each which, at some point in its growth was of the same size, and, from the point at which it reached that size, had the same lag before growth ceased as had a cell in the standard experiment. This should eliminate any relative difference between experiments due to the agar blanket. As can be seen the length of the lag is inversely proportional to the size of the cell with the exception of the very largest cells which have an even longer lag than the smallest. It would thus appear that the fully grown cell is, in fact, behaving as two very small ones.

In spite of this, however, all the cells do not stop growing at the same size as those cells which continue growing for the longest time also grow more slowly.

After growth ceased all the cells underwent one further division. In one or two instances a second division may have occurred.

The timing of the divisions which occurred during this phase was interesting. The generation time got progressively less as the proportion of the life cycle spent in the phase increased, although it increased again fairly rapidly once the period spent in the phase extended/

TABLE 14

Comparison of the time at which growth ceases after the change to N -free medium in three experiments (units of nine minutes).

	Mean	Range	N.
Experiment 28.11	12.25	9 - 16	8
Experiment 7.10	19.72	15 - 25	11
Experiment 12.11	23.33	22 - 26	8

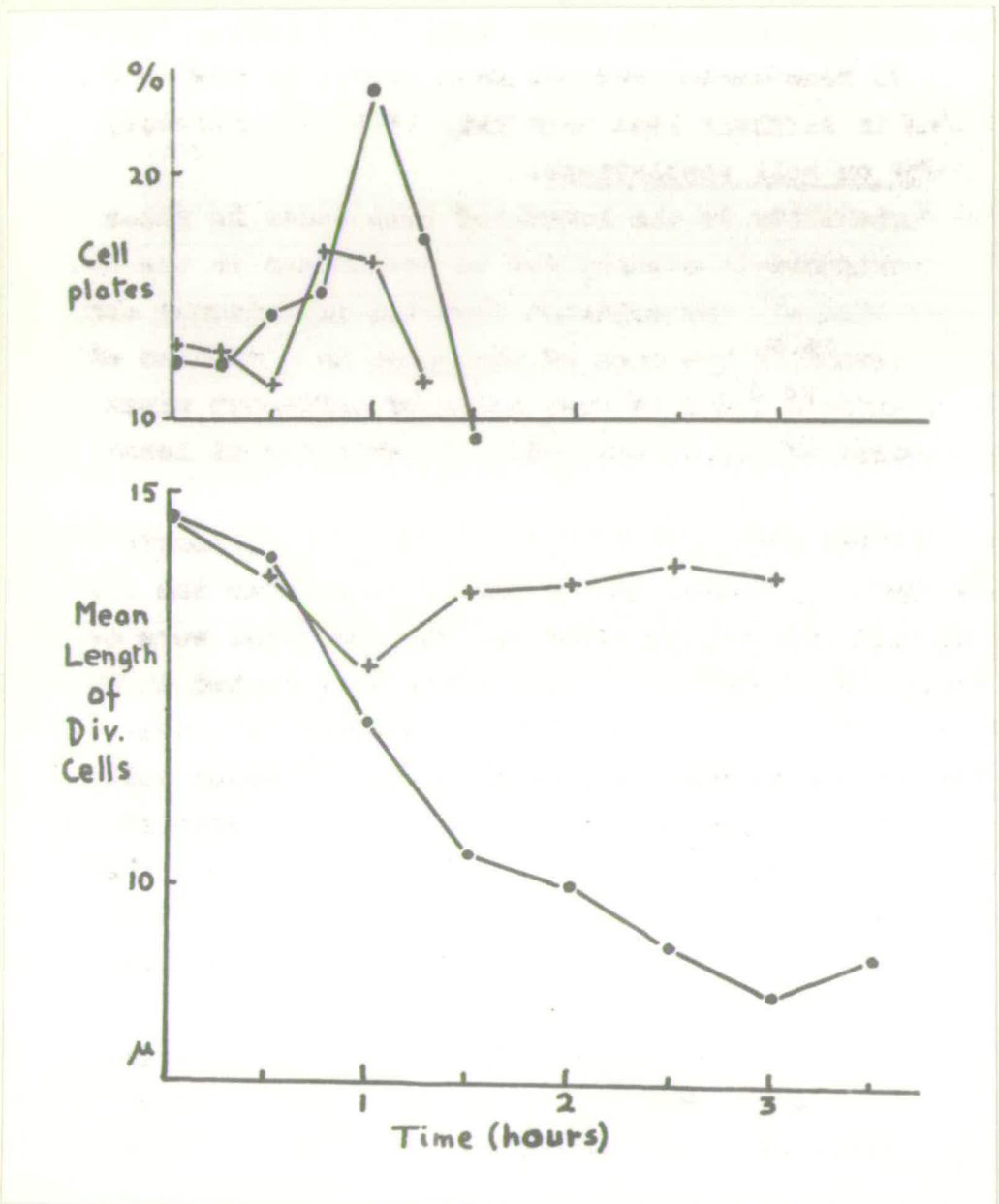

Figure 18. Effect of a change to Nitrogen free medium on the size of the dividing cells in culture (•). A certain degree of synchrony of division is produced by the change. A slight decrease in the mean size of the dividing cells is also found in the controls (+) followed by a small wave of divisions.

tended to include part of the parental cycle also. See (Fig. 17). It is also interesting to notice that the generation time does not start falling until the cell has spent about 5 X 9 minutes in the phase. It will be remembered that the mean length of the normal division plateau is slightly less than this (3.5 X 9 minutes).

Effect on cell populations.

While the variability in the length of time spent in phase 1 in different experiments is clearly due to variations in the thickness of the agar blanket, the evidence from the experiments above does not really determine how much of the phase is a genuine effect, although the variations found between cells of different sizes show that for the smaller cells, at any rate, it lasts for at least an hour.

In order to check this, cells were grown in full strength wort broth for the normal 16 hours, at the end of which time the shaken culture was divided into two portions and both portions were centrifuged, the supernatant removed and the cells twice washed in distilled water at 27.5°C. The cells from one portion were then incubated in 10 mls. of the nitrogen free medium and the other cells in 10 mls. of full strength medium. They were then each sampled immediately and also at intervals of half an hour thereafter. Measurements were made of the mean size of the dividing cells. It can be seen that the sizes of the dividing cells in the nitrogen free medium started decreasing after half an hour but the fall cannot be considered significant for the first hour as the size of the dividing cells in the full strength medium falls in a similar manner. After an hour, however, the dividing cells in the Nitrogen free medium are considerably smaller than the controls.

The decrease between half an hour and one hour in the control can be shown to correspond to a slight degree of induced synchrony. As in all cases this synchrony appeared at the same time after the change of medium it cannot be due to the normal cycling of division which has been shown to occur in culture growth. (Harnden 1957. See also Fig. 1) but must be due to some shock sustained during the centrifuging and washing./

washing. The synchrony of the starved cells is considerably more marked as is to be expected in view of the increased rate of division found after growth ceases.

Thus we can conclude that the lag before growth stops, which was found in the observations on the single cells is, in fact, a genuine result and it is probable that all the cells continue growing for at least half an hour after the change of medium.

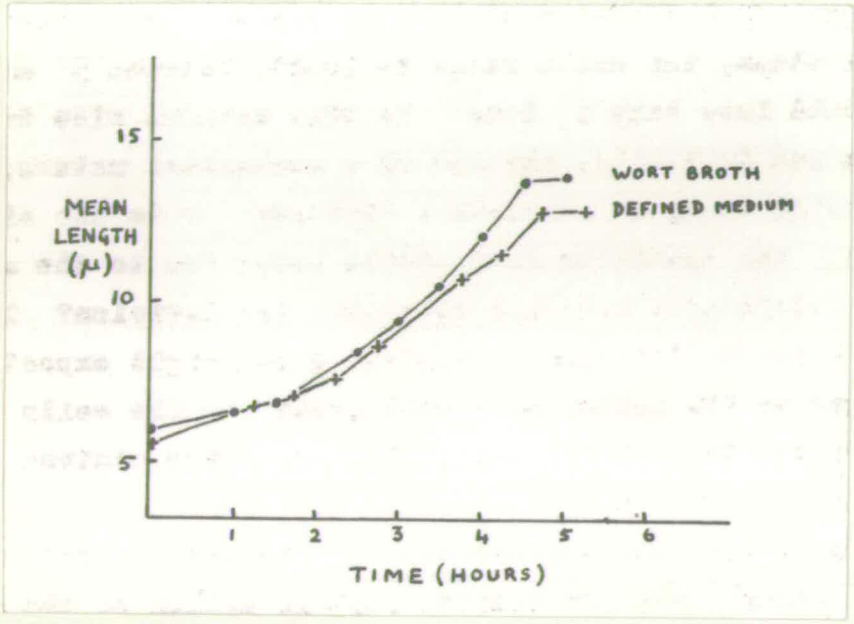

Figure 19. Effect on the mean size of the cells of the return of Nitrogen to starved cells in culture.

E. EFFECT OF A RETURN TO A BALANCED MEDIUM AFTER NITROGEN STARVATION.

(i) Introduction. When division eventually ceases due to the effects of Nitrogen starvation, the cells range in length between 5μ and 9μ. Cells are not found less than 5μ long. Is this minimum size due to some size determined inability, perhaps of a mechanical nature, of a cell of less than 10μ long to complete a division, or is the size itself irrelevant, the cessation of division being due to the cell being unable to obtain some material essential for division? If some mechanical block to division is operating one might expect the return of Nitrogen to the medium to permit growth of the cells to proceed uninterrupted by division until they reach the minimum size at which division is possible.

It would appear from the experiments on Nitrogen starvation which involved a change from the complex peptone medium to the simple Nitrogen free defined medium, that the agar blanket acts as a differential barrier to diffusion thus retaining the large nitrogen molecules in the region of the cells. This makes estimations of the effective time of the change of the medium unduly complex and so to study the effect of replenishment after starvation it was decided to return the nitrogen in the form of Ammonium sulphate, which diffuses readily through the agar.

(ii) Results.

(a) Effect on populations. The medium used throughout the replenishment experiments was identical with that used for starvation with the exception of the 2.0 gms. per litre of Ammonium sulphate added to provide a nitrogen source.

In the initial investigations on the effect of replenishment parallel experiments were run, one culture having full strength wort broth returned to it, and the other the defined medium. A culture which had been starved of nitrogen over night was divided into two equal parts, each was centrifuged, and the supernatant removed, and 10 mls. of wort broth added to one part, and an equal volume of defined/

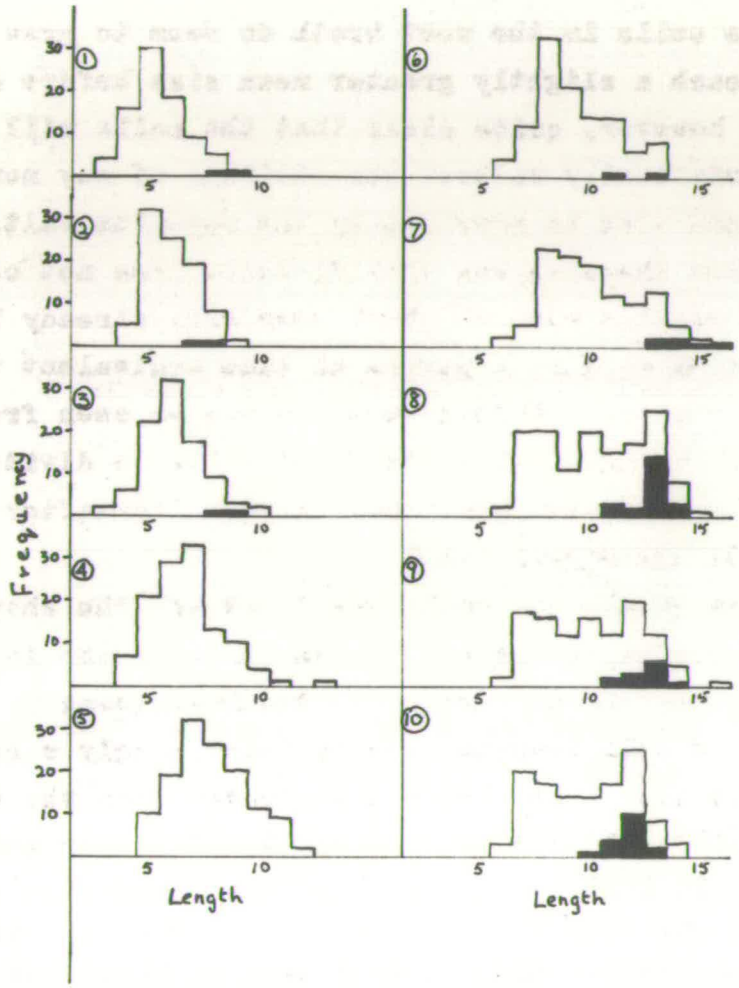

Figure 20. Distribution of cell lengths during Nitrogen replenishment at intervals of forty five minutes after Nitrogen returned. One unit of length equals 1·25μ.

defined medium added to the other. Samples were taken at intervals and the mean size of the cells determined.

As can be seen from Fig.19 the two media do not differ greatly in their effects. The cells in the wort broth do seem to grow slightly faster and reach a slightly greater mean size before division occurs. It is, however, quite clear that the cells will grow and divide quite satisfactorily without the addition of any more complex form of nitrogen than is provided by the ammonium salt.

It is apparent from these curves that division does not occur either when the cells reach a size at which they have already been found capable of division or when a period of time equivalent to a normal generation has elapsed. Furthermore, it can be seen from the cell size distribution in Fig.20 that the first cells to divide are considerably larger than the normal division size. Thereafter there is a gradual decline to the normal level.

If the growth rate of all the cells was identical the shape of the curve in Fig.19 would be equivalent to the shape of the individual growth curve. However, it is clear from the histograms in Fig.20 that the distribution of cell lengths becomes increasingly skewed and this must mean that the larger cells grow faster than the smaller ones. It is not possible, therefore, to determine from the mean length whether the growth of the individual cells is exponential or whether it consists of two or more periods of linear growth, the point at which the rate changes varying from cell to cell. It should be noticed, however, that both curves have an initial period of linear increase lasting slightly more than two hours in the defined medium and rather less in wort broth.

(ii) (b) Observations on individual cells. When the cells had been starved of nitrogen overnight and then replenished with medium containing ammonium sulphate there followed a period lasting about one and a half to two hours during which time growth was exceedingly slow. This has been called the lag period.

At/

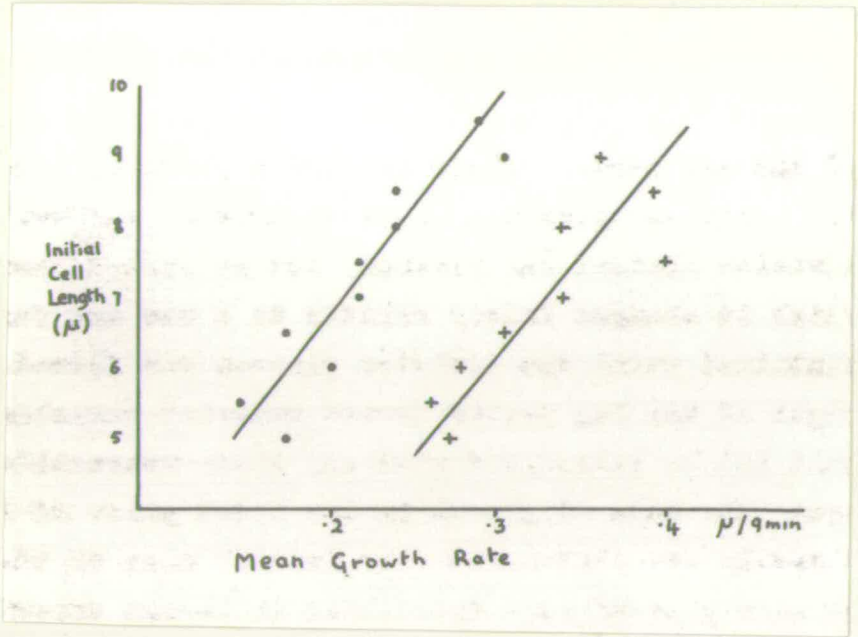

Figure 21. Mean growth rates for each initial cell size in first 'linear' phase (•) and second 'linear' phase (+) after return of Nitrogen.

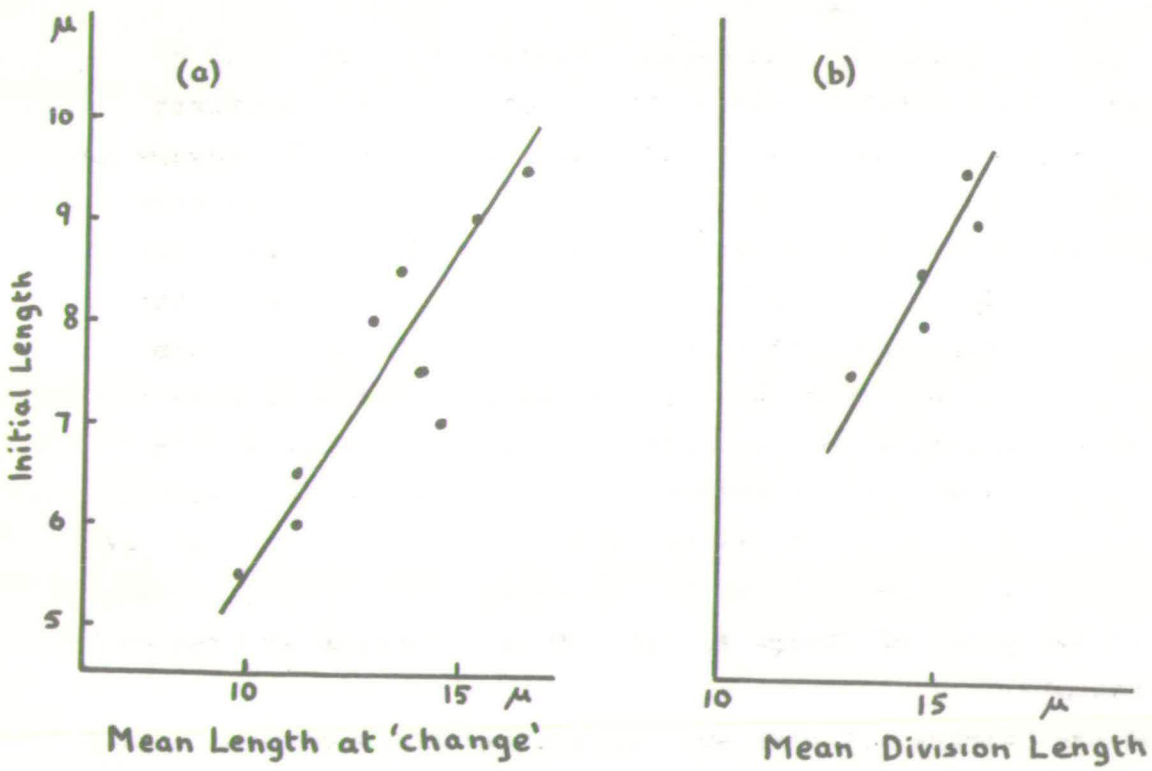

Figure 22. (a) The mean length of the cells at the time that the growth rate doubles. (b) The mean division length for 'normal' cells of different initial sizes. Regression

At the end of the lag period growth entered a phase of linear increase in length. In some cases this rate of growth remained constant until the division plateau was reached, but in others there came a point at which it changed fairly rapidly to a new and faster rate which was maintained until the division plateau was formed.

While the length of the lag period seemed somewhat variable the variations could not be correlated with any other measurable phenomenon. However, the rate of growth in the first phase of linear increase can be directly correlated with the initial size of the cells. The mean rates for each size of cell from three different experiments are shown in Fig.21

The rate of growth in the first linear phase seems to determine whether or not a change of rate occurs. Cells growing at a rate of less than .35μ nine minute period nearly all show a change of rate. Those growing faster than this seem to maintain the same rate until division.

The rate of growth in the second linear phase, can also be correlated with the initial size or the growth rate of the first linear phase. In Fig.22(a) the size at which the change in rate occurs has been plotted against the initial size, and in Fig.22(b) the division size of normal cells grown in wort broth are plotted against their initial sizes. By backward extrapolation of the line describing the relationship between the initial size and division size of normal cells, it would appear that the size at which a change of rate occurs in the cells recovering from nitrogen starvation is more or less equivalent to the size at which they would be expected to divide if they were 'normal' cells. There is, actually, a difference of one micron but it is not possible to decide whether the slightly lower figure for the point of change in rate of the nitrogen starved cells is significant.

When the nitrogen starved and replenished cells eventually divide they are in general considerably larger than the normal size at division./

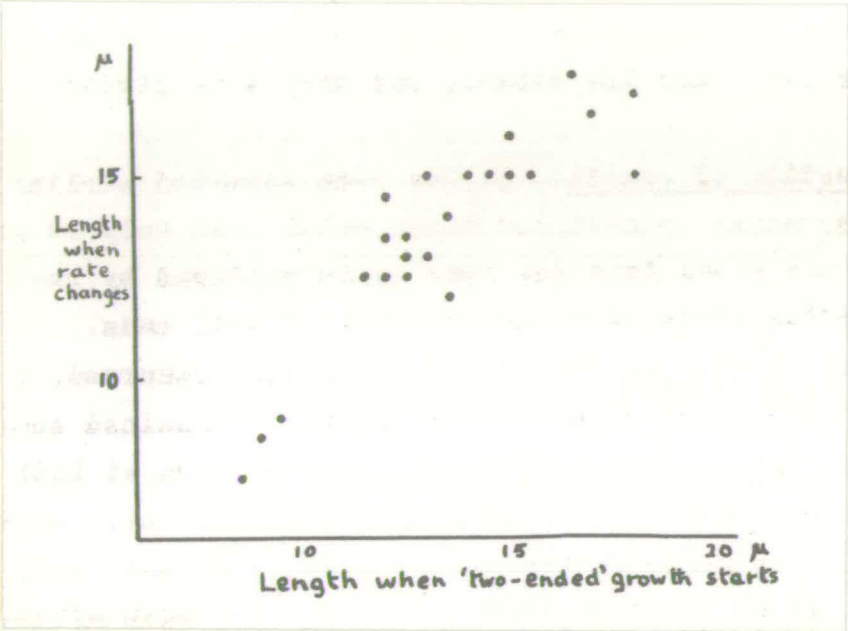

Figure 23. Scatter diagram to show correlation between the size at which growth starts at both ends of the cell and the size at which the cell increases its rate of growth.

division. The cells with the largest initial size are largest at division; those which start small divide at about the normal division size. Not only do the cells which are larger initially divide at a larger size than the others, but they also divide slightly earlier.

Direction of growth. It has been reported earlier (page 27) that under normal conditions these cells grow only at one end. However, it was found that the long cells produced by returning nitrogen after starvation were growing at both ends.

When growth resumed after starvation elongation occurred, as usual, at one end only. If, however, the cells are examined shortly before division, it can be seen that growth is occurring at both ends of the cell. By projecting the photograph of the small starved cell on to an outline drawing of the fully grown adult cell into which it develops, it is usually possible to tell how much of the adult cell length is due to growth at each end. The majority of the increase is due to growth at the end at which the cell started to grow (already found to be the end farthest from the last cell plate). Unfortunately, it is extremely difficult to determine by direct observation the time or size at which growth starts at the other end. In order to calculate this size it has been assumed that growth occurs at the new end at the same rate as at the original growing end. On this basis growth at the new end will start when the cell's length (L_1) is given by the formula

$$L_1 = L_o - 2L_N$$

where L_o is the length of the cell when observed and L_N is the length of that part of it due to growth at the new end. The assumption that growth at the new end is as rapid as that at the old end may not be strictly true as it is conceivable that when growth first starts at the new end it does not immediately achieve the full rate, but the error due to this cause seems likely to be small.

The calculated size for the onset of double ended growth is found to/

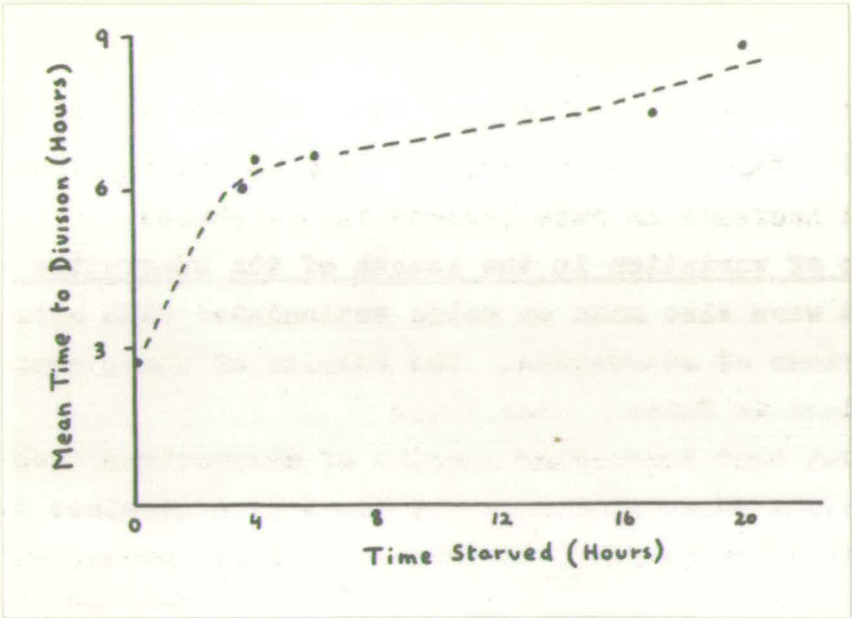

Figure 24. The average time taken by the cells to reach division when replenished with Nitrogen after various periods of starvation. The normal mean generation time is given for zero starvation.

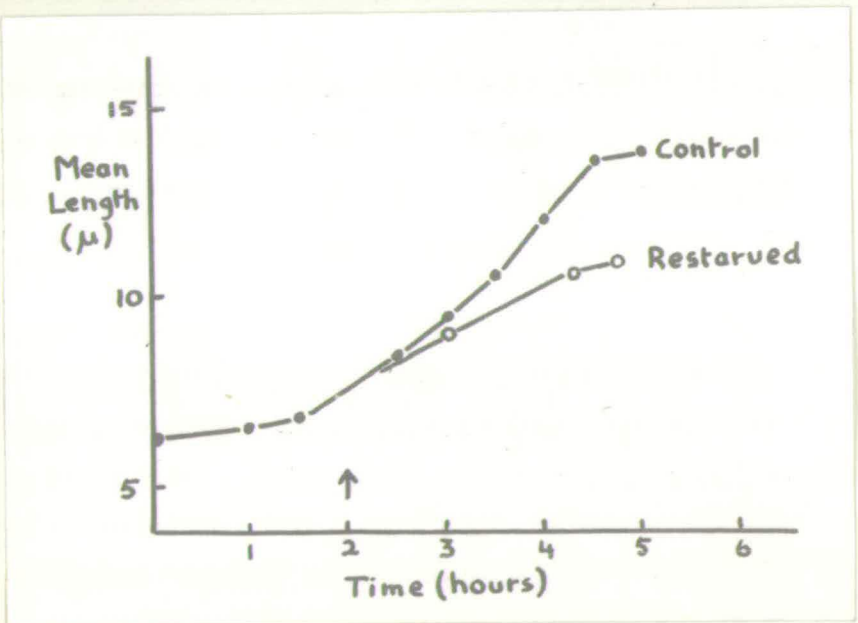

Figure 25. Effect of returning Nitrogen for two hours only after starvation on the rate of increase of mean length. Cells in culture.

to correspond with the size at which a marked increase in the growth rate was observed. Cells which do not show double ended growth do not show a marked increase in rate (second linear phase).

(ii) (c) Effects of variation in the length of the starvation period.

Observations were also made on cells replenished with nitrogen after varying periods of starvation. The results of these observations are summarized in Table 16a and Table 16b

It is apparent that increasing lengths of starvations have no effect upon the adult sizes attained, but there is a distinct increase in the time taken to reach division. In other words, both division and growth are slowed down by increasing periods of nitrogen shortage in such a way as to maintain their normal relationships with one another.

After a short starvation period, sufficient only to reduce the cells to the minimum size, the time until division is just slightly greater than twice the normal generation time. On the other hand, a starvation period some eight times longer increases the time until the first division to only three times the normal generation time. Clearly the first two and a half hours of starvation, during which time division continues without growth, not only decreases the size of the cells but also their capacity for growth. Continuing starvation reduces the cell's capacity for growth more slowly.

After a short period of starvation the smaller cells grow at about half the normal rate during the first linear phase, but cells larger than 7.5μ, that is to say, cells of about the same size as normal newly divided cells, grow at the full rate. The absolute values of the growth rates decrease with increasing length of the starvation period, the effect on all the cells being proportional, so the relative rates of the larger cells to the smaller cells are maintained constant.

(ii) (d) Effect of returning nitrogen for limited periods.

The effect of returning nitrogen for limited periods of time was/

TABLE 16a

Mean length (μ) at division after various periods of Nitrogen starvation.

Initial Length (μ)	Starvation time 4	4.5	6	18	20 hrs.
5.5	17.25	15.8	17.50	15.00	15.00
6.0	18.50	17.6	17.00	---	18.50
6.5	18.00	18.0	19.25	19.25	17.00
7.0	20.00	19.5	24.50	23.50	---
7.5	---	--	19.00	19.50	---
8.0	20.00	--	20.80	---	---
8.5	20.00	--	---	22.00	20.50
9.0	---	--	---	---	25.50
9.5	25.00	--	---	26.00	24.50

TABLE 16b

Mean time(in mins.) to reach division after various periods of starvation.

Initial Length (μ)	Starvation time 4	4.5	6.0	18	20 hrs.
5.5	387	399	414	--	765
6.0	396	414	406	--	558
6.5	396	369	405	495	549
7.0	369	--	396	482	--
7.5	--	414	--	441	--
8.0	369	338	--	387	495
8.5	392	--	--	--	549
9.0	--	--	--	--	501
9.5	369	--	--	450	441

was investigated on cultures of starved cells. Cultures which had been starved over night were replenished with nitrogen and after a period of two or three hours were divided into two equal parts which were centrifuged, the medium withdrawn, and replaced in one part by nitrogen free medium and in the other by fresh ammonia-salt medium. The results can be seen on Fig. 25 Growth continued in the nitrogen free medium for a period of about two hours at the rate which had been established before the change of medium. In the controls the growth rate continued to increase. In the nitrogen free medium the larger cells divided at the end of the two hour period of growth.

Direct observations on individual cells showed that all the cells continued to grow at the rate established before the return to the starvation medium and the effect observed on the cultures was not due to some cells ceasing growth immediately, while others increased their rate.

V DISCUSSION.

Normal growth.

Normal growth, as it is considered in these pages, is the growth shown by the cells when grown in full strength wort broth at a temperature of 27.5°C. When growth in the perfusion chamber is referred to, normal growth also implies a state of exponential or 'balanced' growth. (cf. Schaechter et al. 1958). That is to say, the characteristics of the parameters measured for one generation of cells are identical withthose measured for the preceding or succeeding generations. 'Balanced' growth is not restricted to cells growing at their optimum rate but will occur whenever cells are kept under constant environmental conditions in a medium which provides all materials essential for growth.

Under the conditions considered normal we find that the cell cycle consists of two phases; an initial phase of slightly accelerating volume increase lasting for four-fifths of the cycle followed by a constant volume phase which lasts until division is complete. Division of the central vacuole, which is almost certainly the nucleus, appears to take place at the beginning of the constant volume stage (Mitchison 1957) and the formation of the cell plate must follow soon after. The presence of the cell plate is not generally visible on the film until about half way through the constant volume stage but it can be detected earlier than this by careful visual observation. It is not visible, however, during the first third of the plateau. The plateau period is thus concerned withthe division of the cell into two. Growth in terms of volume increase does not occur during the final fifth of the cycle, but synthesis of cell wall takes place for at least two thirds of this period. The mere containment due to the constant volume, resulting from cell wall synthesis being directed to the formation of the cell plate rather than to increasing the length of the cell, seems unlikely/

unlikely to greatly affect the synthesis of cytoplasm within. By following the increase in dry mass Mitchison (1957) has shown that there is a linear increase in weight between divisions, the new rate for the two daughter cells being established at some point during the constant volume stage. There is no plateau and the calculated concentration rises during the volume plateau and falls over the rest of the cycle. This would indicate that inspite of the fact that the formation of the cell plate is not visible during the first third of the constant volume stage, overall synthesis continues throughout the period at at least the same rate as before. This is, of course, only true if there is not a build up of food materials without corresponding incorporation into cellular material. Unfortunately there is not sufficient evidence to determine whether or not cell wall synthesis continues throughout the whole of the volume plateau. Mitchison has weighed empty cell walls and found that the dry weight of the cell wall accounts for more than twenty per cent of the total dry mass of the cell. But inspite of this relatively high percentage, cessation of wall formation alone for a short period would not be detectable on the curves for mass increase. Furthermore, if the formation of cell wall were to cease because the synthetic centres were engaged in their own multiplication, the dry mass might be expected to continue to rise. On the other hand, the fact that the cell plate is not visible in the first third of the constant volume stage cannot be taken as conclusive evidence that synthesis of cell wall material is not occurring. While the formation of a plate would not be expected until nuclear division is complete there seems to be nothing to prevent the first stages of the synthesis of wall material occurring away from the prospective position of the plate. Even the first stages of plate formation are probably not visible. It thus seems quite feasible that cell wall synthesis continues during the whole of the constant volume stage and if/

if so it continues at a rate similar to that obtaining at the end of the growth phase.

When the volume of the cell is increasing, the rate of increase is logarithmic. If we assume that the thickness of the wall does not alter over the growth period, this means that the rate of cell wall synthesis is increasing over this period. As it appears to continue over the plateau period also, at a rate at least equal to the rate at the end of the growth period, it seems likely that cell wall synthesis is continuous and logarithmic. A point of caution should perhaps be made. As has already been indicated there are insufficient points on the curves to make it possible to decide on the exact shape of the curve. It may not be truly logarithmic.

Mitchison (1957) has shown that the rate of increase of total dry mass is linear between divisions and on the basis of this has suggested that the rate of growth is controlled by a number of active microsomal particles, the number of these remaining constant throughout the cell cycle and doubling their number round about the time of cleavage. The fact that the rate of cell wall synthesis increases logarithmically means that the rate of cytoplasmic synthesis must decrease as the cell grows larger. The diminishing rate of cytoplasmic synthesis fits the microsomal hypothesis well, as it is reasonable to expect that as the cell increases in size the cell's total demands on the synthetic centres for materials required for maintenance rise in proportion, leaving a progressively smaller amount available for growth. Prescott (1955) used a Cartesian diver to measure the reduced weight of single Amoeba over their growth cycle. He found that the rate of increase in mass was greatest when the cell was small and progressively diminished as the size increased, eventually ceasing altogether. He has suggested that this growth is due to the relationship between synthesis and breakdown, the rate of synthesis being constant and determined by the nucleus, while the rate of breakdown is a function of the cell's size. This is precisely/

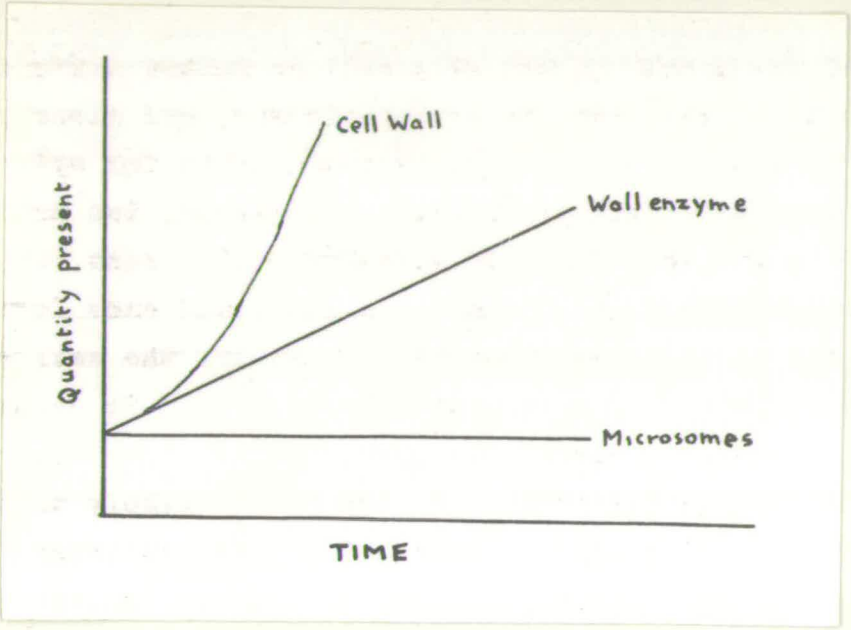

Figure 26. Suggested relationship between microsomal number, the rate of increase in enzyme content in the cell, and the growth of the cell wall. No allowance is made for the effect of cytoplasmic breakdown.

precisely the same argument as that put forward above to account for the diminishing rate of cytoplasmic synthesis in the yeast, except with respect to the nature of the synthetic organelle. There is little evidence on which to base a choice between nuclear and microsomal control although it is perhaps easier to explain continuous growth as a microsomal function than as a nuclear one, as nuclear control might be expected to produce an interruption in growth during mitosis.

The problem of the growth of the cell wall is rather different. The growth rate in this case seems to be logarithmic, and clearly a microsomal form of control such as that discussed above for cytoplasmic growth cannot operate directly. There are, however, two points of difference between the cell wall and cytoplasm which seem relevant. First, the wall is fundamentally a 'dead' structure and once formed probably requires little or no maintenance. Secondly, the wall consists mainly of carbohydrate and there is no real reason to expect microsomes, which are usually associated with protein formation, to be directly involved in its formation. It seems more likely that the 'growth centres' for wall formation consist of enzyme complexes. Now, if the number of microsomes actively engaged in protein synthesis remains constant throughout the cell cycle, and each microsome keeps up a constant rate of formation of each protein which it produces, the amount of each of the individual proteins present in the cell will increase linearly with time. If this is true of the enzymes responsible for wall formation the amount of enzyme available for the purpose of wall building will increase with every unit of time. The rate of wall formation will thus be an increasing function of time (See Fig. 26). As the wall itself probably does not require any maintenance any deviation from a strictly logarithmic growth curve would be due to an alteration in the rate of increase of the enzymes responsible for its formation. The rate of formation of the wall would, however, continue to/

to rise as long as the rate of synthesis of enzyme exceeds its rate of breakdown, but as the difference between these two becomes less the rate at which wall formation accelerates will diminish. More accurate measurements then are available would be required to decide between such a curve and a simple logarithmic one.

Any other cellular material formed in a similar manner by direct enzyme action as distinct from microsomal action would be expected to have a similar curve of increase, but as the bulk of the cell, apart from the cell wall, consists of protein or related compounds, the formation of such other substances is likely to have little effect on the total rate of mass increase.

If, as has been suggested, microsomal multiplication takes place at one particular point in the cell cycle, one might expect to find periodic increases in the amount of RNA in the cell. Burns (1956) quotes James (1953) as stating that RNA synthesis is rapid just before division and Maruyama (1956) finds that cells of Escherichia coli synchronized by a filtration technique increase their RNA content early in the cycle. Barner and Cohen (1956) using a thymineless mutant of E.coli, synchronized by withholding the thymine required, found RNA synthesis to be continuous. More recently Mitchison and Walker (in press) have estimated the RNA content of single cells of S. pombe using a UV absorption technique and have found a fairly steady increase in the amount of RNA throughout the cell cycle. These results are conflicting but one is inclined to put more faith in estimations on single cells than on synchronized cultures, as the methods of inducing synchrony may well upset the balance of the synthetic machinery. At a first glance continuous RNA synthesis is not encouraging to the idea of microsomal multiplication at any one part of the cycle, but, as Mitchison and Walker point out, the RNA formed need not be immediately incorporated into the microsomes. In fact, if microsomal multiplication is to be accomplished rapidly a prior build up of RNA is almost essential.

Theoretically/

Theoretically there seem to be one or two other ways of determining the time of microsomal multiplication. Measurements on the increase of total dry weight will show the time when the rate changes and thus indicate when multiplication is complete, but as the multiplication itself probably implies increase in mass, its onset is unlikely to be apparent. Prescott (1955) has found that the curve for protein increase follows the mass growth closely, although a direct comparison of the two curves does indicate that the initial rate of mass increase is slightly greater than can be accounted for by protein alone. However, the difference is so slight that it is unlikely to be significant. Maruyama's work (1956) on synchronized E.coli, which has already been mentioned for showing an initial rise in RNA, also shows that protein nitrogen increases in the second half of the cycle although total nitrogen increases evenly throughout the whole of the cycle, but bacteria may well be atypical as multinucleate cells frequently occur. Zeuthen (1952) has followed the course of respiration in cells of Tetrahymena pyriformis and has found that the rate of oxygen consumption increases linearly, until a point shortly before division, when a plateau occurs, and at some point during the course of division takes up again at twice the previous rate. Zeuthen has interpreted the plateau as being a period given over to the reduplication of the synthetic centres. If this is the case the synthetic centres must utilise for their own multiplication the energy from an 'energy reservoir' identical with, or comparable to, that suggested by Swann (1954) for cell division. In this respect it is interesting that Maruyama (1956) found that there was a steady increase in Oxygen consumption in E.coli but that Carbondioxide output, although showing an overall general rise also showed two peaks, one corresponding to the time of DNA increase and the other to the time of RNA increase.

It has been found that the volume of single cells of S.pombe increases/

creases logarithmically for four-fifths of the cell cycle and then remains constant until cleavage is complete, but this is not incompatible with the theory that the growth of the cell is controlled by a constant number of synthetic centres, likely to be microsomal particles, which multiply about the time of division. Indirect evidence on the behaviour of the synthetic centres seems to be frequently conflicting, and it appears that only direct observations on the microsomes themselves are likely to clarify the situation. It would seem that volume estimations, at any rate in cells like S.pombe which have rigid cell walls, cannot be translated directly into terms of synthesis as the increase in dry mass follows a different course. Nevertheless, the shape of the curve for cell wall growth can be used as an indication of the amount of active enzyme present and this is itself dependent on the balance between the synthesis and the breakdown of the enzymes concerned. Thus any marked change in this balance, due, for instance, to alteration of the number of centres of protein formation, should have an effect on the rate of cell wall synthesis. Thus linear growth of the cell wall implies that synthesis of cytoplasm is balancing breakdown; a diminishing rate of cell wall formation that breakdown is exceeding synthesis; and an accelerating rate of wall synthesis that synthesis is exceeding breakdown. It seems probable that the balance in favour of synthesis diminishes as the cell grows larger. This would have the effect of reducing the rate of acceleration of the rate of cell wall synthesis over the cell cycle. Unfortunately, the results obtained with the system used, though far from denying this, are not sufficiently precise to confirm it.

Region of growth.

Throughout the phase of volume increase growth occurs only at one end of the cell. At the time of the volume plateau growth ceases at the end of the cell and the cell plate is formed in the centre./

centre. When the cell divides growth is resumed at the end originally growing and is also taken up at the end of the other cell distal to the cell plate. There is no reason to believe that the cell plate differs in structure from any other part of the cell wall and one would thus expect similar structures as are involved in the synthesis of normal cell wall to be responsible for the building of the cell plate. Now if there is always a centre of cell wall synthesis in each of the three regions which show growth at one time or another, an inhibitory mechanism must apply to certain of these at certain times, but inhibitors which work by location rather than specific reaction themselves need some form of control. Rather, it would appear that the centres of growth are located at one end; that at division they move to the centre to form the cell plate and at the same time divide themselves equally between the daughter cells. When the cell plate is complete the centres move to the end farthest from the cell plate. It is possible that the growth centres move to the middle of the cell as a part of the general movement concerned with nuclear division but why or how they move to the extremities on the completion of the cell plate remains obscure.

As the growth of the cell wall is logarithmic the centres of wall synthesis presumably grow or multiply in a linear manner throughout the cell cycle. The microsomes responsible for the formation of the centres would be expected to duplicate themselves at some point during the division of the cell. Under certain conditions growth occurs at both ends of the cell and it seems likely that under these circumstances microsomal multiplication is divorced from cellular division but we shall leave discussion of this to a more appropriate time. (See page 71).

The Relationship of Division and Growth.

The/

The variation found in most of the parameters investigated does not seem to be as random as the variation in size at division might lead one to expect. The variation in increment between divisions is small, of the order of 20 per cent, and such as there is appears to be independent of the cell's initial size. The generation time, on the other hand, is correlated, inversely, with the initial size. Unfortunately, direct measurements of growth rate cannot be made sufficiently accurately to seek a correlation, but in view of the more-or-less constant increment per cell and the correlation of generation time and cell size, it is clear that the growth rate must be directly related to the initial cell size.

The correlation between the initial sizes of sister cells is not significant although the correlation between adult sizes is just significant. This low order of correlation is a reflection on the lack of variation in the population as a whole rather than on the degree of variation between sister cells, but it would indicate that such variations as do occur are not genetically determined, although large parents will divide to give large daughters which will show the usual amount of growth before the next division, thus becoming large mature cells.

The inheritance of generation times and growth rates has been investigated on bacteria by a number of workers. Kelly and Rahn (1932), restricting their remarks to the variations found in generation times, conclude that the variations they found in this parameter were not hereditary. Hughes (1955) found that a bacterium (E.coli) may divide to give daughter cells with different growth rates but that this growth rate is maintained in the progeny when the same culture conditions are maintained. Powell (1956) found some degree of correlation of growth rates and of division rates between related cells but the correlation became less marked as the relationship became less close. Powell also makes the point that the variation found in bacteria/

bacteria might reasonably be expected to be high since the size of the molecules required for growth is not inconsiderable relative to the size of the cell, and during the course of one generation two cells, otherwise identical, may well not receive the same number of food molecules. Considering larger cells, Burns (1956) found that generation times in Saccharomyces cerevisiae were not inherited but Adolph (1929) found that in the protozoan Colpoda there was a strong positive correlation between the size of parents and their offspring at maturity. Such a correlation between parents and offspring, however, does not necessarily imply genetic determination as has already been argued for S.pombe.

The relationship between initial cell size and the rate of growth or division seems to have been less frequently investigated. Adolph (1929) has reported that longer generation times are slightly correlated with larger sizes in Colpoda. This seems to be the reverse of the situation found in S.pombe. Prescott (1956) has found that large cells of Amoeba proteus have shorter generation times than the smaller ones but the average growth rate of a small cell is greater than that of a large one.

It seems necessary to interpret the relation of growth to initial size in the light of what is known of the growth over the cell cycle. If the microsomal hypothesis of the control of growth is true the relation found between the initial size of cells of S.pombe and their growth rate indicates that a small cell must contain fewer microsomes than a large one. It would also imply that microsomal segregation at division is not equal but is a function of the amount of cytoplasm going to each of the daughters. It would be necessary to compare the rates of growth of pairs of sister cells of unequal size to be sure of this last point, but grossly unequal divisions of cells of S.pombe are not sufficiently frequent to make such determinations possible. By subjecting dividing/

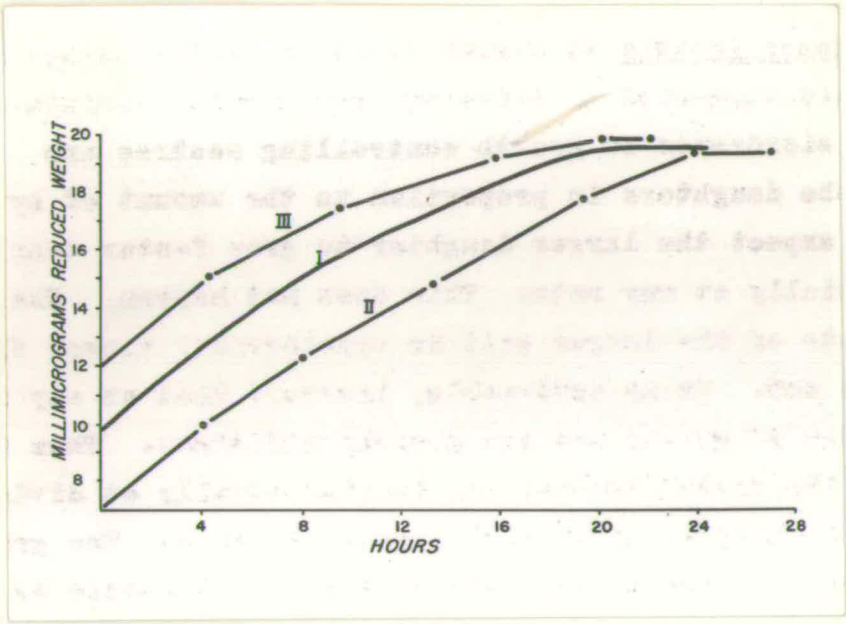

Figure 27. The growth of unequal sister cells (II and III) of Amoeba proteus. Growth of normal sized cells also shown (I). Prescott, 1955.

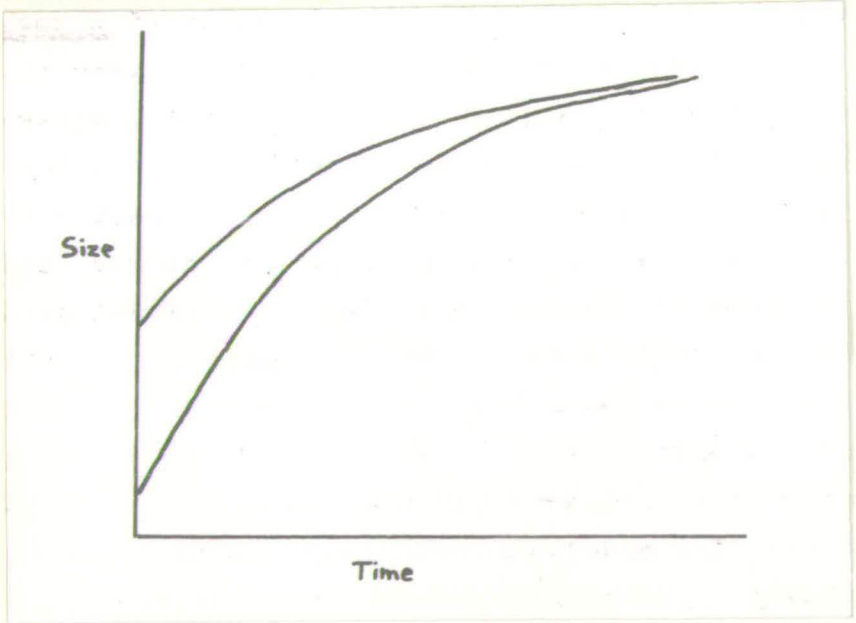

Figure 28. Theoretical curves for cytoplasmic growth assuming a constant rate of synthesis and a rate of cytoplasmic breakdown which is proportional to the amount of cytoplasm present.

iding cells of Amoeba proteus to strong light or gentle agitation Prescott (1956) has succeeded in inducing very unequal divisions. If the number of microsomes or growth controlling centres are divided between the daughters in proportion to the amount of cytoplasm, one would expect the larger daughter to grow faster than the smaller one, initially at any rate. This does not happen. The average growth rate of the larger cell is considerably slower than that of the small one. It is noticeable, however, that at any given cell size the rates of growth are not greatly different. This strongly suggests that the growth centres are divided equally at division irrespective of the inequality of cytoplasmic division. The growth curves expected on this basis are shown in Fig.28 and should be compared with those found by Prescott (Fig.27).

The generation time of cells of S.pombe was found to be shorter for large cells than for small ones and Prescott reports a similar relationship for Amoeba. In Amoeba the total volume increment is not constant; cells which start growth large in size divide only slightly larger than those that start small. In cells of S.pombe, on the other hand, the volume added during the cycle is constant. In the case of Amoeba proteus the growth in volume gets progressively slower as the cell gets larger and eventually ceases altogether, probably, as Prescott has suggested, when cytoplasmic breakdown equals synthesis. A cell which is unduly large when growth begins will reach this size without accomplishing very much actual growth and probably before the build up for division is complete. Cells of the yeast, however, which reach the point of equilibrium in cytoplasmic synthesis and breakdown before the cell is ready to divide will continue to form cell wall and thus the volume will continue to increase but with resulting cytoplasmic dilution. This explanation is, of course, entirely hypothetical, but it should be possible to test it by following the increase in mass of cells of large initial size/

size if these can be obtained sufficiently large with a normal microsomal content, or if division can be delayed, perhaps by lowering the temperature. (Mucibabic, 1956 , found temperature to have differential effects on the rates of division and growth in Chilomonas paramecium).

The constancy of the amount of volume growth achieved between divisions in S.pombe suggests that under normal conditions of growth the final limiting factor controlling the rate of division is the achievement of a level of some material which is built up by a mechanism operating in a similar manner to that whichforms the cell wall. Division is clearly not the result of the cell reaching a certain size although in cells like Amoeba, which have not got cell walls, increase in volume will come to a stop when a certain size is reached, but whether division occurs normally just as this size is reached, or before or after it, will depend on the relationship between growth and division rates and on the initial size of the cell.

Growth in dilute media.

The effect of dilute media on the growth of S.pombe in culture is in line withresults of similar experiments on other organisms (e.g.Phelps, 1936). With decreasing concentration of the medium the rate of multiplication is not affected until the concentration falls very low indeed, but the eventual population level is roughly proportional to the concentration. It was, however, noticeable that with the yeast diminishing concentration increased the efficiency of the conversion of food into cellular material over the culture as a whole. This effect appeared in two stages. As the concentration of the medium decreased the number of cells per unit of food at the end of the logarithmic phase of growth increased, and, initially, so did the mean size of the cells. It seems probable that this is due mainly to the presence of a smaller number of cells, and hence of a lower concentration of alcohol and other end products of metabolism in the medium/

medium at the end of the logarithmic phase, and also to the shorter time taken to reach the final population level thus reducing the total expenditure on maintenance. In the second phase, at concentrations below the .4% level, the overall efficiency continues to rise but it is expressed solely as an increased cell number to food ratio, as the mean size of the cells falls slightly. The generation time remains the same. One can only assume that this second phase is due to the concentration of some substance in the medium useful for growth and not for division reaching a level at which the cell can only obtain it with difficulty. The eventual reduction in the rate of division must be due to a similar situation affecting some substance used in division. The concentration chosen (.13% w/v wort broth) for use in the perfusion chamber was selected on the basis of the results from the mass cultures as being the highest concentration producing an alteration in the generation time which could readily be detected. The extent of the effect was, however, a trifle surprising. First, it should be noticed that even at full strength concentration the generation time of cells grown in culture is measureably faster than cells grown in the perfusion chamber, about 2.1 hours in the former case and 2.7 hours in the latter. The difference in generation times at the lower concentration, however, was even more marked, about 2.8 hours and 4.8 hours respectively. The basic difference in generation time between the cells in culture and those in the perfusion chamber would suggest that the cells in culture have some mutually stimulating effect which conditions in the perfusion chamber do not permit. Such 'auto-catalytic' effects are by no means unknown. (Robertson, 1921). The effect could also be due to the difference in the amount of light to which the cells are subjected. The strong light necessary for filming the cells in the chamber may well have a deleterious effect on the cells. It is, however, rather surprising that the discrepancy in generation time is so much more marked at the lower concentration. The effect of the dilution might be felt in a rather different/

different manner under the two different conditions of growth due to continually changing conditions in the tube cultures on the one hand and the constant concentration in the perfusion chamber. This, however, would be expected to have the reverse effect. The only alternative seems to be that some part of the perfusion system has a deleterious effect on the growth of the cells (in a general sense) to which the cells are more sensitive at low dilutions of the medium. It is impossible to say very much about the cause of this effect though it is presumably not due to some part of the system absorbing the nutrients from the medium, as this would only be effective while the sites of absorption were being filled. It seems more likely that some substance is escaping slowly from a part of the system such as the agar blanket or the rubber connections between the tank and the chamber.

The results from the cells in the tube cultures and the cells in the chamber do, however, seem to be comparable with regard to the relative effect on division and growth rates. The effects of the lower concentration on the cells in the perfusion chamber is to reduce the growth rate to rather less than half the 'normal' rate but the generation time is not quite doubled. This confirms the interpretation placed on the results of the test tube cultures, that the growth rate is adversely affected before the division rate by the decreasing concentration of the medium, perhaps due to some inbalance of the medium which only becomes apparent at critical concentrations and thus offers further evidence that growth and division can be separated on a nutritional basis in this organism.

Work with bacteria seems to indicate that the explanation may be more complex than this. It has been shown for E.coli (Perret, 1958) and more recently for Salmonella typhimurium (Schaechter, Maaloe and Kjeldgaard, 1958) that the mean cell size in various media is related to the rate of growth which the medium permits. The faster the growth rate,/

rate, the larger are the cells. The latter authors found that the DNA content per cell and the RNA content per cell were similarily related to the growth rate. Thus for different growth rates in different media the cells exist in different 'physiological states'. This appears to be true of dilutions of one type of medium as well as of different media. This work will be discussed further in connection with the effects of changing the medium from one concentration to another.

It was concluded earlier that the length of the plateau period depended on the rate of cell wall formation, although it was considered possible that the first part of it represented the time taken for nuclear division to occur. Division processes have been shown to proceed only when all the energy and materials required for them are available (see Introduction) so one would not expect the time taken for nuclear division to vary with the growth rate when this is altered by restricting the nutrients in the medium. If, then, the length of the plateau is determined both by the time taken for nuclear division and by the time for cell wall formation the proportion of the life cycle spent in the plateau period should be less for cells in dilute medium than in media permitting faster growth. However, no significant difference was found. It thus seems that the length of the plateau is solely determined by the rate at which the cell plate can be formed.

Effect of changing the concentration of the medium.

If we are correct in concluding from the observations on the normal growth of cells that the growth rate is determined by the number of synthetic centres and that these multiply at the time of division, it becomes a matter of interest to know how cells will respond to a change to a medium in which they normally show a different rate of growth.

As the cells are clearly capable of growing at normal rates at concentrations/

concentrations well below that used to follow 'normal' growth, the actual rates of growth and division must, in the full strength medium, be limited by the rate at which the internal synthetic processes can function. When, however, the supply of nutrients suddenly becomes limiting do the cells continue to grow at their previous rate relying on stored nutrients 'unaware' of the change in the external environment, either until the stocks run out, or until a certain point in the cycle, such as the time of multiplication of the synthetic centres, when the cell might become 'aware' of the changed conditions? Or does the cell immediately adjust to the changed environment?

The experiments to investigate this had rather unexpected results. When the cells were transferred from full strength medium to a low concentration they did not continue to grow at their original rate nor did they take up the rate characteristic of the new medium rightaway. Instead volume increase ceased almost immediately. After a while growth started again at the new rate. The time spent by the cells in this induced plateau was related to the concentration of the new medium; the lower the concentration the longer was the plateau.

Without the aid of detailed chemical analysis any explanation proferred for the formation of this plateau must be purely speculative. It is clear, however, that the cells are remarkably tolerant to osmotic changes, as periods of up to three hours in distilled water do not in any way reduce the cell's capacity for growth. Furthermore when the osmotic pressure of the dilute medium was increased by the addition of a non-metabolizable sugar the plateau was unaffected. The most likely alternative seems to be that the plateau represents a period of adaptation. Unfortunately there do not seem to be any reports of studies on the effect of adaptation on the cell cycle. Chantrenne (1956) has, however, made a study of nucleic acid metabolism during enzyme adaptation. He has shown that when a cell is presented with a new substrate there is a time lag of about half an hour before synthesis of/

of the necessary enzyme begins. He has also found that if the cells are previously fed with labelled adenine so that all the adenine in the RNA is labelled and the cells then introduced to the new substrate (in this case maltose) in the presence of unlabelled adenine, the amount of free labelled adenine in the cell rises rapidly and falls off again when enzyme synthesis starts, indicating a temporary breakdown of the cell's R.N.A. Chantrenne has tentatively suggested that enzyme synthesis must be preceded by reorganisation of the RNA. In the case of S.pombe the change from a medium of high nutrient concentration to a more dilute medium may well have the effect of inducing the yeast to use an additional energy source or to undertake synthesis of some material which it could previously obtain in sufficient quantities without doing so, and while this adaptation to allow for this is in progress one would expect growth to cease or at any rate to be reduced. It seems necessary to conduct similar change of medium experiments on S.pombe using different concentrations of a medium providing only those substances essential to the growth of the cell. If under these circumstances the plateau appeared adaptation of this kind would be a less likely explanation.

Another form of adaptation is possible. It has been suggested earlier that when different rates of growth occur in different cells in the same medium this is due to the presence in the cells of a different number of microsomes. On this basis one might expect the cells in the medium of low concentration which grow more slowly to have a smaller number of microsomes than the faster growing cells in the richer medium. If this were true a period of adaptation would obviously be required on a change from a low to a high medium concentration before the higher rate of growth could be achieved, but it seems less necessary for the reverse change.

Changing the medium from low to high medium concentrations does not induce a plateau; the change to the new rate is immediate and the rate/

rate is identical to the normal rate in the new medium. This result might be expected from the enzyme adaptation hypothesis as all the enzymes present in the cell growing at the low concentration can presumably be used at the higher concentration. One might expect growth in the dilute medium to induce a greater diversity of enzymes in the cell which could be used to advantage in the more concentrated medium, but the growth rate after the change is no different to that normal for the medium. Thus any idea of the cells in the more dilute medium containing fewer synthetic centres seems unlikely to be true. Rather, the rapid and complete change of the rate of growth would suggest that the microsomal content per cell was independent of the growth rate when this is limited by the concentration of the nutrient in the medium.

Until very recently there have been no reports with which these results could be compared. Within the last year, however, Kjeldgaard, Maaloe and Schaechter (1958) have reported the results of some observations on the cell size and chemical composition during balanced growth of Salmonella typhimurium. The cells were grown by a continuous culture method in a number of different media affording different growth rates. The mean cell mass, DNA content and RNA content and the average number of nuclei per cell were studied in the various media. It was found that the size and chemical composition of the cells differed in the different media, the values of the various parameters measured being exponential functions of the growth rates afforded by the various media. Rather surprisingly, in view of the many reports of cell size varying with temperature (e.g. Mucibabic, 1956) the size and chemical composition of the cells of Salmonella in a given medium were not influenced by temperature to any marked extent over the range studied (25°C to 37°C). When the characteristics of the various 'physiological states' existing in various media had been determined, Schaechter et al. studied the course of events when the medium was changed./

changed. They found that on a 'change down' that is, a change from a medium permitting fast growth to a medium permitting only slow growth, increase in total mass and RNA ceased immediately and there was no further increase for thirty to forty minutes. At the end of this time synthesis started again at the rate characteristic of the new medium. After the change down synthesis of DNA and the rate of cell division continued at their original rates, in the case of DNA for about ten minutes and for cell division slightly longer. At the end of this time the cells had attained their new 'physiological state' and the new rates were established.

In so far as the behaviour of the yeast can be compared with these results it would seem to be similar. Growth in volume of the yeast shows a similar plateau to that found for mass in Salmonella after the change of medium. On the evidence available for the yeast division does not appear to continue either at the old rate or at the new rate over the plateau period. It appears to cease. It should be noticed, however, that the difference in the 'physiological state' between the Salmonella cells in the 'fast' medium and the 'slow' medium is considerably greater than that for S.pombe as the size of the dividing cells of the organism were only just significantly different in the two media used, whereas the Salmonella cells in the 'fast' medium were about twice the size of those in the 'slow' medium. The difference in the division rates in the two media is not greatly different for the two organisms and the difference with respect to the other parameters is explained by the fact that the bacterium is binucleate in the 'fast' medium and uninucleate in the 'slow' medium. Thus the change in 'physiological state' per nucleus is not great so the maintenance of the division rate, if it occurs in the yeast, is unlikely to be detectable, at any rate by single cell determinations.

In/

In the case of a 'change up' Salmonella shows an immediate increase in the rate of RNA synthesis, soon followed by the new rate of increase in mass and, after about twenty minutes followed by the change to the new rate of DNA synthesis. The new rate of increase in cell number does not start for some eighty minutes but as the number of nuclei per cell doubles during this time this is hardly relevant for comparison with the yeast. As for the change down the similarities in the effect on the mass growth of Salmonella and the volume growth of S.pombe are striking. Changes in the other parameters cannot be compared.

Schaechter et al. make no attempt to explain the mechanism by which the rates of division and DNA synthesis are maintained after the change of medium. The time for which this rate maintenance occurs depends only on whether the change of medium is 'up' or 'down' and is independent of the growth rates in either of the media. The plateau found for mass growth and RNA synthesis on the change down, like that for volume growth in the yeast, is dependent on the difference in rates between the two media, and is similarily explained as being the time required for the formation of enzyme systems required in the slower medium but not in the faster.

Effects of imbalance of the Nitrogen-Carbon ratio.

Considered simply, the requirements of a cell are twofold. It requires materials for the cell substance, and energy for the maintenance and formation of the cellular material and for the processes associated with the division of the cell. The bulk of the energy, in yeast at any rate, might be expected to come from carbohydrates, nitrogenous substances being mainly required as building blocks for the cellular material. Undoubtedly, in yeast carbohydrates are also used for 'building'. The cell wall, for instance, consists largely of mannans and glucans (Trevelyan and Harrison, 1956). No doubt too, nitrogenous substances can be used as energy sources, but this does not alter the basic premise that the bulk of the cell's energy will be provided by the carbohydrate fraction.

The period of adaptation which the yeast showed when the medium was changed from a high to a low nutrient concentration might be required for the development of enzymes either to utilize an additional energy source or to add to the possible sources of Nitrogen. Once balanced growth was established in the new medium, the relation between division and growth rates was found to be different to that obtaining in the more concentrated medium. This difference might be due either to a priority of division over growth for the available energy, or to a differing degree of availability of the nitrogenous materials required for growth and for division.

The experiments involving a change from full strength medium to one of low Nitrogen concentration with excess glucose gave essentially the same results as the change from full strength to Nitrogen free medium and so will be considered together.

In neither case was there an immediate effect on the rate of growth such as was found on the simple change from full strength to dilute medium. In the majority of cells growth continued at its previous/

previous rate until the cell divided. After division, those cells that continued to grow did so at about half the rate of the parent cell. In the case of the change to the medium containing some Nitrogen growth continued at this rate indefinitely but in the Nitrogen free medium growth eventually ceased altogether. A number of cells did not reach division before changing their rate (dilute N + glucose medium) or ceasing to grow (N free medium). These cells were all small when the change of medium took place and growth continued for at least an hour before a change occurred.

It is quite clear that the plateau period found in the straight forward change from high to low medium concentration is absent when carbohydrate is supplied, and so must be due to an adaptation which enables the cell to obtain carbohydrate from other sources. It is interesting that the rate of growth after such a period of adaptation is such that balanced growth occurs immediately in the new medium. Whether this is a fortuitous result of the concentration of the materials in the medium or whether the cell only adapts to an extent necessary to balance the uptake of Nitrogen cannot be determined from the results available. The cell clearly does not take account of the balance of the medium if glucose is provided in excess, as growth continues after the change at a rate considerably faster than that which the Nitrogen in the medium can sustain.

There seem to be two possible explanations for growth continuing after the change to Nitrogen free media. Either total growth continues utilizing Nitrogen from an amino acid pool, or growth of the cell wall alone continues with a resulting decline in cytoplasmic concentration. It has been shown by Halvorson and Speigelman (1953) that Nitrogen starvation of Saccharomyces cerevisiae (grown at 30°C) reduces the level of the free amino acids in the cell to about twenty per cent of its normal level in two and a half hours. However, the free amino acids in the cell only represent seven per cent of the total/

total Nitrogen of the cell (Ingram 1954). Clearly this cannot account for an hour's growth which represents forty per cent of the generation time. Growth of the cell wall, which is primarily carbohydrate, need not be limited by the absence of Nitrogen. In fact, if the mechanism proposed earlier to account for cell wall growth is the true one, the growth in volume would be expected to continue, showing a linear rate of increase, even when cytoplasmic growth has ceased entirely. Such an arrangement would, of course, mean dilution of the cytoplasm and it is probable that cell wall growth ceases when the dilution of the cytoplasm affects the operation of the enzymes concerned in wall formation. The appearance of the cells towards the end of this growth period would encourage the idea that dilution of the cytoplasm occurs. Under the phase contrast conditions used normal cells contrast strongly with the background and internal structures can only be discerned with difficulty. At the end of the growth period, however, granules are clearly visible in the cytoplasm and the overall contrast between the cells and their background seems to be less.

The hypothesis also explains two other characteristics of the growth period. It was found that when division occurred soon after starvation the rate of growth of each of the two daughter cells was half that of the parent. This would be expected if growth of the cytoplasm, and hence of the centres of wall formation, had ceased. Each of the daughters would thus contain half the number of centres of wall syntheses possessed by the parent and could grow at half the rate. It was also observed that the smaller a cell was at the onset of starvation, the longer it continued to grow. The cytoplasm in the normal cell, it will be remembered, becomes less concentrated as the cell grows larger. Thus a cell which is 'caught' by the starvation at the beginning of the cycle when it is in its most concentrated state can afford more dilution than a cell 'caught' later in the growth phase. It will also grow more slowly.

After/

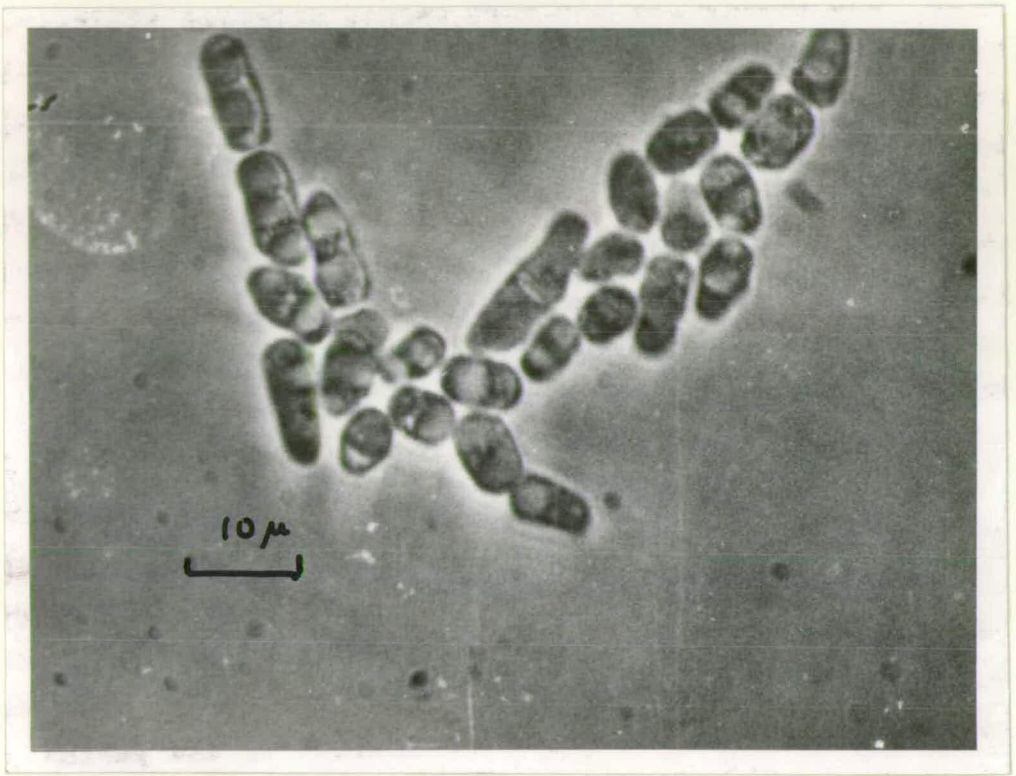

Figure 29. Cells after six hours of Nitrogen starvation. Note vacuolated appearance.

After the change of media division continues normally while volume growth continues. One further division also occurs after the growth ceases. The energy for division during Nitrogen starvation presents no problem as carbohydrate is present in excess. Materials for building the cell plate are also available and as its formation does not increase the volume of the cell, the concentration of the cytoplasm will not be further reduced. There is thus no reason for the plate not being formed. The duplication of the chromosomes is quite another matter. There is no question of a reduction division occurring as the cells are already haploid. The Nitrogen for chromosomal re-duplication must come from internal sources. Schmitz (1954) has shown that there is a nucleotide pool in yeast comparable to the amino acid pool. Cowie and Bolton (1957) have studied the incorporation of labelled purines in the cells of the yeast, Candida albicans. They conclude that the purines first enter a purine pool, the concentration of which is proportional to, though higher than, the exogenous purine concentration. The purines then enter a nucleotide pool the level of which is independent of the concentration of the purine pool. The nucleotides are then incorporated into the nucleic acids. The existence of such a system of pools should enable the cell to continue forming nucleic acids for a period after the onset of Nitrogen starvation. Walker and Yates (1952) have studied the UV absorption of the nuclei of various tissue culture cells. They found that the nuclei of dividing cells contained twice as much absorbing material as non-dividing ones. This extra absorbing material was not stained by Feulgen and they concluded that it consisted of DNA precursors. The DNA and these suspected precursors were built up during interphase. It would thus appear that a normal cell at division has enough DNA precursors to complete a further division. This seems to be the most likely explanation for division in the yeast continuing after the change to a Nitrogen free medium.

The/

The rate of division is normal until growth stops. However, the generation time of cells dividing after the time at which volume growth ceases gets progressively less. This effect reaches its maximum when the whole inter-division period has been spent in the phase of 'no growth'. Such cells which have undertaken no growth between divisions have a generation time of about two-thirds of that which is normal. This would seem to indicate that when growth is not occurring the processes leading to division can obtain additional energy or materials not normally available to them. If it is true that the volume growth shown after the change to Nitrogen-free medium is due only to continued wall growth, the decrease in the generation time when wall growth ceases, suggests that it is the energy normally used for Wall growth which is utilized to decrease the inter-division period.

There is, of course, nothing to indicate whether the division processes and wall synthesis are sharing some structural material or an energy source. It merely seems less likely that the nucleus and the wall would have a common building material than share a common source of energy. In either event, it is an extremely interesting phenomenon as it follows that competition normally occurs between division and wall growth for the factor common to them both. When this common factor is alone limiting, division and growth might be expected to fluctuate together, but when it is not limiting there will be an inverse relation between the rate of division and the rate of cellwall growth. Cell wall growth, in a medium supporting 'balanced' growth, will be ultimately dependent on the rate of cytoplasmic synthesis but there is no real reason to expect the rate of formation of nuclear materials to be related to cytoplasmic growth. It seems more likely that the nuclear material is truly self-replicating and that its capacity for growth is determined by the number of functional chromosomes, which is a genetic characteristic and independent of the state of the cytoplasm. The finding that DNA synthesis ceases during division (Barner and Cohen, 1956; Walker and Yates, 1952) would support this suggestion./

suggestion. The rate of division will thus be dependent on the availability of materials required for nuclear reduplication and the energy which the division processes can obtain. Some factor required, probably the energy, is shared with wall growth. Thus if any of the other requirements of cytoplasmic growth become limiting more of the factor will be available for division, and the mean size of the cells will decrease. On the other hand, there is no evidence that the reverse applies. On the contrary, Barner and Cohen (1956) found that when DNA synthesis was blocked in a thymine requiring mutant of E.coli. by withholding the thymine, other forms of synthesis continued normally. This rather suggests that when all the materials required for growth are available it exerts a priority over division for those materials which the two processes share, and only when growth is in some way inhibited can division increase its rate.

It is perhaps unwise to ascribe undue significance to the actual values of the different rates of division found in the yeast when the cell wall is growing and when it is not, but an interesting parallel has been reported. Scherbaum and Zeuthen (1954) subjected Tetrahymena pyriformis to repeated shifts of temperature from the optimum (29°C) to the sublethal value of 34°C. By this means division was prevented from occurring and the cells eventually reached a size about three times normal. When these cells were returned to the optimum temperature, division of eighty-five per cent of the cells occurred together after about an hour and a half, which is about two thirds of the normal generation time. Two further divisions occurred after this, also at intervals of an hour and a half. During this period of synchronous divisions the growth rate was considerably reduced. This seems to provide another instance of the rate of division being increased when the growth rate is reduced and it is interesting that the same relative difference is found between the division rates in Tetrahymena as in the yeast, though this is perhaps fortuitous. It may indicate that the normal/

normal relationship between growth and division is such that the division processes are working at a rate one third under capacity and that when growth is no longer making demands on the energy supply, division can draw enough energy to function at its maximum rate.

The return of Nitrogen to starved cells. When division eventually ceased the size of the cells ranged between a half and a quarter of the normal size at division. On the return of medium containing Nitrogen these cells started by growing exceedingly slowly. This 'lag' phase lasted for about an hour and a half. At the end of this phase growth became faster, the actual rate of growth being related to the size of the cell. When the Nitrogen had been returned as soon as divisions had stopped, the rate of growth taken up by the large cells at the end of the lag phase was slightly less than that shown by normal cells and in most cases this rate was maintained until division. The smallest cells, on the other hand, took up growth at a rate of about half the normal rate. This rate was maintained for a while but it eventually changed to twice its previous rate. Leaving aside, for the moment, the question of the lag period, the capacity for growth of the larger cells is clearly little impaired by the period of Nitrogen starvation. These are the cells which underwent their last division very soon after growth stopped. The smallest cells are those which divided after having spent the whole of the previous inter-division period in the phase of 'no growth' and the capacity of these cells for growth is reduced by half. In terms of the microsomal hypothesis this means that the larger cells have their full microsomal complement while the smaller ones have only half. It would thus seem that although chromosomal reduplication can take place in the absence of external Nitrogen microsomal multiplication cannot. It has already been argued that a certain number of microsomes can only be expected to support the growth of a certain amount of cytoplasm and one would therefore expect the cytoplasmic growth of the small cells to stop when this critical size is reached. Instead, however, the rate of growth doubles at that size at which/

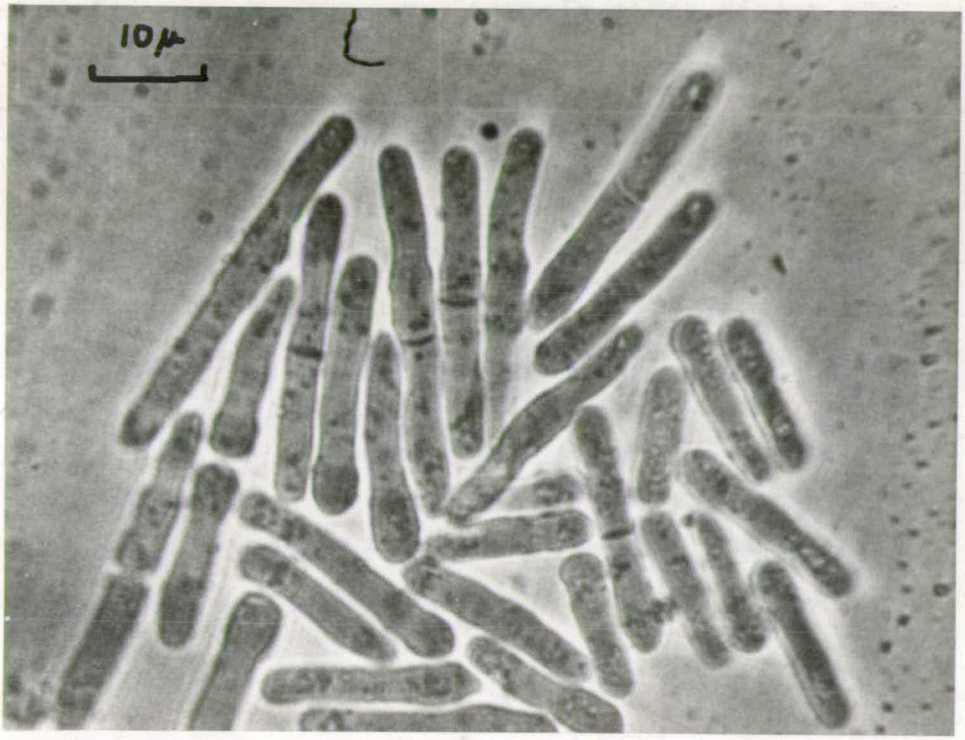

Figure 30. The first divisions occuring in cells replenished with Nitrogen after eighteen hours starvation.

which a normal cell with the same initial size would be expected to divide. This increased growth rate must be due to microsomal multiplication unaccompanied by nuclear division. Up till the point of this change in rate growth takes place at one end of the cell. After the change, however, growth occurs at both ends. It would seem that inspite of the fact that no cell plate is formed the multiplication of the microsomes involves separation of equal numbers of centres of wall synthesis to opposite ends of the cell.

It has been suggested that much of the continuing growth in volume found after the change from full strength to Nitrogen free medium is due to wall growth without corresponding synthesis of cytoplasm. This results in a dilution of the cytoplasm and the eventual cessation of wall growth. The most likely explanation of the lag period found when Nitrogen is returned to the cell is that the cell is re-adjusting this balance and that growth of the cell wall does not occur to any appreciable extent until the normal cytoplasmic concentration is achieved.

When the period of starvation is greater than that required to reduce the cells to their minimum size the cell's capacity for growth is reduced. This reduction affects all the cells equally and is a gradual effect proportional to the length of the starvation and is probably due to the RNA of the cell being slowly metabolized. Starved cells are well known to have a reduced RNA content (for example, see Harris, 1953).

When replenished with Nitrogen after a short period of starvation the cells start dividing after about six hours. The normal generation time is two and three quarter hours. These first cells to divide do so at a size considerably greater than the normal size at division and are the cells with the largest initial sizes. Most of the other cells divide within the next hour, those with the smallest initial size dividing last at a size about equal to the normal division size. The average time taken to reach division thus seems to be rather more than twice/

twice the normal generation time. The conclusions that can be drawn from this are severely limited by lack of biochemical information over the period. The time taken to reach division does, however, suggest that a cell starting with raw Nitrogen in the form of ammonium salts requires a period equivalent to two normal generation times to build up enough DNA to complete the chromosomal duplication. One might suggest that the formation of the nucleotide pool takes one generation time, as it must normally be formed at this rate in order to keep pace with division, and that it takes a further generation time to incorporate the nucleotides into the DNA. The larger cells divide a little earlier than the smaller ones. It seems probable that this is due to the fact that the smaller cells, which divided after a considerable time in the Nitrogen free medium, have completely drained their stocks of DNA precursors, while the larger ones still have some in reserve. However, the timing of division cannot be entirely independent of the rate of growth as it was found that increasing periods of starvation not only reduce the growth rate but also proportionally increase the time taken to reach division. In view of the widely different increments shown by cells of different sizes the relationship cannot be direct. It seems more probable that continuing starvation impairs some basic cellular mechanism such as the respiratory system and thus reduces the rate of all cellular activities.

A return of Nitrogen for a period of two or three hours was sufficient to return to many cells the ability to divide. Division, under these circumstances, followed at more or less the same time as division in those cells which had not been re-starved, although the rate of growth was maintained at the rate existing at the time of the re-starvation, and did not rise in the manner characteristic of the cells kept in the medium containing Nitrogen. The continuing linear growth is presumably comparable to that found on the Nitrogen starvation of normal cells. As a return of Nitrogen to the cells for only two or three hours was sufficient to bring many cells to division, it is clear that the materials/

materials accumulated during this period are sufficient for the eventual multiplication of the chromosomes. This lends strength to the idea that the formation of DNA from ammonium salts takes at least two normal generation times.

CONCLUSIONS.

The work which has been reported consists of straight forward observations on the behaviour of single cells with respect to their rates of growth and division under various definable environmental conditions. Such a study is essential before any attempt can be made to understand the mechanisms which control the rates of growth and division and their relation to each other.

There are, however, a number of difficulties about such observations. The number of cells that can be observed is necessarily small and any irregularities of growth assume unduly large proportions. The technical difficulties are also considerable. It is essential to keep the same cells under observation throughout an experiment and the cells must be prevented from showing Brownian movement. These two objectives were achieved by anchoring the cells under a thin agar blanket. Such a technique has its disadvantages. The agar blanket acts as a differential diffusion barrier and there is an inevitable variation in its thickness which makes it difficult to determine the time at which a change of medium becomes effective. The cells also showed a tendency to grow upwards into the agar rather than along the agar-glass interface. Such cells could not be accurately measured and so had to be ignored. In a field which in any case contained only a few cells such losses were regrettable. In the balance, however, the technique proved successful. Balanced growth was readily achieved and effects due to the agar blanket could usually be estimated.

The results of the observations made invite much speculation on the synthetic machinery involved. In the absence of comparable biochemical data the conclusions based on such speculations must be viewed with reserve. Nevertheless, they seem valuable as they indicate more clearly than the bare results the most profitable lines for further research. I therefore, offer themwithout apology.

Observations/

Observations on the growth of normal cells show that the volume growth accelerated for four-fifths of the life cycle and it seems likely that cell wall growth does so over the whole cycle. Mitchison (1957) has found that growth in total mass during the life cycle increases linearly. It follows, by substraction, that cytoplasmic growth must decrease over the cycle. Such a decreasing rate of cytoplasmic growth is suggestive of the inter-action between a mechanism which forms cytoplasm at a constant rate and of cytoplasmic breakdown, which would be expected to occur at a constant rate per unit of cytoplasm. The increasing rate of wall synthesis suggests that wall formation is a function of the cytoplasm, its rate of formation being dependent on the amount of cytoplasm in the cell. If this is the case the formation of new wall would not be expected to cease when cytoplasmic synthesis does, but would continue linearly until the increase in volume of the cell so diluted the cytoplasm that the operation of the enzymes responsible for wall formation was affected. This situation was found to arise when normally growing cells were transferred to a Nitrogen free medium, as the growth in volume after the change continued for a longer period than can be accounted for by utilization of the internal amino acids. A similar mechanism seems to apply to bacteria as Hancock and Park (1958) found that the synthesis of wall material in cultures of Staphylococcus aureus became linear when protein synthesis was blocked by Chloramphenicol. It would therefore seem that wall synthesis can occur within fairly wide limits without corresponding cytoplasmic synthesis but its rate is determined by the amount of some enzyme present in the cell.

It was found that cells grown under identical conditions did not grow to a particular size before dividing, although the range of division sizes was not great. All the cells showed a similar volume increment between divisions irrespective of their initial size. This would/

would tend to reduce the variability in cell size which might result from random variation in the food supply, as the daughters of an unduly large cell would not double their sizes before the next division, and daughters of an unusually small cell would more than double their size before the next division. This would account for the well known lack of variability in the size of the dividing cells in a logarithmically growing culture at any one time (Hinshelwood, 1946).

The volume increment between divisions, although constant for the cells in any one medium, varies with the medium in which the cells are grown. In Nitrogen free medium division can occur although no growth at all has been accomplished since the last division. Clearly, then, the occurrence of division can not be dependent upon growth. It seems more reasonable to suppose that a cell's capacity for growth and for division is genetically determined, and that different media, by making available different materials in different amounts limit the division mechanism or the growth mechanism separately. Thus for any one medium growth will be able to proceed at a certain rate and division will be able to proceed at a certain rate, and the growth shown between divisions will simply be the result of these two processes functioning at independent rates. This, however, is clearly not the whole story, for when the formation of cell wall is blocked the rate of division rises. There must, therefore, be some internal mechanism limiting the rate at which something required for wall growth and for division is supplied. As enough precursors for the doubling of the DNA appear to be present within the cell at the previous division (Walker and Yates, 1952) and as the cell wall is likely to have little in common withthe nucleus with respect to materials, it seems probable that the factor common to both is energy. Whether this extra energy acquired by division speeds the formation of nuclear materials from their precursors, or whether it increases the rate at which the energy reservoir used to effect division is filled, or both, cannot be decided nor is it particularly relevant to our argument./

argument.

Such evidence as there is suggests that the growth rate does not increase if division is blocked (Nickerson and Sherman, 1952; Barner and Cohen, 1956). It would thus appear that growth proceeds at the maximum rate of which it is capable and only such energy as remains goes toward division. On this basis one would expect the division rate to decrease before the growth rate when energy becomes limiting. This appears to be true of bacteria as filaments are formed when the concentration of carbohydrate in the medium is low (Hinshelwood, 1946; Nickerson and Sherman, 1952).

When balanced growth occurs the multiplication of the growth centres appears to coincide with cell division as this is the time at which the new rates of growth are established. Under conditions of Nitrogen starvation nuclear division occurs, apparently without corresponding microsomal multiplication as the growth rate of such cells on the return of Nitrogen is considerably reduced. However, this reduced growth rate is not maintained until division. The growth rate doubles before reaching division, and the doubling is accompanied by a change from growth at one end only, to growth at both ends. The size at which this change of rate occurs seems to be dependent on the initial rate of growth. The cells with a small initial size thus change their rate at a smaller size than do the larger ones. This suggests that the smaller cells, which grow slowly, and thus probably have a smaller number of microsomes, can undertake microsomal multiplication after less growth than can the larger, faster growing cells. This seems not unreasonable if it is assumed that microsomal multiplication takes place when there is enough material present for them to double their number. Chromosomal reduplication, on the other hand, would not be expected as soon as this, as the small cells have a full chromosomal content and enough material for their multiplication would not be present until considerably later.

It therefore seems that microsomal multiplication is dissociable/

ciable from nuclear division, but this does not mean that microsomal number is not controlled by the nucleus. It seems likely that RNA synthesis is directly controlled by the nucleus as the RNA content of the cells of polyploid series of yeast is proportional to ploidy (Ogur et al. 1952). If this is true, under conditions of balanced growth the number of microsomes per nucleus in any one medium would be constant and their multiplication would tend to coincide with nuclear division.

Thus a system can be envisaged in which the nucleus, by determining the rate at which RNA synthesis takes place, controls a cell's capacity for growth. The state of the cells in any one medium will, however, depend on the provision that the medium makes for the various synthetic activities. The matter of the energy supply appears to be somewhat special, as growth exercises a priority over division for the energy available. The cell, moreover, even under optimum conditions, appears to limit its own internal supplies of energy to an extent that all the demands of the cell are not satisfied.

SUMMARY.

1. A method was developed for observing the growth of single cells of the fission yeast, Schizosaccharomyces pombe, under controllable environmental conditions. The cells were grown in a perfusion chamber which was a modification of that described by Christiansen et al (1953). The growth of the cells was recorded by time lapse cine-photography.

2. The normal growth of the yeast in wort broth at 27.5°C was studied. It was found that the rate of growth in volume of the cell increased for the first four fifths of the life cycle. For the remaining fifth of the cycle there was no increase in volume. This constant volume period was concerned with the division of the cell. Growth of cell wall material appeared to be continuous throughout the life cycle.

3. In any one medium permitting balanced growth, the cells were found to divide after a certain amount of growth. They do not divide at a specific size.

4. The growth of the yeast was also studied in wort broth fifteen times more dilute than that used for normal growth. The characteristics of the growth curve were unaltered but growth was reduced to a greater extent than was division.

5. When the medium was changed from one concentration to another the behaviour shown by the cells depended on whether the change was from a high to a low concentration or the reverse. After a change 'down' volume growth ceased for a while before taking up the rate characteristic of the new medium. After a change 'up' the cells immediately took up the new rate of growth. The behaviour on a change down seems to be brought about by the necessity for the cells to adapt to the new medium.

6. When the medium was changed from full strength broth to a medium unbalanced in favour of carbo hydrate there was no apparent immediate change in the rate of growth. The continuing growth is considered/

considered to be due mainly to continued wall growth with resulting cytoplasmic dilution. The growth rate changes when dilution of the cytoplasm becomes excessive.

7. When the change was to a medium completely lacking in Nitrogen, division continued normally until cell wall synthesis ceased. After wall synthesis ceased there was usually one further division. The time between the last two divisions was shorter than usual, being least for those cells which did not grow at all between the divisions.

8. The cells were much reduced in size after Nitrogen starvation. When Nitrogen was returned to them there was a period of very slow growth usually lasting for about an hour and a half. The growth rate after the lag period varied with the size of the cell, being slowest for the smallest cells. This growth rate, however, doubled at some point before division, and cell growth, which normally occurs only at one end of the cell started at both ends.

9. These results were discussed in terms of the mechanisms controlling division and growth and their inter-relations with each other.

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