1) The radiation effect which has been investigated is the inhibition of cell division by ultra-violet radiation. The fission yeast *Schizosaccharomyces pombe* was a natural choice of experimental organism because its cell cycle has been extensively investigated in this Department. A short review of the literature on division delay revealed that many of the earlier investigations are inadequate. For various technical reasons *S. pombe* is ideally suited to the investigation of this effect.

2) The normal growth and division behaviour of *S. pombe* was investigated by time-lapse photography. The design of the division delay experiments was based on the results obtained.

3) Ultra-violet induced division delay was investigated by time-lapse photography of irradiated cells. Complex changes in sensitivity during the cell cycle were found. A period of major sensitivity extends from about the time of nuclear division to shortly after the time when cytoplasmic division is completed; at the end of this period there is a distinct critical point at which sensitivity falls dramatically. A period of moderate sensitivity follows which is terminated at a second critical point at about the time of nuclear division. The results suggest that two radiation sensitive processes are involved in the preparations for cell division. The period of major sensitivity corresponds with the most commonly reported critical point at prophase.

4) The changes in sensitivity during the cycle to division delay and lethal mutational killing are very similar and suggest that the two effects are related. A revised analysis of the data of another worker on the distribution of deaths among the progeny of irradiated cells indicated that the period of major sensitivity to killing and division delay is also the period of gene replication. Gene replication may be equivalent to DNA synthesis.

5) Another worker has shown that when stationary phase cells are transferred into fresh growth medium, a burst of DNA synthesis occurs almost immediately. An investigation of division delay in this situation showed that cells become sensitive to division delay at the time of the burst of synthesis; similarly they become resistant when the burst of synthesis is completed. The results lend weight to the hypothesis that the period of major sensitivity is the period of DNA synthesis.

6) An investigation of division delay at various temperatures showed that the two sensitive periods have different temperature dependencies, and that the period of major sensitivity occupies a smaller fraction of the generation time at lower temperatures. The results support the view that distinct processes are involved in the two sensitive regions and they may be consistent with the suggestion that the major sensitive period is the period of DNA synthesis.
7) The pattern of sensitivity found in *S. pombe* was compared with the data on division delay in other organisms. Although a simpler pattern seems to prevail in some cases, there is good evidence that complex changes in sensitivity occur also in the budding yeasts and it is possible that the pattern found in *S. pombe* is typical of all higher cells. No definite conclusions can be drawn about the mechanism of division delay but the suggestion is made that the mechanism underlying the major sensitive period is the inhibition of DNA synthesis and the second sensitive period is explained in terms of interference with nuclear division.
The Effects of Ultra-violet Radiation during the Cell Cycle

by

Brian F. Gill

Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Science

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PART I

Introduction

The radiation effect with which this thesis is chiefly concerned, is the inhibition of cell division, and the organism chosen for study is the fission yeast Schizosaccharomyces pombe. This phenomenon of division delay is a characteristic effect of radiation on living cells, and if it were better understood, we would gain not only considerable insight into the problems of radiation biology, but also much valuable information about the mechanism of cell division. My own attitude to the problem is to use division delay as a tool for the investigation of cell division and the cell cycle. This approach is a natural result of the climate of research in this Department where, for a number of years, a great deal of work has been devoted to the cell cycle of S. pombe. The investigations reported here are in many ways a continuation of this corporate research effort, and so the first part of this introduction will be taken up with a description of this background work; in the second part the phenomenon of division delay will be briefly reviewed.

The Cell Cycle of Schizosaccharomyces pombe

There are several excellent reviews of this work available (Mitchison, 1963a, 1963b) so only a brief summary is needed here. In 1957 Mitchison initiated the programme of research
into the cell cycle of this yeast which has continued ever since. He began by repeating, more extensively, the earlier work of Knaysi (1940) on the pattern of volume growth of single cells, but he combined with this, measurements of growth in dry mass, using an interference microscopy method (Mitchison, 1957).

This yeast is particularly convenient for measurements of this kind. It is a cylindrical cell with hemispherical ends; its size, 3.5 microns x 6-18 microns, is convenient for microscopy, and as it grows only in length, the increase in volume of a cell is directly proportional to the increase in length. Its generation time, about 2½ hours in a defined medium at 32°C, is convenient experimentally, and as the medium is relatively acid, the growth of bacterial contaminants is discouraged. It divides by the formation of a median cell plate, a type of division which more nearly resembles that of higher plants and animals than the budding yeasts. A central nucleus can be demonstrated by phase contrast microscopy and by staining methods (Ganesan and Swaminathan, 1958) and there is evidence of a nuclear envelope and nucleolus (Maclean, 1964), which again points to similarity to higher cells. However, nuclear division does not resemble that of higher cells, as there is no visible mitotic apparatus, and the nucleus divides without any marked changes in appearance.

The growth measurements showed that growth in volume follows a gently upward sloping curve for the first three-quarters of the cycle: volume growth then ceases and a plateau follows. At the beginning of this plateau stage the nucleus
divides and shortly afterwards the cell plate appears. Fission into two separate daughter cells follows. By contrast, growth in dry mass was shown to be linear throughout the cell cycle, the rate doubling to that of two cells around the time of fission. Cell concentration is thus at a minimum at the beginning of the constant volume stage, and rises to a maximum at about the time of cleavage.

In further experiments advantage was taken of the pattern of volume growth. As the cell grows only in length, a measurement of the length of a cell will define fairly accurately its stage in the cell cycle. Asynchronous growing cultures of the yeast were pulse labelled with various radioactive precursors of the main macromolecular cell constituents. The cells were then fixed and grain counts made on autoradiographs to determine the rate of incorporation. The mean grain count of large numbers of cells at various cell lengths, gave a rate of synthesis curve through the cell cycle. In this way it was found that the synthesis curves for protein, carbohydrate, and RNA, are approximately exponential (Mitchison and Walker, 1959; Mitchison and Wilbur, 1962). In view of the earlier dry mass measurements it was concluded that the pool of low molecular weight compounds must fluctuate in size during the cycle. This has recently been confirmed (Mitchison and Cummins, 1964).

Mitchison has also studied growth at various temperatures (Mitchison, Kinghorn and Hawkins, 1963). Down to 23°C the patterns of volume and dry mass growth are unaltered, but below
The synthesis of DNA has proved to be very difficult to locate, partly because of the extremely low content of DNA relative to RNA (1:100), but mainly because potential DNA labels, e.g. tritiated thymidine, are incorporated into RNA. Recently the timing of DNA synthesis was tentatively established by a method involving bulk measurements on partially synchronised cultures (Boetock, 1964). A more successful synchronous system has now been developed (Mitchison and Vincent, 1965) and Bostock has established the timing of synthesis with some certainty (Bostock, 1965). This work will be referred to in greater detail later.

The sensitivity to ultra-violet radiation during the cell cycle has been measured by Swann (Swann, 1962). He followed the fate of irradiated cells by time lapse photography, and found a period of maximum resistance to killing by UV approximately in mid cycle. He also estimated by an indirect method the timing of gene replication; and he noted the occurrence of a division delay following irradiation. It was this work which indicated the potential interest of an investigation of division delay and it will be considered in greater detail later.

There are a number of other investigations in which this yeast has been used but only one seems particularly relevant here. This is the detailed study of growth and division, under carefully controlled nutritional conditions, which was made by Faed (Faed, 1959). He studied particularly, the
effect of changes in the medium on growth and division, but his data on the normal cell cycle were of considerable use in the initial design of division delay experiments.

Division Delay

The extensive literature on division delay has been reviewed several times (Lea, 1955; Giese, 1947; Kimball, 1955; Carlson, 1954). The general picture which has emerged, is that radiation, both ionising and ultra-violet, specifically inhibits cell division. The resulting division delay only occurs if the cell is irradiated at a stage prior to mitotic prophase: irradiation at a stage later than this results in no delay, although the following division may be delayed. The usual interpretation is that one of the preparations for division, which is completed by about the time of prophase, is radiation sensitive. A further comprehensive review of the literature is unnecessary here, but it is worth pointing out that this general picture must be accepted with some hesitation: a brief survey of the literature will show why.

Radiation produces a variety of effects in living cells, and it is unfortunate that one of these effects is the inhibition of growth. If the growth of a cell is temporarily slowed or stopped after irradiation it is clear that division will be delayed, but this is quite different in character from true division delay, which results from the specific inhibition of cell division. These two effects must not be confused. It
appears that in most cases cell division is rather more sensitive to radiation than is growth, so that providing low radiation doses are used the division delay effect can be investigated. However it must not be assumed that this is always the case. For example Kimball has shown that in Paramecium caudatum growth is relatively sensitive to radiation (Kimball, Geckler and Gaither, 1952; Kimball, 1957) and in this organism division delay is attributed largely to an effect on growth, superimposed on a specific inhibition of division at an unstated stage in the cell cycle. Clearly then, the ideal investigation of division delay must include measurements of cell growth. When to this is added the problem of measuring changes in sensitivity during the cell cycle, it is clear that the investigation of division delay presents severe technical difficulties, and it is not surprising that most of the reported investigations are to some extent inadequate. The survey of the literature which follows is designed primarily to illustrate this point; as far as possible detailed discussion of individual papers has been deferred until the final part of this thesis.

The classic work on division delay is now twenty-five years old. Henshaw and Cohen irradiated the eggs of the sea urchin Arbacia punctulata with X-rays at various times after fertilisation (Henshaw and Cohen, 1940). They found that early in the cleavage cycle sensitivity was high but it soon dropped dramatically, and by the time of prophase X-rays did not delay cleavage. This work has been repeated using UV in
Arbacia and other species of sea urchins (Marshak, 1949; Blum and Price, 1950; Rustad, 1960). Although there are some differences, the sensitivity changes are essentially the same. A similar pattern has been found in other invertebrate eggs (Chase, 1938). These investigations of division delay are probably the best available; in no other material have the changes in sensitivity during the cell cycle been measured with such accuracy. It must be admitted though that a cleaving egg is a very unusual cell: it certainly isn’t growing in any normal sense and its huge size and the complications associated with fertilisation suggest that it may be of limited use as a model of normal cell division.

There are numerous reports of division delay in microorganisms. In bacteria several workers have noted the specific inhibition of division and the consequent appearance of filamentous cells, but in general the sensitivity during the cycle has not been measured (e.g. Lea, Haines and Coulson, 1937; Roberts and Aldous, 1949; Wyss, Haas, Clark and Stone, 1950; Errera, 1954; Harold and Ziparin, 1958; Grinevich and Talipov, 1963). Deering and Setlow have also noted the formation of long filaments after UV radiation in Eschericia coli (Deering, 1958), and from preliminary work on synchronised cultures they suggest that sensitivity is greatest just before and after cytoplasmic division (Deering and Setlow, 1957), but they do not present their results very clearly and in some ways their approach to the problem is unusual.

A number of workers have investigated division delay in
the protozoa. Kimball and his co-workers studied division delay in Paramecium induced by both UV and X-rays. They followed the retardation of cell division over several generations but they do not clearly describe the changes in sensitivity during the cell cycle (Kimball, Geckler and Gaither, 1952). Kimball's comments on the radiation sensitivity of growth in Paramecium have already been mentioned, and one wonders if this is a general feature of the protozoa.

Certainly, other workers have not checked for an effect on growth, and the significance of their results is in some doubt. For example Daniels observed division delay in the giant amoeba Pelomyxa carolinensis after high doses of X-rays (Daniels, 1952). His observations on the effect of fusion of unirradiated protoplasm with irradiated cells are interesting, but he did not measure sensitivity during the cell cycle, and it seems quite likely that his division delay involved the inhibition of growth. This criticism probably cannot be applied to the investigations of Mazia and Hirshfield who studied the effect of cytoplasmic amputation on division delay in Amoeba proteus. They used low doses of UV which probably did not affect growth but they did not report changes in sensitivity during the cell cycle. Other workers have investigated division delay by observing the lag in population growth following irradiation. Typical of these are the experiments of Ducoff on X-ray induced division delay in Chilomonas paramecium (Ducoff, 1957) and in Tetrahymena pyriformis (Ducoff, 1956). He followed population growth by
observing the optical density of cultures. Needless to say this method cannot distinguish between division delay and growth inhibition and does not reveal changes in sensitivity during the cell cycle.

Division delay has been investigated in a great variety of metazoan cells (e.g. Canti and Spear, 1929; Carlson, 1940, 1941, 1944, 1950, 1954; Dewey and Humphrey, 1961; Mak and Till, 1963; Neary, Evans and Tonkinson, 1959; and many others which will be referred to in the final part of this thesis). In most cases the approach has been indirect; what in fact has been measured is the proportion of cells visibly in mitosis at various times after irradiation. Although a depression of the mitotic index obviously indicates the inhibition of cell division it does not reveal growth effects and in most cases parallel measurements of growth have not been made (notable exceptions are Kohn and Fogh, 1959; and Klein and Forssberg, 1954). The mitotic index method is also a rather feeble tool for measuring the changes in sensitivity during the cell cycle. In some cases greater accuracy has been attained by combining mitotic counts with tracer labelling of DNA: in this way the position of a cell relative to the period of DNA synthesis at the time of irradiation can be estimated. More detailed comments on this method of studying division delay will be found in Appendix A.+

Broadly speaking, these investigations have shown that division can be delayed throughout interphase, but at prophase the cell becomes resistant to division delay; in this respect

+ It will be more appropriate to read this appendix in conjunction with the final discussion. p. 70.
the picture is essentially the same as that found in cleaving invertebrate eggs. However, there is evidence that sensitivity is not uniform throughout interphase and it is possible that there are more subtle changes in sensitivity that cannot be detected by the methods employed.

Finally there is some work on division delay in yeasts. In most cases the usual criticisms apply. For example Brace noted the appearance of large cells following irradiation. He showed that in these cells there was no change in specific gravity or nitrogen content per unit volume. He concluded that cell enlargement was due to the inhibition of cell division but he did not follow sensitivity during the cell cycle (Brace, 1950). A similar criticism applies to the work of Spoerl and his colleagues. They showed that division was selectively inhibited and made extensive observations on the metabolism of irradiated cells, but they too present no data on sensitivity during the cell cycle (e.g. Spoerl, Loveless, Weissman and Balske, 1954; Spoerl and Looney, 1958 a and b).

The most thorough investigation of division delay in yeast is that of Burns (Burns, 1956). He followed irradiated yeast by photography. Division was specifically inhibited by X-rays and there were changes in sensitivity during the cell cycle. Unfortunately he only measured sensitivity at selected stages in the cycle but he reports that cells become resistant to division delay when the bud is about half the size of the parent. This is a little before the time of nuclear division and might therefore be consistent with the sea urchin work.
This survey of the literature has shown that the accepted picture of division delay is based largely on the work on cleavage delay in invertebrate eggs. It receives virtually no support from work on bacteria or protozoa although the situation in yeast may be comparable. Experiments on higher cells have certainly confirmed the existence of a critical prophase step, but they also suggest more complex changes in sensitivity. In many cases division delay may have been confused with the inhibition of growth, and even when specific inhibition of division has been demonstrated, adequate measurements of sensitivity during the cell cycle have rarely been made.

In view of these comments it is not surprising that the mechanism of division delay remains in doubt. The various hypotheses will be discussed in some detail in the discussion at the end of this thesis but it may be helpful at this early stage if some of the main lines of argument are pointed out.

The critical prophase step suggests that it is in this region of the cell cycle that a clue to the mechanism of division delay will be found. Lea has suggested on the basis of Henshaw's data (Lea, 1955; Henshaw and Cohen, 1940) that radiation interferes with the condensation of the chromosomes which occurs at this stage. The hypothesis still has its advocates (Whitfield and Rixon, 1962). Another long standing suggestion is that division delay is brought about by interference with the cleavage mechanism (Giese, 1938). A related hypothesis has been proposed more recently by Rustad (1959a, b and c, 1961; convenient review, 1964).
Observations of the biochemical behaviour of irradiated cells led Mitchell many years ago to suggest that division delay resulted from the inhibition of DNA synthesis (Mitchell, 1942), and in later years this hypothesis gained much support. In recent years though it has been shown that DNA synthesis is restricted to a limited period of the cell cycle, and observations that division can be delayed by irradiation in the post-synthetic period have led many workers to reject this hypothesis. This is a controversial issue and further discussion of it will be postponed until later, but it is worth pointing out now, that the suggestion that division delay in higher cells is a complex effect makes it possible that more than one mechanism is involved.

It should now be clear that the fission yeast *Schizosaccharomyces pombe* is particularly suitable for the study of division delay. In this organism it will be possible to measure changes in sensitivity during the cell cycle with considerable accuracy, and it will be comparatively easy to make parallel measurements of growth. It will be shown that under these relatively ideal conditions the picture of division delay which emerges is rather different from that revealed by many of the earlier investigations.

**Summary**

Research into the cell cycle of the fission yeast *Schizosaccharomyces pombe* has been proceeding for a number of years
in this Department. The investigation of ultra-violet induced division delay in this yeast is a natural extension of this work. In most organisms the study of division delay is technically difficult and much of the earlier work is to some extent inadequate. For various reasons these difficulties are not encountered in *S. pombe* and the study of division delay in this organism is therefore particularly promising.
PART II

Observations on the Normal Cell Cycle of
Schizosaccharomyces pombe

Introduction

Many of the investigations on the cell cycle of S. pombe have made use of the fact that the length of a cell gives a good indication of its stage in the cell cycle. For example, Swann estimated the variations in sensitivity to UV killing during the cycle, by irradiating cells growing on an agar medium, and following their subsequent fate by time lapse photography. He then compared the sensitivity of cells in various length groups to find the changes in sensitivity during the cell cycle (Swann, 1962). It seemed reasonable to apply this technique to the study of division delay, but a preliminary investigation of the problem (Gill, 1962) suggested complex changes in sensitivity and it was felt that a more accurate method of assessing the stage in the cell cycle was required.

The main deficiency of the cell length convention is the inherent variability in the relationship between cell length and stage in the cell cycle, and the fact that cells do not grow in length during the last quarter of the cycle introduces a further complication. The latter objection is less serious if the beginning and end of the cycle is defined not by fission (Mitchison's convention) but by cell plate formation (Swann's
convention). In this way the errors due to the constant volume stage are divided between the beginning and end of the cycle. Clearly though, in a study of division delay it is important to decide which criterion of division is preferable, on less arbitrary grounds. In order to make this decision and to devise an accurate system for measuring the stage in the cell cycle, a more detailed investigation of the normal cycle was undertaken. The general lines of this investigation were suggested by some observations of Faed (1959) on smaller numbers of cells and under slightly different conditions.

Material and Methods

Stock cultures of *Schizosaccharomyces pombe* (N.C.Y.C. 132) were maintained in Edinburgh Minimal Medium (EMM). The composition of this medium is given in Appendix B. The cultures were maintained at 32°C in 10 ml. of medium in McCartney bottles and were subcultured once every 7 days using a 0.05 ml. micro-pipette. Standard sterile precautions were observed.

For experimental purposes 10 ml. of medium were inoculated with 0.05 ml. from a fully grown stock culture (3-7 days old), and incubated at 32°C for 12 hours. At the end of this time the cells are in the logarithmic phase of growth (Concentration = 1-2 x 10^6 cells/ml. Optical Density = 0.1 - 0.25 measured on a Unicam, S.P. 500. Measured at 595 µm). A bacteriological loopful of this culture was then transferred to an agar pad
(2% agar made up in EMM) lying in the well of a simple perfusion chamber. Details of the chamber are given in Appendix C. The cells were gently spread over the surface of the agar and left for a few moments to dry down. They were then covered with a quartz coverslip, and the chamber sealed with a mixture of wax and vaseline. The chamber was then transferred to the stage of a microscope in a warm room at 32°C ± 0.5°C, and connected to the perfusion system. This consisted of a 100 ml. reservoir, containing pre-warmed medium, attached to the chamber with poly-ethylene tubing. The exhaust side led through a pasteur pipette, selected to give a drop of 0.02 ml., into a second reservoir at a lower level. The rate of flow was adjusted by means of a tap on the inlet side to deliver 2 drops/minute on the exhaust side, and from time to time the reservoir was topped up to maintain the rate of flow as constant as possible. The reservoir was sterilised by steaming for 30 minutes and the tubing by boiling for the same period. The perfusion chamber was not sterilised and sterile conditions were inevitably broken in setting up the chamber. Nevertheless contamination was noted only once in two and a half years.

The cells were allowed to equilibrate for 5 hours and then they were photographed at five minute intervals for a further 6 hours. A X10 Gillet and Siebert 'Experimental' objective was used in conjunction with a X15 'Kelner' eyepiece and dark ground illumination. Between 50 and 200 cells were included in the field. Details of the time lapse apparatus are given
in Appendix D. The photographs were brought to a final magnifica-
tion of 500X so that a distance on the photograph of 0.5 mm. represented 1 micron. The parameters of growth and division were measured on the photographs.

Results and Discussion

a) The criteria of division

There are two ways of looking at this problem: one is to choose the criterion which is most convenient experimentally, the other is to choose the criterion which seems biologically correct. The formation of the cell plate is the first easily visible sign that a cell is about to divide, and it also signifies the physiological separation of the two daughter cells. On these grounds it might be judged the better criterion of division. On the other hand division is certainly not completed until the time of fission. The crucial question is, is fission an inevitable consequence of cell plate formation, or can the two effects be dissociated? Very occasionally in irradiated cells fission is dissociated from cell plate formation; the cell plate forms but fission does not follow, even though the daughters of such a division form cell plates and undergo fission normally. The fact that the daughters continue to divide normally does suggest that this situation may be of minor importance and there are reasons for believing that it is a result of a disturbance in the control of growth rather than in the control of division. It has been shown
that cells usually grow only at one end, usually the end away from the last point of division (Paed, 1959; May, 1962). This means that to form a cell plate, growth has to be switched from the end of the cell to the middle. Fission seems to be a further consequence of this growth. Growth is then rather surprisingly switched back to the opposite ends of the daughter cells. A slight disturbance in this switching mechanism could lead to the formation of a cell plate without fission. The evidence is hardly conclusive one way or the other but on the whole the adoption of cell plate formation as the criterion of division seems more reasonable. Some of the data on the normal cell cycle support this view.

Figure 1 shows the distribution of generation times using both conventions. The range of generation times is the same for both conventions and they are approximately normally distributed, but measuring the generation time from cell plate to cell plate does result in less variability. This suggests that it is cell plate formation which is subjected to tighter control. The distribution of cell plate times (time from appearance of the cell plate to fission) is shown in Figure 2. This is not normally distributed and suggests that the control of cell plate times is rather different from the overall control of generation time. In Figure 3 cell plate time is plotted against generation time: there is some degree of positive correlation, cells with a long generation time have a long cell plate time. In Figure 4 cell plate time is plotted against what may be called growth time (the time from fission
Figure 1. Frequency histograms of generation times.

a) Generation time measured from cell plate to cell plate.

b) Generation time measured from fission to fission.
Figure 3. The relationship between the duration of the cell plate stage and generation time. The slope of the regression line is significant at the 1% level. Variance ratio test.
Figure 4. The relationship between the duration of the cell plate stage and growth time.
Figure 2. Frequency histogram of the duration of the cell plate stage.
at the beginning of the cycle to the first appearance of the cell plate). There is no correlation. The correlation of cell plate times and generation times is therefore due to the addition of cell plate times on to normally distributed growth times. The duration of the cell plate period is therefore rather uncontrolled.

These observations suggest that cell plate formation is a better criterion of division than fission because it is over cell plate formation that control seems to be exercised. Fission follows cell plate formation as a result of further growth, but it can probably be influenced by physical factors like shaking, etc. In experiments involving the use of data on generation times, cell plate formation is a better criterion experimentally because there is less variability in generation times by this convention. Clearly division delay falls into this category.

b) The estimation of stage in the cell cycle

Figure 5 shows the distribution of lengths of cells at the end of the cell cycle, i.e. at the cell plate stage. Presumably this variability extends throughout the cell cycle and is an obvious source of error in the cell length convention. An alternative convention would be to base the stage in the cell cycle on the age of a cell, but as already seen in Figure 1 there is considerable variability in generation times. This can be reduced by taking into account the initial length of the cell. Figure 6 shows the relationship between generation time
Figure 5. Frequency histogram of the lengths of cells at the cell plate stage, i.e. at the end of the cell cycle.
Figure 6. The relationship between generation time and the length at which cells start the cell cycle.
and initial length: they are negatively correlated.

The system for assessing stage in the cell cycle is therefore to measure the age and initial length of a cell. The age of the cell is then divided by the mean generation time of cells of that initial length, to give a figure for the stage in the cell cycle. For example, a cell which is 80 minutes old and had an initial length of 8 microns would be scored at stage $\frac{80}{140} = 0.57$. Such a cell would be expected to divide in a further 60 minutes, so that if, after irradiation, it was observed to divide after 100 minutes, a division delay of 40 minutes would be recorded.

It would be very tedious to take photographs every five minutes in the more extended division delay experiments and in practice the time lapse interval has been lengthened to 20 minutes, and the median generation time has been used for assessing the stage in the cell cycle rather than the mean. The convention is still more accurate than the cell length convention: the sensitivity changes which will be described in the next section are characterised much less clearly if the cell length convention is used.

There is still variability within the initial length groups and an interesting consequence of this variability is that the method will inherently give a better measure of the stage in the cell cycle for young cells, than for old cells. For example, the median generation time of cells initially 8 microns long is 140 minutes and most cells fall within the range 120 - 160 minutes. A cell 20 minutes old will be scored
at stage 20/140 = 0.14 but it could be at stage 0.166 (20/120) or stage 0.125 (20/160). Similarly a cell 100 minutes old will be scored at stage 0.715 (100/140) but it could be at stage 0.833 (100/120) or 0.625 (100/160). Clearly the method is less accurate for older cells. It will be shown in the next section that the most dramatic change in sensitivity to division delay is near the beginning of the cell cycle and can therefore be characterised very well; but there are also changes near the end of the cycle, and when interpreting data about these changes the greater error in staging must not be forgotten.

Summary

A preliminary investigation of division delay in *Schizosaccharomyces pombe* suggested complex changes in sensitivity during the cell cycle. The usual convention, by which the length of a cell is taken as an indication of its stage in the cell cycle, did not seem accurate enough to characterise these changes properly. There was also some doubt about whether cell plate formation or cell fission should be taken as the beginning and end of the cycle. In order to solve this problem and to devise an accurate system for assessing the stage in the cell cycle, a detailed investigation of the normal cycle was undertaken. It was decided that the formation of the cell plate is the preferable criterion of division and a system was devised by which the stage in the cell cycle is based on the age and initial length of the cell.
PART III

Division Delay

Method

The methods of culturing and mounting the cells have been described in the last section. After transfer to the perfusion chamber the cells were allowed to equilibrate for five hours at 32°C. Time lapse photography (20 minute intervals) was then started. After a further three hours the cells were irradiated on the microscope stage with a Phillips 6 watt TUV Mercury Resonance lamp; photography was then continued for a further twelve hours. The UV lamp gives 98% of its output in the 2537 A line and the intensity on the microscope stage, measured with a calibrated thermopile, was 58 ergs/mm$^2$/minute. As a precaution against photoreactivation, the warm room was lit only by a dull red lamp for a few minutes immediately before and after irradiation, and an orange filter was used in front of the microscope lamp. At the time of irradiation all the cells had undergone between two and three divisions and were thus growing exponentially. The parameters of growth and division were measured on the photographs both before and after irradiation. For each cell the stage in the cell cycle at the time of irradiation and the division delay were calculated by the method described in the last section.
Results

Preliminary experiments suggested that a dose of 62 ergs/mm.\(^2\) (64 seconds irradiation) would be suitable. This dose produces considerable division delay but does not affect growth. In Figure 7 the logarithm of the total length of a group of 20 cells is plotted against time before and after irradiation. There is no effect on growth in the first 80 minutes following irradiation but after this time there is a slight downwards curvature. This apparent slowing of growth is misleading. In Figure 8 the growth of a sample of 20 cells which were at the beginning of the cell cycle at the time of irradiation is compared with a similar group of unirradiated cells. It will be shown later that cells at the beginning of the cell cycle are very sensitive to division delay, and in fact in this group no division occurs up to 400 minutes after irradiation. In the first 120 minutes after irradiation there is no appreciable difference between the two groups: the cells are following the upward sloping curve of normal volume growth. At the end of this period cell division is occurring in the control group and growth continues on its approximately exponential course. It is at this time that the difference appears between the two groups, and the difference can be attributed to the lack of division in the irradiated group. In the irradiated group the cells continue to grow as single cells; the growth rate continues to increase but more slowly than in the dividing cells. Eventually the growth rate stops
Figure 7. The growth of a group of 20 cells before and after irradiation. The total length of the cells is plotted on a log. scale against time. The cells were irradiated with a dose of 62 ergs/mm$^2$ UV at the time indicated by the arrow.
Figure 8. The growth of irradiated and unirradiated cells. The total length of two groups of 20 cells is plotted against time. All the cells were at the beginning of the cell cycle at time zero. Group-b was irradiated at time zero with 62 ergs/mm$^2$ UV; in this group no division occurs up to 400 minutes after irradiation. In the unirradiated group-a division occurs at approximately 140 minute intervals.
increasing but growth itself never stops nor does it slow down. Observations of these irradiated cells suggest that they continue to grow only at one end, though it is difficult to be certain about this. It is possible then that there is a limit to the rate of growth which can be maintained by one growing point. The important point about these growth measurements is that at no time after irradiation does growth slow, and for the first 120 minutes after irradiation there is no perceptible difference between irradiated and unirradiated cells. There is no possibility therefore that the division delay results from an effect on growth.

Figure 9 shows the changes in sensitivity to division delay during the cell cycle. This graph represents observations on about 600 cells derived from several experiments. Each point represents observations on one cell. The variability is large but the changes in sensitivity during the cycle are fairly clear. Early in the cycle the cells are sensitive and suffer a large division delay, but at about stage 0.2 there is a dramatic change in sensitivity, and the cells become resistant. Around the middle of the cycle division is delayed, but much less than in the early part of the cycle. Near the end of the cycle the cells become resistant and their division is not delayed.

The statistical analysis of this graph presents difficulties. For example in the regions of the cell cycle where sensitivity is changing there appear to be bimodal distributions. These would suggest that in these regions sensitivity
Figure 9. The sensitivity to division delay during the cell cycle. UV dose = 62 ergs/mm².
changes very quickly, a cell is either sensitive or resistant; there are in fact 'critical points' in the cell cycle. The bimodality could result from error in staging the cells or it might also indicate a variability in the stage at which cells reach the critical point. Unfortunately there are no completely satisfactory methods of analysing bimodal distributions in cases where the two populations overlap. Probably the best method available is that described by Harding (1949). The details of this method are given in Appendix E. When applied to a bimodal distribution this method gives an estimate of the mean and standard deviation of the two populations. Figure 10 shows the division delay data analysed by this method. The position of the critical points can now be estimated by counting the number of cells falling within the range of the standard deviations, and estimating the point at which there would be equal numbers of cells in the delayed and non-delayed populations. It cannot be claimed that this method is completely reliable but it does illustrate the changes in sensitivity more clearly.

Early in the cycle cells are very sensitive to division delay: a critical point is reached and the cells become resistant. Sensitivity then increases as the cycle progresses until a further critical point is reached and the cells again become resistant. Thereafter division is not delayed. One point which may seem puzzling is that cells at the end of the cycle are apparently delayed slightly. In fact this is due to the method of staging. Cells which are scored at stage 1.0
Figure 10. Sensitivity to division delay during the cell cycle. The data of Figure 9 have been analysed by the method described in Appendix E. The open circles mark the means of the separated populations; standard deviations are indicated by the vertical bars. The arrows indicate the estimated positions of the critical points.
have already been growing for the mean generation time of their initial length group, and therefore even though they may divide within the normal range of generation times, they will be scored as delayed.

In Figure 11 division delay at the second post-irradiation division is shown. Division delay in this cycle is estimated by comparing the length of the cycle with the normal mean generation time which is 140 minutes. This system is adopted because after irradiation most cells are longer than normal at the time of division, and the system which takes into account the initial length of the cells cannot be applied. There are clear changes in sensitivity during the cycle. Early in the cycle, the cells (which were greatly delayed at the first division) divide in less than the normal time. Some cells are again delayed at the second division but these cells are in fact destined to die. In the middle part of the cycle most cells undergo a slightly accelerated second division: the few cells which are delayed either escaped delay at the first division or are dying. In the last third of the cycle most cells are considerably delayed but a bimodal distribution is evident. In fact the cells which are delayed at the second division are the cells which escaped delay at the first division and vice versa. This again illustrates the critical point in this region of the cell cycle: cells which are past the critical point are not delayed at the first division but suffer a considerable delay at the second division.

A comparison of Figures 9 and 11 will show that the delay
Figure 11. Division delay at the second post-irradiation division. Sensitivity during the cell cycle. Dose as before.
Figure 12. Sensitivity to division delay during the cell cycle. The period of major sensitivity extends from stage 0.22 to stage 0.62. The second sensitive period from stage 0.22 to stage 0.62 has been drawn by eye.

Dose as before.
Figure 13. The relationship between the first and second cycle division delays. Delay at the second post-irradiation division plotted against delay at the first division. Dose as before.
to the second division of cells irradiated at the end of the cycle, is the same as the delay to the first division of cells irradiated at the beginning of the cycle. This would be expected for they are in fact two measures of the same thing from opposite viewpoints. The period of maximum sensitivity to division delay therefore extends over the last part of one cycle and the beginning of the next. The growth of sensitivity in this region can be illustrated by joining together the graphs of the first and second cycle delays. A regression line fitted through the delayed populations illustrates this growth in sensitivity (Figure 12). The method of calculating this regression line is discussed in Appendix F. This region of the cell cycle which ends at a critical point early in the cell cycle will from now on be referred to as the period of major sensitivity, to distinguish it from the other period of sensitivity which ends at a critical point about two thirds of the way through the cycle. Figure 12 defines the limits of the period of major sensitivity and one other new point emerges which is that the variability in division delay increases as the sensitivity increases.

In Figure 13 the relationship between first and second cycle delays is shown. With the exception of a few dying cells division is not delayed in two consecutive generations. Delay in the first generation is accompanied by acceleration in the second.

The accurate measurement of division delay at different doses presents difficulties. Above about 100 erg/mm$^2$ there
is an increasing effect on growth and estimates of division delay may have little meaning. At higher doses too lethality becomes a problem and because of the variations in sensitivity during the cycle selectively removes cells from certain regions of the cycle. It is unfortunate that these regions are the ones which are of particular interest in the study of division delay. The range of doses at which division delay can be properly investigated is therefore strictly limited. At lower doses it becomes very difficult to separate out bimodal distributions especially in the middle of the cell cycle. The curves of division delay at various doses shown in Figure 14 are not very useful. At the lower doses it has not been possible to separate the delayed from the non-delayed populations and the curves are based on mean division delays.

Discussion

The most significant feature of the data so far presented is the complex nature of the changes in sensitivity to division delay during the cell cycle. Clearly there are two periods of sensitivity and two critical points. This suggests that there are two processes involved in the preparations for division which are UV sensitive; these processes are completed at the times of the critical points. The second critical point seems to coincide with the most commonly reported critical prophase step for it is at about this time that the nucleus divides. The position of the critical point at stage 0.62 is perhaps a
Figure 14. Sensitivity to division delay during the cell cycle at various doses.
little early for nuclear division but it must be remembered that there is a considerable error in staging in this region of the cycle. Recent observations by Bostock (1965) have also indicated that nuclear division occurs rather earlier than had been thought previously. The form of the second peak suggests a continuous recovery from radiation damage; the delay increases steadily as the critical point approaches. The fact that the second division following irradiation is not delayed suggests that recovery has been completed by the time division occurs, and in fact cells irradiated in this region all take about the same time to complete division. Thus a cell at stage 0.2 which is not delayed completes division in about 120 minutes; a cell at stage 0.7 which is delayed 80 minutes also takes this time to complete division.

At the beginning of the cycle the delay is much greater and recovery therefore takes much longer but once again the fact that cells are not delayed in two consecutive generations suggests that recovery has occurred by the time of division. It is not possible, on the evidence so far presented, to make any speculations about the process which is involved in this region of the cell cycle, and the rest of this thesis is largely concerned with the identification of this process.

The accelerated second division of cells delayed at the first division is most easily explained in terms of the relationship between cell growth and cell division. Although older ideas that the onset of cell division is controlled by a critical nuclear-cytoplasmic ratio have largely been discarded
it is clear that cell division is not absolutely independent of growth; there is a certain amount of feedback between the two processes (discussed more fully by Mazia, 1961). Cells must normally be a certain minimum size before they divide and they do remain roughly the same size from generation to generation. Thus after a normal division some growth is necessary before further division can occur. When division is delayed however, cells grow to much larger sizes than normal before dividing and the daughters of these cells may be as long or longer than the normal length at which cells divide. Further growth as a preparation for division is not then strictly necessary. The length of the division cycle in these circumstances may give an indication of the time required to complete the growth independent preparations for division. It is probably more meaningful to consider the shortest cycle length that is observed rather than the mean cycle length, and in practice the second cycle length was never observed to be less than 60 minutes. This is about 40% of the normal division cycle and agrees fairly well with observations of division without growth in other organisms (e.g. *Amoeba*, Prescott, 1957; *Tetrahymena*, Scherbaum and Zeuthen, 1954).

The curves of division delay at different doses are not reliable and apart from the obvious point that division delay increases with dose, no other conclusions can be drawn.
Summary

In *Schizosaccharomyces pombe* division is particularly sensitive to radiation and can be considerably delayed by doses which do not affect growth. The changes in sensitivity during the cycle are complex. At the beginning of the cycle sensitivity is high and cells are greatly delayed. At about stage 0.2 there is a dramatic fall in sensitivity. Sensitivity then increases as the cycle progresses until a second 'critical point' is reached at stage 0.62. Thereafter division is not delayed. In the second generation after irradiation those cells which were delayed at the first division undergo an accelerated second division; cells which escaped delay at the first division are delayed at the second division. Division delay increases with dose. The existence of two critical points suggests that two radiation sensitive processes are involved in cell division. The second critical point resembles the usually reported prophase step but the process involved at the first critical point cannot yet be identified.
PART IV

UV Killing, Gene Replication and Division Delay

The Argument

The method by which Swann estimated the variations in sensitivity to UV killing during the cell cycle have already been described and it will be profitable now to compare his results with the data on division delay (Swann, 1962). In making this comparison it must be remembered that different conventions were used for measuring the stage in the cell cycle. Figure 15 allows comparison between Swann’s data on lethality and the data on division delay. In both cases there is a marked increase in resistance quite early in the cycle, but the timing of this increase is slightly different in the two cases. In fact this difference is entirely due to the different staging conventions. In Figure 16 sensitivity to killing as measured on the ‘age-initial length’ convention is shown. There is not enough data available at higher doses to plot LD50° but the curve shown of percentage deaths against stage in the cell cycle illustrates the point adequately. Sensitivity to UV killing and to UV induced division delay alter dramatically at the same point in the cell cycle. This suggests that the two effects are closely related.

The similarities are even more striking when the whole cycle is considered. From the second critical point to the
Figure 15. Sensitivity to killing and division delay during the cell cycle. Upper graph $LD_{50}$, lower graph division delay.
Figure 16. Sensitivity to UV killing during the cell cycle. Percentage deaths against stage in the cell cycle. UV dose = 124 ergs/mm².
end of the cycle cells are not delayed but they have not escaped radiation damage for they are delayed at the next division. To get a true picture of the sensitivity to division delay throughout the cycle therefore, this delay to the second division should be substituted for the last part of the curve of first division delay. This has been done in Figure 17. It would have been better to have plotted the dose required to produce a constant division delay during the cycle but this would have required data at higher doses which is undesirable in division delay experiments. It is reasonably certain that if this could be done the result would be very similar to Swann's curve.

This similarity is fairly strong evidence that killing and division delay are closely related, and as the killing curve is based on deaths due to lethal mutations it is reasonable to suggest that division delay is a result of damage to the genetic material. There are objections to this simple argument, for example, the similarity of the two effects might only indicate that the site of the primary damage and/or specific repair mechanisms are shared. The primary damage might not be on the genetic material. On the whole though modern opinion is turning towards the view that the primary site of UV damage leading to mutations is in the genetic material, i.e. DNA. This opinion is based on action spectrum evidence (Zelle and Hollaender, 1955) and on recent work which has shown that molecular changes can be induced directly in DNA by UV (reviewed by Wacker, 1963). Action spectrum evidence
Figure 17. Sensitivity to division delay (1st and 2nd cycle delays) during the cell cycle.
also suggests that damage to nucleic acid is involved in division delay (Giese, 1947). This subject will be returned to in the discussion at the end of this thesis but it is interesting to note now that there is some other evidence that division delay and killing are closely related. Burns reports that in budding yeast the changes in sensitivity to X-ray induced division delay and killing are very similar and he also concludes that they result from damage to the same cell organelles (Burns, 1956). On the other hand Kimball has claimed that there is no correlation between the extent of division delay and genetic damage in Paramecium (Kimball, 1950). This evidence may not be strictly relevant though, for Kimball has not measured the changes in sensitivity to division delay during the cell cycle. His curve of sensitivity to mutation induction during the cycle is very similar to the one found in S. pombe (Kimball, Gaither and Perdue, 1961; reviewed in Kimball, 1962) and comparable data on division delay might be very interesting.

At the moment it seems reasonable to assume as a working hypothesis that division delay results from damage to the genetic material. It has been shown that two radiation sensitive processes are involved in the preparations for division and one is therefore drawn to the interesting conclusion that the genetic material is involved in both of them.

Swann correlated the dramatic change in sensitivity early in the cycle with the occurrence of gene replication. He based his estimate of the time of replication on the distribution
of deaths among the progeny of irradiated cells. His argument was that if a lethal mutation is induced in a cell prior to the replication of its genes, then when gene replication occurs the lethal mutation will be replicated, and when the cell divides both daughters will receive the mutation and they or their progeny will eventually die. The whole pedigree will die, and as all the cells die from the same cause they may be expected to die in essentially the same way. On the other hand if a cell is irradiated after it has replicated its genes the chances are that a lethal mutation will be induced in only one gene set, or at least that mutations induced in both sets will be different. In this case it might be expected that only one side of the pedigree will die or that the two sides will die in demonstrably different ways. Swann's argument was that at some stage in the cell cycle there should be a change over from predominantly the former type of deaths (similar deaths) to the latter type (differential deaths). In fact his expectation was borne out very well in practice and he was able to show a marked change in pedigree type quite early in the cell cycle. The timing of this change corresponded quite well with the rise in resistance, which suggested that the increase in resistance was associated with the period of gene replication.

There were, however, a number of complications, perhaps the most important being that at the beginning of the cycle 50% of all deaths were of the differential type. Theoretically, at this stage, before replication, there should have been no differential deaths. It was also observed that isolated
deaths occurred in the third, fourth and fifth generations. Swann explained these results in terms of gene multistrandedness.

In the investigation of division delay the importance of observing the delay in the second post-irradiation division has been pointed out. Observation of this delay showed that a division could be delayed by irradiating a cell back in the previous cycle. One possible explanation of this observation might be that even when a cell is approaching a period of cell division, preparations (radiation sensitive preparations) might already be under way for the next but one division. With this in mind it seemed possible that the period of gene replication might extend back into the previous cycle, and the figure of 50% differential deaths at the beginning of the cycle might in fact mean that either the genes are replicated in 50% of the cells at this time or that the genes are only partly replicated in all the cells.

This hypothesis is easy to check and involves only a fresh analysis of Swann's data. What is required is not only information about the type of pedigrees which result when a cell is irradiated at all stages of its cycle, but information too about the type of pedigrees which result when the cell is irradiated back in the previous cycle. This analysis which I have called an $F_1$ analysis is illustrated in Diagram I and was carried out on data made available by Swann.

It was expected that the $F_1$ analysis would reveal one of two alternative results. The first, consistent with Swann's
Diagram I. In the parental analysis a comparison is made between cells B and C (or their progeny) and the pedigree is scored as either differential or similar. In the $F_1$ analysis the comparison is made between cells D and E (or their progeny) and between cells F and G (or their progeny).
original interpretation, would have the form shown in Diagram IIa; the second, consistent with the revised hypothesis, would have the form shown in Diagram IIb. In fact the analysis revealed a curve which is not consistent with either hypothesis (Figure 18). This is a very interesting curve but one difficulty is the discontinuity between the end of the $F_1$ curve and the beginning of the parental curve. One would not expect to find perfect continuity because cell length is not a perfect measure of stage in the cell cycle, but the discontinuity seems to be too large to explain in these terms. The two curves are, however, based on different data because the parental curve includes cells which die in the $F_1$ which will not be included in the $F_1$ curve. If these cells are excluded from the parental analysis the curves shown in Figure 19 are obtained.

Two points arise. First, the curves nowhere return to zero; differential deaths are apparent at all stages. This points once more to Swann's suggestion of multistrandedness. Secondly, the change from similar to differential behaviour extends over the last part of one cycle and the first part of the next. This period coincides quite well with the period of maximum sensitivity to ultra-violet (division delay and killing). At first sight the curve suggests that gene replication is a somewhat extended process occupying perhaps two-thirds of the cell cycle, but this is almost certainly not the case. The cell length convention tends to give an expanded estimate of the first and last parts of the cycle because the
Diagram II. Expected results of an F₁ and parental analysis. The vertical dashed line is the point of transition from the F₁ to the parental analysis. In IIa the period of gene replication (change over from similar to differential deaths) is early in the cycle. In IIb the period of gene replication extends over the last part of one cycle and the first part of the next.
Figure 18. Proportions of differential deaths plotted against cell length. A - PI analysis; B - Parental analysis.
Figure 19. Proportions of differential deaths plotted against cell length. Deaths in the P₁ have been excluded. The curves from the P₁ and parental analyses have been joined.
length at which cells begin and end their cycle varies considerably. This has been seen in the lethality curve where the change in sensitivity which Swann estimated at about stage 0.4 has been shown on the age-initial length convention to be at stage 0.2. It should be remembered too that the age-initial length convention is liable to similar errors in the last part of the cycle. The period of gene replication and sensitivity to UV may therefore be quite short but it does extend over the last part of one cycle and the beginning of the next.

On the basis of this information a model of the cell cycle can now be constructed (Diagram III). The essential point of this model is that gene replication is initiated shortly after nuclear division, proceeds during the period of cell division, and is completed by about the time of cleavage.

It is now widely accepted that the genetic material is DNA and one might therefore substitute the term DNA synthesis for gene replication. There are grounds for objecting to this simple view, for example the separation of the two strands of the DNA helix might constitute gene replication but this could precede synthesis. A comparable situation is the one where chromosomes begin to react to radiation as though double, before synthesis has started (Evans and Savage, 1963; Wolff, 1961; Wolff and Luippold, 1964). Nevertheless the simple view is basically reasonable. The timing of DNA synthesis in the cell cycle of *S. pombe* has therefore been tentatively established and a period of major sensitivity to UV has been
DIAGRAM III

A MODEL OF THE CELL CYCLE
correlated with it.

In the next part of this thesis evidence will be presented which links the period of major sensitivity more firmly to the period of DNA synthesis and it may be helpful to clear up one or two problems of terminology raised by the proposed model. It has become customary in recent years to use the terminology of Howard and Pelc (1953) by which the cell cycle is divided into a number of phases: a $G_1$ phase which follows mitosis and precedes DNA synthesis, an $S$ phase in which synthesis occurs and a $G_2$ phase which follows synthesis and precedes mitosis, the $M$ phase. The application of this terminology to the proposed model presents a number of difficulties. The crucial point is whether the $M$ phase is to be regarded as nuclear or cytoplasmic division. If nuclear division is adopted the model suggests a very short $G_1$ and a long $G_2$ but if cytoplasmic division is adopted the $G_2$ is very short (fission convention) or the $S$ periods and $M$ periods coincide (cell plate convention). It seems more reasonable to adopt nuclear division as the $M$ phase and the terminology which will be used in the following sections is therefore a short $G_1$ following nuclear division, an $S$ period extending over the time of cytoplasmic division and a $G_2$ extending from about the time of fission to nuclear division.

Summary

The changes in sensitivity to division delay and to UV killing during the cell cycle are very similar and suggest that
the two effects are closely related. The dramatic change in sensitivity to UV killing early in the cycle had been correlated by Swann with the occurrence of gene replication. A more elaborate analysis of Swann's data suggested that the period of gene replication extends over the last part of one cycle and the first part of the next and thus coincides not with the change in sensitivity but with the period of major sensitivity to killing and division delay. A model of the cell cycle has been proposed on this basis in which gene replication begins shortly after nuclear division and is completed by about the time of fission. It is suggested that gene replication may be equivalent to DNA synthesis.
PART V

Division Delay in a Partially Synchronised Division System

Introduction

In the early part of 1964 Bostock (1964) obtained the first data on the timing of DNA synthesis during the cell cycle. His method consisted essentially of bulk measurement of DNA in stationary and log. phase cultures. Stationary phase cells are all very short and there is some evidence that as cells come into stationary phase, division takes precedence over growth, so that cells come to rest at the beginning of the cell cycle. In this case it might be expected that if DNA were synthesised early in the cycle most log. phase cells would have the double amount of DNA; stationary phase cells would have only the single amount. On the other hand if DNA were replicated late in the cycle most log. phase cells would have the single amount and there would be little difference between stationary and log. phase cells.

Bostock found a mean stationary phase value of 0.018 pico grams DNA/cell and a log. phase value of 0.037 pico grams DNA/cell. This suggested an early doubling of DNA (he took fission as the point of division). However, the situation was more complicated than this. In a second series of experiments he estimated DNA content at intervals after re-inoculating stationary phase cells into fresh medium. He found that
within a few minutes of re-inoculation the DNA value increased from 0.018 pico grams to 0.028 pico grams and stayed at this level for something over 200 minutes. At about 240 minutes after re-inoculation, which is about the time the cells begin to go through a first partially synchronised division, the DNA value increased again to very high values, probably to over 0.050 pico grams. This result was rather puzzling but Bostock's final model system was as follows: the 1 c. amount of DNA is 0.014 pico grams/cell, cells come into stationary phase as a mixture of two sorts of cells, some which have replicated their DNA and have a 2 c. value of 0.028 pico grams, and some which are arrested prior to synthesis with a 1 c. value of 0.014 pico grams/cell. The mixture of these two populations gives a mean value of 0.018 pico grams/cell. Shortly after re-inoculation the 1 c. cells double their DNA to the 2 c. amount of 0.028 pico grams/cell. They remain at this value until shortly before division when the DNA doubles to a value of 0.056 pico grams for cells showing cell plates. This is immediately halved by fission into two cells with a value of 0.028 pico grams. In an asynchronous log. population two groups of cells will be present: one group with a 2 c. value of 0.028 pico grams/cell, the other with a 4 c. value of 0.056 pico grams/cell giving a mean log. phase value of 0.037 pico grams/cell.

There are a number of complications which Bostock deals with but the final model system which he proposes is very similar to the model proposed on the basis of pedigrees: gene
replication/DNA synthesis begins shortly after nuclear division. Bostock has recently repeated this work (Bostock, 1965) on the rather better synchronous system developed by Mitchison and Vincent (1965). His conclusions are essentially the same.

Bostock's re-inoculation system provides an opportunity to test the hypothesis that the period of major sensitivity to division delay is linked to the period of DNA synthesis, for in this system a burst of DNA synthesis occurs shortly after re-inoculation. This period of DNA synthesis has been shifted from its normal location in the cell cycle for it now occurs at the beginning of the cycle instead of between nuclear division and fission; if sensitivity to division delay could be demonstrated in the early minutes after re-inoculation it would be rather good evidence that sensitivity is linked to DNA synthesis, or something closely connected with it, and not to some other process which in the normal cycle occurs at the same time. Sensitivity to division delay was therefore investigated following re-inoculation.

Control

Methods

In order to measure division delay it is necessary to know more about the division behaviour of cells after re-inoculation. Cells from a stationary phase culture (10 ml. EMM inoculated with 0.25 ml. log. culture and incubated for 3 days at 32°C) were therefore transferred on to agar medium in the usual way
and their growth and division followed by time-lapse photography. The transfer in this case was carried out in the warm room at 32°C to avoid temperature shock.

Results

Figure 20 shows the increase in cell number following re-inoculation and the cell plate index (cell plates/100 cells). The first cell division occurs at 210 minutes but the peak cell plate index is at 270 minutes. The synchrony is not very good but it can be seen from Figure 21 that cells within a size group exhibit quite good synchrony. The method of measuring division delay is therefore to compare the time taken for a cell to divide after irradiation with the mean control time to division of cells in the same size group.

Division Delay

Methods

Cells were transferred from stationary phase on to agar as described. They were irradiated with a dose of 62 ergs/mm.² at intervals after re-inoculation and their growth and division followed by time-lapse photography. The division delay was found for each cell by subtracting from its observed time to division the mean control time to division for cells in the same size group. In separate experiments the cells were irradiated at fifteen different times following re-inoculation.
Figure 20. Lower graph - increase in cell numbers with time, following re-inoculation of stationary phase cells on to fresh growth medium. Upper graph - cell plate index (cell plates/100 cells) following re-inoculation.
Figure 21. Increase in cell numbers with time, following reinoculation of stationary phase cells of various size groups on to fresh growth medium. a = 9 microns; b = 8 microns; c = 7 microns; d = 10 microns; e = 6 microns.
Results

Figure 22 shows the sensitivity to division delay at intervals after re-inoculation. Cells become sensitive to division delay very quickly, maximum sensitivity is reached ten minutes after re-inoculation with a mean delay of 120 minutes. By 25 minutes after re-inoculation the cells are becoming resistant and this trend is complete by about 40 minutes. Thereafter the cells become sensitive again. From about 170 minutes on there is growing evidence of two populations, one of which is delayed and the other which isn't. By 200 minutes the delayed population is suffering a mean delay of about 300 minutes.

Discussion

The cells clearly become sensitive just when a burst of DNA synthesis has been demonstrated in the early minutes after re-inoculation, and equally clearly they become resistant when this period of synthesis is completed. The hypothesis that sensitivity to division delay and DNA synthesis are linked has thus gained support.

The second wave of sensitivity was also to be expected as it is reasonable to suppose that in this partially synchronous system one should observe most of the changes characteristic of a normal cell cycle, though in a somewhat extended form. The sensitivity from 60 - 240 minutes can therefore be taken as the
Figure 22. Sensitivity to division delay at intervals after re-inoculation of stationary phase cells on to fresh growth medium. The group of cells shown to the left of zero time is a control group which was not irradiated.
second peak of sensitivity to division delay. There are, however, a number of difficulties in this simple interpretation.

In the first peak the mean delay is only about 120 minutes in the re-inoculation system, compared with about 280 minutes in the normal cycle. The most obvious explanation of this is that in both cases the cells are taking about the same time to divide, i.e. about 420 minutes, giving a mean delay of $420 - 140 = 280$ minutes for normal cells and $420 - 300 = 120$ minutes for cells in the re-inoculation system. Some observations of my own and a more detailed investigation by Vincent (1965) indicate that cells grow very slowly for the first two hours following re-inoculation, and only adjust to the normal growth rate at the end of this time. This might suggest that the progression of the irradiated cell towards division depends more on temperature than on the general metabolism of the cell.

It is interesting too that in the early minutes after re-inoculation the cells respond to radiation as a unimodal population. There are a few odd cells which are delayed about $250 - 350$ minutes but these are dying. Bostock proposed that cells entered stationary phase as two separate populations only one of which replicated its DNA in the early minutes after re-inoculation. On the hypothesis that sensitivity is linked to DNA synthesis one might have expected to find two populations, one sensitive, the other resistant, but this is not apparent. There are several alternative explanations for this. In Bostock's original experiments he used a complex medium: in the division delay experiments a minimal medium was used.
Bostock has, however, repeated his work using the minimal medium and has found exactly the same situation. One rather worrying explanation is that the coincidence of UV sensitivity and DNA synthesis is purely fortuitous and that sensitivity to UV marks the timing of some other process not related to DNA synthesis but occurring at the same time. It is surprising though that this other process, whatever it might be, should preserve its temporal relationship to DNA synthesis in the re-inoculation system. Another alternative is that Bostock's original hypothesis was wrong and that all cells enter stationary phase with a DNA content of 0.018 pico grams, that is that cells come to rest having partially replicated their DNA. This is a rather surprising suggestion but it must be seriously considered.

The second peak in this re-inoculation system also presents some difficulties. In the normal cycle the maximum delay in the second peak is about 100 minutes but in the re-inoculation system much greater delays are observed. In this peak there are, however, clear indications of two populations, at all stages there is a small group of cells which is extensively delayed, at 40 minutes after re-inoculation the delays in this group range from 120 - 225 minutes. This group seems to be progressively delayed more as the end of the cycle approaches, culminating in delays of around 300 minutes at 200 minutes post-inoculation. The lower population fits quite well into the normal pattern of the second peak, with mean delays of about 100 minutes or a little more. If one accepts
the hypothesis that sensitivity marks synthesis, the presence of some synthesising cells at all stages is indicated. This could be interpreted either as a group of cells which slowly synthesise their DNA throughout the re-inoculation cycle, or it could mean that different groups of cells are coming into a period of synthesis at various times after re-inoculation. This latter interpretation seems unlikely as it would result in steadily rising DNA values throughout the cycle: Bostock's data do not rule out a slight increase but they certainly do not show a rise of this kind.

The question that now arises is what sort of synthesis this is. If all cells synthesise in the early minutes after re-inoculation sufficient to raise the mean DNA value from 0.018 pico grams to 0.028 pico grams, is this second group of synthesising cells to be taken as a group which immediately initiates a new round of synthesis, or is it to be taken as evidence of two distinct populations in stationary phase, one of which has already partially replicated its DNA and consequently finishes synthesis quickly and the other which has not replicated its DNA and consequently takes longer to do so? If this second possibility is to be accepted Bostock's estimations of the 1 c. and 2 c. amounts of DNA will require modification.

In the absence of more information there is little point in pursuing these speculations further. Bostock's recent work on a rather better synchronous system has in general confirmed his earlier conclusions but work is still proceeding and it will probably be some time before a definitive interpretation
emerges. The observations on division delay have, however, upheld the prediction that the burst of synthesis in the early minutes of re-inoculation should be accompanied by sensitivity to division delay and this can only be taken as good evidence of a strong link between sensitivity and DNA synthesis.

Summary

Another worker has shown that when cells in stationary phase are re-inoculated into fresh medium a burst of DNA synthesis occurs almost immediately. Observations on division delay in this system show that cells also become sensitive to division delay shortly after re-inoculation. They become resistant again at about the time when the burst of DNA synthesis is completed. The results strongly suggest that the correlation between sensitivity and the period of DNA synthesis which was indicated by the work on logarithmically growing cells was not fortuitous. There are, however, a number of complications which cannot be convincingly explained at the moment but work in progress may eventually lead to a satisfactory interpretation.
PART VI

Division Delay at Different Temperatures

Introduction

Data on the temperature dependence of complex radiation effects like division delay is of very limited value. It is probable that in such effects a large number of reactions are involved each with their own particular temperature dependence; and the overall temperature dependence therefore has little meaning. In the case of *S. pombe*, however, there are some reasons for believing that a study of temperature dependence might be interesting. First, it might be hoped that the two radiation sensitive processes, which the division delay data indicate, would have markedly different temperature dependencies. This might at least confirm that independent processes are involved. Secondly, it would be interesting to know if the observations of Mitchison, Kinghorn and Hawkins (1963), which showed that the pattern of dry mass growth alters below about 23°C, have any relevance to the pattern of sensitivity to division delay.

Methods

The experimental set up was essentially as before. Stock cultures were maintained at various temperatures. Cultures
for experimental purposes were incubated until an optical density of 0.1 - 0.25 was reached as in the experiments at 32°C. After transfer to agar the cells were equilibrated until they had undergone between two and three divisions. Irradiation and photography were as before except that at the lower temperatures it was found necessary to increase the time lapse interval to 30 minutes in order to get a complete experiment on to one film.

Some difficulty was encountered in controlling the temperature accurately particularly at temperatures near to 20°C which is close to the ambient temperature of the laboratory. In practice these experiments were carried out on cold summer days when there was no artificial laboratory heating. The equilibration period was arranged as far as possible to be overnight when the temperature control was most effective. During the day time it was impossible sometimes to avoid temperature fluctuations of as much as ± 1°C. This is a fairly serious temperature fluctuation especially at these low temperatures where a small variation in temperature alters the growth rate considerably. This variation should only affect the extent of the division delay, it should not affect the position of the critical points, etc., as one would expect these to be fixed during the equilibration period when the temperature control was adequate.
Results

Figure 23 shows the relationship between generation time and temperature; the line has been drawn by eye. In Figure 24 the sensitivity to division delay at various temperatures is shown; a regression line has been fitted through the major peak, in the second peak the line has been drawn by eye. The position of the critical points has been estimated in the usual way. The general picture of division delay is essentially the same at all temperatures but there are some differences. The first critical point shows a slight tendency to move towards the beginning of the cycle as the temperature is lowered; the second critical point moves markedly towards the end of the cycle as the temperature is lowered from 32° - 24° C but below this temperature it moves very sharply in the opposite direction. The critical point defined by cells becoming sensitive to delay in the second post-irradiation division is in all cases in the same position as the second critical point in the parental cycle. At all temperatures, therefore, no cells escape delay and it also turns out that as at 32° C division is not delayed in two consecutive generations. A consequence of the movement of the critical points is that the period of major sensitivity occupies a smaller fraction of the cell cycle as the temperature is lowered to 24° C, below this temperature the trend is reversed and the sensitive period occupies a larger fraction of the cycle (Figure 25).

In Figure 26 the relationship between division delay and
Figure 23. The relationship between generation time and temperature.
Figure 24. Sensitivity to division delay at various temperatures. The critical points, etc., are indicated as in Figure 12. A - 21°C, B - 24°C, C - 28°C, D - 32°C.
stage in the cycle

division delay

(minutes x 100)
Figure 25. The position and duration of the period of major sensitivity at various temperatures.
Figure 26. The relationship between division delay and temperature. The mean time from irradiation to division at the first and second critical points is plotted against temperature.
temperature is shown; the generation time curve has been included for comparison. In fact the mean time to division at the critical points has been plotted rather than the division delay as this probably gives a better indication of the time required for recovery. The points for 19.5°C may be more inaccurate than the others for they are based on a rather small number of observations in an unrepeated experiment. The time to division at the second peak follows the generation time curve quite closely, but the first peak shows quite different behaviour: it diverges from the generation time curve markedly, especially at the lower temperatures. The temperature dependence of these effects is shown better by an Arrhenius plot in Figures 27 and 28. This type of plot is used to reduce the relationship between the rate of a process and temperature to a straight line, the slope of which indicates the temperature dependence in terms of a value for the critical thermal increment . In the range of biological temperatures this is directly comparable with the Q_{10} (Heilbrunn, 1937).

In the major peak the slope is slightly different from that of generation time, although in view of the errors involved in estimating it the difference may not be significant. Below 24°C, however, the slope is quite clearly different, and it may be safely concluded that at these lower temperatures recovery is not closely linked to the growth rate of the cell. For the second peak the situation is less clear. Figure 25 suggested that time to division at this peak is fairly closely tied to generation time but on the Arrhenius plot this is much
Figure 27. The temperature dependence of division delay.
Arrhenius plot. A - time to division at the first critical point. B - generation time.
Figure 28. The temperature dependence of division delay. Arrhenius plot. A and B - time to division at the second critical point (alternative lines). C - generation time.
less obvious and no one line fits the points really convincingly. It should be noted that in this sort of plot discontinuities are to be expected, and there is therefore little point in fitting a regression line. Two alternative lines have been drawn: the one which comes closest to all the points suggests a rather greater temperature dependence than generation time, the other following on from Figure 25 has been drawn with the same slope as the generation time curve. It is perhaps pointless to try to choose between these alternatives but it does seem clear that the two peaks of division delay have different temperature dependencies, at least below 24°C.

Discussion

It has already been pointed out that little importance can be attached to the values of the critical thermal increment of division delay. The two peaks do show different behaviour with temperature, however, and this helps to confirm that they involve independent processes. It has been suggested that in both peaks the site of the fundamental UV damage may be the same, so perhaps it is fair to conclude that it is the recovery processes which are different. The sharp change in temperature dependence below 24°C in the first peak is interesting although not particularly informative. High temperature coefficients of this order are usually associated with de-naturation-re-naturation phenomena but it is difficult to imagine what might be undergoing de-naturation-re-naturation in this particular
range of temperatures. It must be remembered too that the point for 19.5°C may be inaccurate so that although a change of temperature dependence certainly occurs it may not be as great as the graph suggests. It is interesting too that the change in temperature dependence occurs at about the temperature that the pattern of dry mass growth was found to alter by Mitchison, Kinghorn and Hawkins (1963). Taken at face value this might suggest a link between the mechanisms which control the pattern of dry mass growth and the processes involved in recovery from UV damage, but until more is known about the significance of dry mass growth this sort of suggestion is not going to lead very far (see discussion in Mitchison et al., 1963).

The change in the position of the critical points as the temperature is lowered is very striking, particularly for the second critical point. It has already been suggested that this second critical point corresponds with the usual prophase step and may therefore be closely related to the timing of nuclear division. It is possible then that the results indicate that nuclear division changes its position in the cycle as the temperature is lowered. Nothing is known about nuclear behaviour at lower temperatures but recent observations of Bostock (1965) have at least indicated that the position of nuclear division is not fixed under all conditions. He has shown that its position alters during the logarithmic phase of growth, moving first towards the beginning of the cycle and then towards the end of the cycle as the logarithmic phase progresses. The magnitude of the changes he has estimated are
of the same order as the changes in the position of the critical point. The possibility must be considered, therefore, that the observed changes in the position of the critical point may be attributable not to temperature changes but to different positions in the population growth cycle. The criteria by which cells were judged to be in the same stage of population growth at the time of irradiation, i.e. the same cell concentration and two to three cell divisions after transfer to agar, may not be reliable. Cells grown at 24°C, for example, suffered a much smaller temperature shock on transfer to agar than cells grown at 32°C. This possibility is not very likely and it is difficult to see how it could account for the changes in position which were observed, but it must be considered and clearly an investigation of the position of nuclear division at various temperatures is desirable.

The temperature at which the critical point moves most sharply towards the beginning of the cycle is about the temperature that a plateau appears in the dry mass growth curve. It is possible that these two effects are related but it is not easy to offer a rational explanation. It could be perhaps that nuclear division occurs during the last part of dry mass growth, and thus below 25°C, when dry mass growth is completed earlier in the cycle, nuclear division also occurs earlier, but this does not explain why nuclear division should move to a later position in the cycle between 32°C and 24°C. Nor does it explain why dry mass growth and nuclear division should be connected in this way especially as dry mass growth
may bear little relation to synthesis (discussed by Mitchison et al., 1963).

The first critical point also changes position, but only slightly, and it tends to move fairly constantly towards the beginning of the cycle as the temperature is lowered. The rather surprising consequence of this is that as the temperature is lowered, the period of major sensitivity occupies a smaller fraction of the generation time down to 24°C; below this temperature it once more expands. In the earlier part of this thesis the idea was developed that the period of major sensitivity is the period of DNA synthesis and it is interesting to note that there are some grounds for believing that the S period would show a similar contraction on lowering the temperature. It was in fact partly in the hope of observing such a contraction that these experiments were initiated. This was at a time before Bostock established the timing of synthesis directly and evidence was needed which would link the period of sensitivity to the period of synthesis more firmly.

There is evidence that the variability of generation times of cells within tissues reflects mainly variability in the $G_1$ period; the S and $G_2$ periods remain relatively constant (Mendelsohn, 1960). This has been confirmed experimentally for cells in tissue culture where changes in the growth conditions affect principally the $G_1$ period (Richards, Walker and Deeley, 1956), and there is a growing amount of evidence that a similar situation occurs in bacteria (Schaecter, Maaloe
and Kjelgaard, 1958; Maaloe, 1963). In these experiments the generation time was lengthened by changing the growth medium but there is at least one naturally occurring case where the differences in generation time might be ascribed to differences in temperature (Quastler and Sherman, 1959; Quastler, 1960; Sherman, Quastler and Wimber 1961.)

It might then have been expected that in *S. pombe* the S period and thus the sensitive period would occupy a smaller fraction of the generation time at lower temperatures. There are some difficulties in this interpretation though. Firstly, all the evidence suggests that it is the G₁ period which is extended when the generation time is lengthened. In *S. pombe* the G₁ is very short at 32°C, and one might therefore have expected that at lowered temperatures a pronounced G₁ period would appear between nuclear division and synthesis, that is, that a gap would appear between the critical point defined by delay in the first division and the critical point defined by delay in the second division. This gap would be detected by the presence of a group of cells which showed no delay in either division, but such cells have not been found. This means either that cells are also delayed in the G₁ or in *S. pombe* it is the G₂ period which is extended. The latter suggestion seems more likely for if there was delay in an extended G₁ period no contraction of the sensitive period would be expected and it is already clear that there are difficulties involved in applying the usual G₁, G₂ terminology in *S. pombe*.

This interpretation is highly speculative and open to
criticism but it does offer a neat explanation for the observed contraction in the sensitive period. Underlying this interpretation though, there is a rather surprising basic assumption that the rate of DNA synthesis is relatively temperature independent. In many ways this seems unlikely although there is very little experimental evidence which points one way or the other. Some years ago Lark and Maaloe (1956) found that in *Salmonella typhimurium* the rate of synthesis at 25°C was half the rate at 37°C but they did not measure the rate at intermediate temperatures. Over a comparable range, 32°C - 21°C, the division delay data are also consistent with a halving of the rate. There is some rather weak evidence that DNA synthesis may not be temperature dependent coming from work on division synchrony induced by temperature shocks. For example in *Bacillus megaterium* it has been reported that on transfer from 34°C to 15°C DNA continues to be synthesised at a rapid rate for a short period (Falcone and Sybalski, 1956). This suggests that at least part of DNA synthesis is not temperature dependent. A similar sort of situation has been reported in *Tetrahymena* after heat shocks (Zeuthen, 1964). This is not very good evidence; the behaviour of cells after a sudden change of temperature may have no relevance to their behaviour in balanced growth at the new temperature.

On theoretical grounds one might expect that DNA synthesis would be rather temperature dependent for it now seems clear that it is an enzymic process (Kornberg, 1957; Bollum, 1963). On the other hand if the rate limiting process in synthesis
were a physical one, for example the unwinding of the double helix, a low temperature coefficient would be expected. Various workers have proposed mathematical models for the unwinding of the helix (Fong, 1964a, b; Longuet-Higgins and Zimm, 1960) which in general suggest that unwinding is a fairly rapid process, but so many assumptions are involved in their calculations that it would be unwise to accept their conclusions too readily. The question remains open, therefore, and it is clearly a field which deserves attention.

It is apparent that the interpretation of the data on the temperature dependence of division delay is very difficult. None of the interpretations suggested can be regarded with much confidence but there is clearly some interesting information here which in the light of future work may be valuable.

**Summary**

The investigation of division delay at different temperatures has shown that the two peaks of sensitivity to division delay in the cell cycle have different temperature dependencies. This may indicate that different recovery processes are involved in these regions of the cell cycle. The position of the critical points in the cell cycle alters with temperature and the period of major sensitivity passes through a phase of contraction and then expansion as the temperature is lowered. No definitive interpretation is possible but a speculative model system consistent with all the data is as follows. The
period of major sensitivity is the period of DNA synthesis. DNA synthesis is relatively temperature independent in the 32°C to 24°C range. Synthesis is initiated at all temperatures shortly after nuclear division, which changes its position in the cell cycle with temperature.
PART VII

Discussion

The picture of division delay which has emerged is rather different from that outlined in the introduction to this thesis, and it may be helpful now to briefly summarise the information that has been obtained. There are two critical points in the cell cycle marking the ends of two separate periods of sensitivity: a major sensitive period which extends over the time of cell plate formation and a second sensitive period which ends at a critical point around the time of nuclear division. The period of major sensitivity to division delay is also the period when cells are most sensitive to UV mutational killing and it also appears to be the period of DNA synthesis. The first part of this discussion is taken up with a re-examination of the literature in the light of this information, the second part is devoted to speculations about mechanisms.

The Literature

It would be desirable to confine discussion to work on division delay induced by ultra-violet as opposed to ionising radiation but as there is comparatively little work on UV induced delay this would limit the scope for discussion severely. The two types of radiation do have, outwardly at
least, very similar effects and until the time comes to deal with mechanisms it is perhaps not necessary to differentiate between them.

Work on cleavage delay in invertebrate eggs has in general revealed a simpler pattern than the one found in *S. pombe*, but there are two pieces of work which may suggest the more complex pattern. The first is the classic work of Henshaw and Cohen (1940) on X-ray induced cleavage delay in the eggs of the sea-urchin *Arbacia puctulata*. Although these workers found that sensitivity declined to zero by the time of prophase, the fall in sensitivity was not quite continuous, there was a small but distinct discontinuity just before prophase. They made very little comment about this discontinuity but they did suggest that it indicated a distinct mechanism. In comparison with *S. pombe* this is an interesting observation for if one regards the sea-urchin cleavage cycle as a rather condensed normal cycle, the discontinuity could well represent a distinct second peak. The other piece of work is the investigation of Gross on UV induced cleavage delay in the eggs of *Chaetopterus* (Gross, 1950). This work has only been reported in a short abstract and is therefore rather difficult to interpret, but it appears that in a normal cycle of 57 minutes the eggs are sensitive for the first 30 minutes; sensitivity then falls off, but rises again in the period from 40 - 50 minutes. The sensitivity in the period from 50 minutes to the end of the cycle was not determined. In the absence of more information it is difficult to be critical about this work but one very striking
feature is that for the dose used (3000 ergs/mm\(^2\)) the delays observed were very small (6-7 minutes in the most sensitive region of the cycle). This does indicate that Chaetopterus is very resistant to this type of UV damage and therefore may not be entirely typical. The failure to establish the sensitivity in the period from 50 minutes to the end of the cycle is unfortunate; if for example sensitivity did not decline after 50 minutes it would be fairly good evidence that this high UV dose was interfering directly with the progression of mitosis rather than with the preparations for mitosis. Finally, it seems rather surprising that delays of the order of five minutes could be established accurately relative to a normal cycle of 57 minutes.

Other investigations of UV induced cleavage delay have not confirmed this picture. Blum and Price (1950), working with the same species as Henshaw and Cohen, found that sensitivity was high at the beginning of the cell cycle and then fell fairly quickly to zero at a critical point at about the time of prophase. There was no evidence of a discontinuity. These workers did not state the dose used but they observed delays of 1-2 generation times comparable to those observed by Henshaw and Cohen. A very similar pattern was found by Rustad in Strongylocentrotus purpuratus (Rustad, 1960). He found a plateau of sensitivity in the early part of the cycle followed by a transition to resistance at the beginning of prophase (delay at the plateau 50 minutes; GT 90 minutes 400 ergs/mm\(^2\)). He did find though that in the first cycle following
fertilisation there was a period of high sensitivity early in the cycle which soon declined to the plateau. As this high sensitivity was not apparent when cells were irradiated early in the second cycle following fertilisation he concluded that it is an effect associated with fertilisation and is not a feature of the normal mitotic cycle. It should be noted that Henshaw and Cohen, and Gross only observed cleavage delay in the first cycle following fertilisation; this could be the explanation of the more complex pattern which they found.

A similar picture of high initial sensitivity declining to a plateau was reported many years ago for UV induced cleavage delay in the eggs of the sand dollar *Dendraster excentricus* and the marine worm *Urechis caupo* (Chase, 1938). Again irradiation was only carried out in the first cycle following fertilisation. Unfortunately Chase did not state the absolute dose used but there were changes in the pattern of sensitivity as the dose was altered. At a dose of 60 seconds, the pattern was essentially the same as that found by Rustad, but at lower doses the plateau disappeared, only the high initial sensitivity remained. Rustad, on the other hand, found no change in the pattern of sensitivity with changing dose.

It is clear that apart from the existence of a critical point at prophase there is no entirely consistent pattern. The observed differences may be attributed to the use of different species and/or different radiations and doses. There is no very strong evidence that there are two peaks of sensitivity in the cell cycle but there is at least a
suggestion of it. It could be argued that in the highly contracted cleavage cycle it would be unlikely that two peaks could be detected, and it must always be remembered that cleavage may be an inadequate model of cell division.

On one point there is almost complete agreement with the results on *S. pombe*: that is that after the critical point irradiation produces no delay in the immediate division but delay is observed in the following division. Even here though there are some differences, for example Chase found no acceleration of division in the second division of cells which were delayed at the first division, all cells were again delayed. However, less delay at the second division was observed for those cells which had been highly delayed at the first division. The accelerated second division of *S. pombe* has been interpreted in terms of division without growth, and as cleaving eggs are not in fact growing it is perhaps not surprising that no acceleration is observed. However, the fact that further delay is apparent does suggest that the cell has not completely recovered from the UV damage by the time cleavage occurs, and one wonders if this also applies to *S. pombe*; could it be, for example, that there is a residual delay in the second cycle which is masked by the acceleration in division attributed to division without growth?

There are no really adequate investigations of UV induced delay in yeasts but X-ray induced delay in budding yeast has been very thoroughly investigated by Burns (1956). There are difficulties involved in making comparisons between the budding
and fission yeasts, and at first sight Burns' results seem rather different from those obtained with *S. pombe*; on close inspection, though, there are striking similarities. Burns' method was to select cells at the same stage in the cell cycle by micromanipulation, transfer them to agar, irradiate them and follow their fate by photography. He says that there is a change in the pattern of response to radiation at only one stage in the cell cycle, at the time when the bud is about half the size of the parent. Accordingly he only presents data on division delay for cells at two stages:

1) When the bud is mature, i.e. the same size as the parent. This is a few minutes before division occurs, in budding yeast usually taken as the first appearance of the next bud.

2) About 15 - 25 minutes after the inception of budding when the bud is about one-third the diameter of the mother cell.

It will be helpful to consider some of his data in detail. When cells were irradiated at stage 1 with a dose of 7000 r, the first division following irradiation was only slightly delayed (he does not say how much), but the second division following irradiation was greatly delayed (mean delay 234 minutes, normal cycle 67 minutes). On the other hand, cells irradiated at stage 2 with the same dose suffer a delay at the first division of 63 minutes. The transition from one pattern to the other occurs when the bud is about half the size of the parent and this becomes interesting when one realises that it
is at about this time that the nucleus is thought to replicate (Williamson, 1964). The similarity to the situation in *S. pombe* now begins to become apparent; it would seem that irradiating a cell before the time of nuclear replication produces only a moderate delay to division. Irradiation later than this point causes only a slight delay, but there is a major delay to the second division. The great difference between the budding and the fission yeast is that in the budding yeast the major delay is never observed in the same cycle as the radiation was administered, but this rather curious situation clearly arises from the choice of different criteria of division in the two cells. In *S. pombe* the period of major sensitivity ends at about the time of fission, and it is clear that if fission had been chosen as the criterion of division the major delay would not have appeared in the same cycle as irradiation, it would have appeared as a delay to the second division following irradiation. In fact this is not quite true for although most cells which have undergone fission have become resistant some remain sensitive for a little longer. This may be a point of difference between the budding and fission yeasts, but more probably it simply indicates that the preparations for cell division, which are radiation sensitive, take place more than one cycle ahead of division, and are therefore quite independent of the cell division which is concurrently in progress. The picture of division delay in the budding and fission yeasts is therefore strikingly similar: in both cases there are two critical points in the cell cycle at
which the pattern of response changes. In the budding yeasts one of the critical points occurs just at the time of division and this is undoubtedly why Burns failed to recognise it as a point of transition. It is unfortunate that Burns has not presented data on delay at other stages; it would be interesting to know, for example, if there is a growth of sensitivity as the critical points approach as in *S. pombe*. One suspects that Burns' preliminary study, which indicated one of the points of transition, was probably not detailed enough to provide this sort of information.

In *S. pombe* the period of major sensitivity is also the period of sensitivity to killing and it appears to be the period of DNA synthesis. In the case of the budding yeast there is also evidence that division delay and lethality are closely related; the changes in sensitivity to the two effects are similar and Burns has suggested that they result from damage to the same cell organelles. As they seem to be affected differently by ploidy, however, Burns concludes that the damage is qualitatively different in each case. DNA synthesis in budding yeasts is stated to take place shortly after division (Williamson, 1964) but this is based on evidence from synchronised cultures in which the rise in bulk DNA parallels the increase in cell numbers. One feels that workers may have been influenced to some extent by the preconceived idea that DNA synthesis must occur between one cell division and the next, rather than between one nuclear division and the next. As far as one can see, the data do not exclude
the possibility that DNA is synthesised slightly before or around the time of division. It is possible then that the sensitive period in the budding yeasts includes the period of DNA synthesis but on present evidence the duration of the DNA synthetic period could not account for the entire sensitive period. There are two possible explanations for this: first that the synchronisation procedures have accentuated the step-like nature of DNA synthesis, or secondly that the sensitive period includes also the $G_1$ which in $S. pombe$ is very short.

The problem of division delay in the cells of higher plants and animals has been approached mainly by indirect methods. The interpretation of experiments based on these methods is extremely difficult and there are good reasons for believing that many of the interpretations that have been proposed are inaccurate or misleading. In Appendix A the difficulties involved in these methods are pointed out and two typical papers are discussed in detail; the reader is recommended to read this appendix before proceeding further.

The problem has been investigated in a great variety of cell types. Individual workers have used slightly different approaches but the criticisms outlined in Appendix A are generally applicable and it is not now proposed to discuss other papers in detail. It is perhaps not surprising that no entirely consistent pattern has emerged and a survey of the literature is liable to be confusing. There is quite good agreement that cells in tissue culture irradiated with ionising radiation suffer a delay when irradiated in the $G_2$ and $S$ periods
with a possible delay in the $G_1$, e.g. mouse fibroblasts (Dewey and Humphrey, 1961); Ehrlich Ascites tumour cells (Kim and Evans, 1964); L cells (Mak and Till, 1963); Chinese hamster cells (Hsu, Dewey and Humphrey, 1962); human kidney cells (Bootsma, 1965). A similar situation has been reported in Hela cells by Terasima and Tolmach (1963a). As this report was based on observations on synchronised cells it must be accepted more readily. A $G_2$ delay is also suggested by the work of Carlson on Chortophaga neuroblasts (Carlson, 1950, 1954). In this case division delay was observed directly but the neuroblast is not a very typical cell for prophase occupies about a half of the cell cycle. Carlson found a critical point in late prophase; irradiation before this point produced a delay the magnitude of which depended on the nearness of the critical point. This is certainly a $G_2$ delay but Carlson does not present his results in a way which makes comparison with other cells easy; on the whole he is more concerned with the prolongation of the various phases of mitosis. There is fairly good evidence that the Chortophaga neuroblast is most sensitive to delay induced by UV radiation when irradiated in interphase although delay is also induced by irradiation in prophase (Carlson and Hollaender, 1944, 1948; Carlson, 1954). In this cell DNA synthesis extends over the whole of interphase (Gaulden, 1956) so this is a delay in the $S$ period. Chinese hamster cells irradiated with UV are said to be most sensitive in the late $G_1$ early $S$ (Dewey and Cork, 1963) but there is a small delay to $G_2$ cells. This is similar to the pattern
deduced by Vant Hof for X-ray induced division delay in *Pisum sativum* (Vant Hof, 1963) and it could be consistent with the observations of Neary, Evans and Tonkinson (1959).

To attempt a summary is hazardous but one or two features are clear. Division can be delayed in all cases by irradiation in the G₂. Division can also be delayed by irradiation earlier in the cycle; the bulk of the evidence suggests that the S period is most sensitive but the possibility of sensitivity in the G₁ cannot be ruled out. The suggestion that sensitivity is greatest in the last part of the G₁ and early S fits in with what little evidence there is on sensitivity to killing during the cycle which suggests that the change in sensitivity to killing is linked to DNA synthesis. Whether it is the initiation or completion of DNA synthesis which is important is not really clear (Sinclair and Morton, 1963, 1965; Terasima and Tolmach, 1963a and b).

There is a fair amount of work on the sensitivity to chromosomal damage during the cell cycle. This work must be approached with caution for in most cases the interpretation of the results is dependent on a knowledge of division delay. In some cases maximum sensitivity is reported in the S period (Dewey and Humphrey, 1961; Humphrey, Dewey and Cork, 1963) and in others in the G₂ (Hsu, Dewey and Humphrey, 1962; Koller, 1953; see also comments by Evans and Taylor in discussion Smith, 1963).

The work on higher cells can only be said to be inconclusive; there is good evidence that division delay in these
cells is a complex effect but as yet it has not been accurately characterised. It is entirely possible that the pattern found in *S. pombe* might apply to higher cells.

**Summary**

There is fairly strong evidence that the pattern of sensitivity to UV induced division delay in the fission yeast applies also to X-ray induced delay in budding yeast. In both cases the sensitivity to lethal damage parallels the sensitivity to division delay and there is some evidence that the period of major sensitivity is related to the period of DNA synthesis. Work on cleavage delay in invertebrate eggs provides some support for the idea that the pattern found in the fission yeast may be of general application, although on the whole a simpler pattern seems to prevail. Work on higher cells is difficult to interpret but it is clear that the pattern is complex and may resemble that found in the fission yeast. There is some evidence that changes in sensitivity to lethality in higher cells are related to the occurrence of DNA synthesis but the exact timing is in some doubt.

**The mechanism of division delay**

In the past there have been two main approaches to the mechanism of division delay. One approach is based on the finding of a critical point at prophase and seeks for a mechanism which would operate in this region of the cell cycle.
The other is based on observations of the biochemical behaviour of cells after irradiation.

It seems fair to apply the interpretations of the critical prophase step to the second peak in the cell cycle of \textit{S. pombe} which ends at a critical point at about the time of nuclear division. The biochemical evidence could apply to both peaks but it will be suggested that it is more relevant to the first peak.

It was suggested earlier on the basis of the similarity in the pattern of sensitivity to division delay and lethal mutation that the same fundamental UV damage is involved in division delay at both peaks, and it was suggested that this is damage to the genetic material. A great deal of the older work at least supports the view that division delay is a nuclear effect. The main arguments have been reviewed by Giese (1947) and Kimball (1955); briefly they are based on the following observations: Cleavage delay can be produced by irradiation of the sperm as well as the egg (e.g. Henshaw and Francis, 1936; Henshaw, 1940). Cleavage delay can be induced by irradiating nucleated half eggs but not enucleate halves (Henshaw, 1938; Blum, Robinson and Loos, 1950, 1951). Action spectrum evidence suggests a nuclear effect for sperm but a cytoplasmic effect for the egg (reviewed by Giese, 1947) and work on photoreactivation of cleavage delay also indicates the involvement of cytoplasm (Blum, Robinson and Loos, 1950). It may be concluded that the site of damage is in the nucleus but that the cytoplasm is involved in recovery.
The two main hypotheses for the mechanisms involved at the critical prophase step have already been briefly described in the introduction to this thesis. One suggests an interference with the condensation of chromosomes at prophase, the other, in its most recent form, an interference with centriolar reproduction. Modern exponents of the first hypothesis cite as evidence that chromatin condensing agents prevent mitotic delay (Whitfield and Rixon, 1962). Their argument seems basically sound although their estimations of division delay are based solely on cell counting, and thus a growth effect is not excluded. The basis of this objection, which is that they may be artificially condensing the chromosomes and thus inducing the growth blocked cells to undergo mitosis, seems extremely unlikely, but it ought to be considered. The hypothesis of Whitfield and Rixon is backed up by a number of cytological observations which have shown that delayed cells are held up in prophase with the chromosomes in the expanded state (Henshaw, 1940) and in some cases even a reversion from the condensed to the expanded state has been observed (Carlson, 1940, 1941, 1950). At the moment this hypothesis is little more than a refined description of the effect; no explanation has been offered for the mechanism of radiation induced chromosome expansion. A great deal more experimental work of the Whitfield and Rixon type is required but this should preferably be conducted on material in which division delay can be accurately characterised.

In recent years the principal devotee of the second
hypothesis, interference with centriole reproduction, has been Rustad (1959a, b and c, 1961a and b; briefly reviewed in Rustad, 1964). He bases his arguments on two lines of evidence. In his work on UV induced cleavage delay in the sea urchin he tied down the period of transition from sensitivity to resistance very accurately to just before the streak stage, which is when the asters migrate to opposite sides of the nucleus. The second line of evidence is that at high doses of X-rays and UV, multipolar cleavages occur which clearly indicate that the normal replication of the centrioles has been disturbed. This hypothesis is slightly difficult to reconcile with the older observations which show that it is the sperm which contributes the functional centrosome for the first cleavage (Henshaw and Francis, 1936) and yet irradiation of the unfertilised egg results in a cleavage delay. Rustad does suggest, however, that it might be a nuclear trigger for centriole reproduction which is interfered with, and this is a way around the difficulty. The hypothesis clearly has merits but at the moment it is difficult to see how it could be tested. Quite certainly it cannot be applied in its present form to division delay in plant cells, yeast, etc., all of which complete division in the absence of an observable centriole. The hypothesis also has its opponents. Levis and Marin, for example, take the view that multipolar cleavages are a result of division delay and not the cause of it (Levis and Marin, 1963).

The known biochemical effects of radiation are far too
numerous to be listed here but they have been comprehensively reviewed by Bacq and Alexander (1961). The great majority of these effects cannot be invoked as the mechanism of division delay however, for division delay is characteristically an effect of low radiation doses; the biochemical process which is involved in division delay must be extremely radiation sensitive. It has long been recognised that one of the most sensitive processes in cell metabolism is DNA synthesis and inhibition of this process has often been suggested as the mechanism of division delay. In recent years though, this idea has met with vigorous opposition, at least for division delay induced by ionising radiation. The main arguments have been reviewed by Kelly (1957) and have been neatly summarised by Bacq and Alexander (1961). While the inhibition of DNA synthesis is not denied, they claim that this inhibition is a result of mitotic delay and not a cause of it. Either cells are arrested before DNA synthesis, or they are arrested at a later stage than synthesis but the mitotic block prevents another round of synthesis from being initiated. In both cases bulk measurements will indicate an apparent inhibition of synthesis. They cite, too, experiments in which DNA synthesis has been found to continue in the absence of cell division.

The argument is fairly convincing but there are one or two weak points in it, especially if the pattern of sensitivity found in *S. pombe* applies to all cells. The first point that cells may be arrested before DNA synthesis is really only making a distinction between the inhibition of synthesis and
the postponement of synthesis; the end result is the same in both cases, the completion of synthesis is delayed. There is certainly some evidence that much higher doses are required to block DNA synthesis once it has started than in the pre-synthetic period and this may be interpreted as an interference with enzymes required for synthesis (Lajtha, Oliver, Kumatori and Ellis, 1958; Howard and Pelc, 1953; other references in Bacq and Alexander, 1961). On the other hand Terasima and Tolmach (1963a) have clearly demonstrated that maximum inhibition occurs when the cells are irradiated in the S period, and they find no inhibition in the G1. They cite too other work which supports their observations. The second point that mitotic delay may prevent cells from initiating a new round of synthesis is based on experiments like those of Whitfield and Rixon (1959) in which it was found that DNA synthesis proceeded after irradiation up to the pre-mitotic level and then stopped until cell division restarted. These are valuable experiments for they clearly show the interdependence of DNA synthesis and cell division, but the main conclusion may not be justified. The experiments involve bulk measurements of DNA and it is unlikely that they could detect the presence of a fraction of cells in which DNA synthesis was inhibited. The duration of the period of inhibition of DNA synthesis has in many cases seemed too short to account for division delay but if there are two separate mechanisms this may not be a difficulty; the crucial point is the relative importance of the two mechanisms in different cell types. For example Terasima and Tolmach
(1963a) found a prolongation of the S phase in synchronised Hela cells of about 0.6 minutes/rad but division delay in this period was rather greater. They conclude that irradiation in this period induces a G₂ delay but they recognise that the total delay observed is not solely attributable to a G₂ delay, the inhibition of synthesis is also involved. In this cell it is clear that the G₂ delay is greater than the S delay but this may not be so in all cells. The presence of two mechanisms might also explain the observations of division delay without an effect on DNA synthesis.

One final technical point. The inhibition of DNA synthesis is often inferred from the reduced uptake of labelled precursors and the point must be made that in the absence of further evidence uptake cannot be equated to synthesis. Terasima and Tolmach (1963a) have pointed out that reduced uptake ³H-thymidine could result from breakdown of nucleic acids with consequent dilution of the labelled precursor. They exclude this possibility in their experiments but it may occur in others. It must also be demonstrated that the precursor is being incorporated into DNA, in the fission yeast, for example, a ³H-thymidine label is eventually incorporated into RNA (Mitchison, 1963a). It may be pedantic to raise objections of this sort but in the present confused state of the literature on radiation effects the value of rigorous methods cannot be overemphasised.

As the evidence stands at the moment there is a case to be made out against the involvement of the inhibition of DNA
synthesis in division delay induced by ionising radiation but it is not an indisputable case and if it does turn out that the pattern of sensitivity found in the yeasts is of general application, some re-thinking may be necessary. For division delay induced by UV radiation the involvement of the inhibition of DNA synthesis seems quite likely, for whereas it is possible that ionising radiations act upon the genetic material indirectly there is a growing amount of evidence that for UV the action is direct, and considerable progress has been made towards an understanding of how the action might work at the molecular level.

It is now clear that low doses of UV cause the formation of thymine dimers in DNA; dimers may be formed between adjacent thymines along the helix or across it. Deering has written an excellent introductory article to the subject (Deering, 1962) and a more detailed treatment and further references are to be found in a review by Wacker (1963). Although it is clear that dimer formation is not the only action of UV upon cells, there is good evidence that it is a major one, and it does offer an explanation for the inhibition of DNA synthesis and for at least the first steps in the formation of mutations.

There are one or two reports of division delay induced by UV in the absence of an effect on DNA synthesis and these must be considered. First there is evidence that in UV irradiated L strain mouse cells DNA synthesis proceeds until the normal pre-mitotic level is reached and then stops (Whitfield, Rixon
and Youdale, 1961). As in their work with ionising radiation (1959) the presence of a fraction of cells in which synthesis is inhibited could probably not be detected. Deering and Setlow (1957) have reported the formation of long filaments in *Eschericia coli* after UV radiation; DNA synthesis was not affected. However, the filaments were said to be multi-nucleate which suggests that the phenomenon may have been rather different from the usual division delay effect. *E. coli* is not a very suitable organism for the study of division delay, for in normal growth the formation of cross walls is a rather haphazard affair and the number of nuclei is extremely variable.

DNA synthesis can certainly be inhibited by low doses of UV (Kelner, 1953; Kanazir and Errera, 1956; Dendy and Smith, 1964) but as yet there is very little evidence on the time course of the inhibition and there is no data which allows comparison between the extent of inhibition and the extent of division delay. The work of Doudney (reviewed in Doudney, 1962) has shown that the extent of inhibition is dependent on dose and in *E. coli* at least could account for delays of the order of 1 or 2 generation times after quite low UV doses. Comparable data from non-bacterial cells is urgently required. *S. pombe* is now a promising material for this sort of study.

The hypothesis that UV induced division delay results from the inhibition of DNA synthesis clearly then merits attention; it is also clear that it cannot be invoked as the mechanism of the critical prophase step for this occurs much later than the
period of synthesis. In *S. pombe* therefore the inhibition of DNA synthesis is proposed as the mechanism which operates at the first peak, the period of major sensitivity. It remains now only to construct a model system on this basis which can be reconciled with all the information that has been obtained.

There are five main points which have to be considered:

1) Delay is induced when cells are irradiated in the S period. It is possible that delay is also induced by irradiation in the very short G_1.

2) The delay increases as the S period progresses. Maximum delay is induced in cells near the end of the S period.

3) Variability in division delay increases as the S period progresses.

4) Sensitivity to division delay is closely paralleled by sensitivity to killing.

5) The temperature dependence of division delay must be considered.

If the inhibition of DNA synthesis is attributed to the formation of thymine dimers the explanation of the first point may be quite straightforward; as Wacker has pointed out "...if dimerisation occurs between the two individual strands of DNA (intermolecular) the dividing mechanism of the cell may be completely blocked. Although an intermolecular dimerisation in native DNA appears improbable, during cell duplication there are phases with cleaved DNA strands in which intermolecular dimerisation is more likely." The suggestion here is that the
formation of between strand dimers is more likely at the time when DNA is actually replicating. Thus cells would be more sensitive to the inhibition of DNA synthesis and division delay in the S period. Division delay in the G₁ might still be possible but as between strand dimer formation seems less likely in this period one might expect that delay would be less; a sharp increase in sensitivity would be expected at the beginning of the S period. It is worth pointing out that in *S. pombe* although, for convenience, a straight line regression was fitted in the first peak there is a suggestion that a sigmoid curve would have fitted the points better. This could be taken as the growth of sensitivity in the S period, but it is possible that the first part of it might represent sensitivity in a very short G₁. The possibility of a separate mechanism operating in the G₁ cannot, however, be excluded and in some ways seems more likely. A destruction of enzymes required for DNA synthesis is one possibility.

The second point that sensitivity increases as the S period progresses is less easily explained; it seems rather curious that DNA synthesis should be maximally inhibited at a time when it is nearly completed. It is rather strange, too, to find supporting evidence from work with ionising radiation. Smith (1963) has found that in mouse L cells the inhibition of DNA synthesis increases as the S period progresses. Perhaps the most likely explanation revolves on the fact that DNA synthesis is not synchronous in all the chromosomes of a cell. Doudney has concluded that the extent of the inhibition depends
on the number of lesions formed in the DNA and the rate at which these lesions can be repaired (Doudney, 1962). The fact that the extent of the inhibition depends on the dose, that is, on the number of lesions formed, implies that the repair mechanism is limited; if all the lesions could be repaired simultaneously there would be no dose effect. In higher cells the number of lesions would depend not only on the dose but on the number of sites available for damage, i.e. the number of chromosomes engaged in synthesis. Maximum delay would occur when all the chromosomes were synthesising. It is necessary to make the assumption that the chromosomes are switched on to synthesis at different times, but there is much less variability in the time at which synthesis is completed. The chromosomes of yeasts are rather mysterious bodies and certainly do not resemble those of higher cells, but in *S. pombe* genetical studies have shown that there are about seven linkage groups which probably represent chromosomes (Auerbach, 1965) and so this explanation is feasible.

The increase in variability as the delay increases can be explained in similar terms. The assumption here is that the time of switching off of synthesis has a slight variability from cell to cell. Thus considering cells at one particular stage, e.g. the cell plate stage, in some cells all chromosomes will be synthesising and delay will be maximal, in others most, but not all, chromosomes will have finished synthesis and so the delay will be small. Cells will also be present at all intermediate stages. This is a somewhat contrived argument
but it does fit the facts.

The fourth point, the close relationship between lethality and division delay, is not easily explained. If it were assumed that mutation resulted from the formation of inter-molecular dimers the explanation would be straightforward, but this may not be so, and some workers have taken the view that there are two distinct kinds of UV lesion: replication blocking (intermolecular dimers) and pre-mutational lesions (intramolecular dimers?) (Doudney, 1962). The mechanism by which pre-mutational lesions are stabilised into mutations is the subject of great research interest at the moment. For a complete account the work of Doudney (1962) and Witkin (review and references in Witkin, 1962) should be referred to, but briefly it appears that mutations are only stabilised at the time of synthesis. Thus the number of mutations which are eventually expressed in an irradiated cell can be reduced by limiting protein synthesis after irradiation. This treatment prevents cells from initiating a new round of synthesis. It is not the inhibition of synthesis which reduces the number of mutations but the attainment of the non-DNA synthetic state. In simple terms, the reduction in the number of mutations depends on the time available before the next round of synthesis at which the pre-mutational lesions will be stabilised. Cells irradiated during synthesis might be expected to suffer maximum mutational damage. This work is as yet far from complete and the explanation which has just been given is an oversimplification, nevertheless it is probably
near enough to the truth for our present purposes.

Finally an attempt must be made to incorporate the data on temperature dependence into the model system. It is already apparent from the comments in Part VI that any explanation proposed will have to be highly speculative. If the simplest explanation is accepted that DNA synthesis is comparatively temperature independent down to about 23°C a reasonably consistent explanation can be offered. The assumption is that DNA synthesis is switched on shortly after nuclear division. The position in the cell cycle of nuclear division varies with temperature in a way which cannot be easily explained: from 32°C - 23°C it moves towards the end of the cycle but below this temperature it moves to an earlier position. The observations of Bostock (1965) at least show that nuclear division can shift in this fashion in some circumstances. The duration of DNA synthesis and hence of the sensitive period remains constant down to 23°C, but below this it increases sharply; this implies that there is a critical temperature below which the temperature coefficient of DNA synthesis increases. This would also explain the increased temperature coefficient of division delay itself if it is assumed that the mechanism of synthesis after irradiation is similar to the normal synthetic mechanism. The greatest difficulties in this explanation are associated with the assumption that DNA synthesis has this very curious temperature dependence. If the comments made in the preceding few paragraphs are accepted it may not be necessary to make this assumption, for the
duration of the synthetic period would then depend not only on the rate of synthesis, but also on the spread of times at which synthesis was switched on in the chromosomes. It is conceivable that such a switching on process might have an unusual temperature behaviour.

The conclusions and speculations presented in this discussion can now be summarised by taking a short journey through the cell cycle. At the beginning of the cycle the cell plate has just formed, each daughter cell has a nucleus and within each nucleus the DNA is already replicating. In some cells every chromosome is engaged in synthesis, in others synthesis has been completed in some or all of the chromosomes. Irradiation at this time with UV causes the formation of thymine dimers in the DNA, some of these dimers are formed across the DNA strands and block replication, others are formed along the strand and will give rise to mutations. The block to replication is confined to the DNA which is actively replicating at the time of irradiation and results in a division delay. The cells in which all the chromosomes are engaged in replication suffer the greatest delay. Mutations are stabilised at the time of replication and thus the maximum yield of mutations is obtained by irradiation at this time. The cell is thus maximally sensitive to lethal mutational killing.

Shortly after fission into two separate daughter cells the replication of DNA is completed and thus cannot be blocked by radiation. Irradiation therefore produces no division delay.
The yield of mutants is also reduced at this stage for a long interval is available before the next round of replication in which repair can occur. As the cycle progresses it becomes evident that the cells are not escaping radiation damage for once more division is being delayed. The damage now appears as an interference with nuclear replication, but it evidently can be repaired for the delay becomes greater as the time of irradiation approaches the time of nuclear division. When nuclear division has passed division cannot be delayed but within the daughter nuclei DNA synthesis shortly resumes. This synthesis can be blocked by radiation and the next but one division can therefore be delayed. The delay increases as more and more chromosomes take part in synthesis. The onset of synthesis is accompanied by a sharp rise in the yield of mutations and the cell therefore becomes sensitive to killing.

Conclusions

This investigation has shown the value of direct observations. A complex pattern of sensitivity changes during the cell cycle has been found which could not have been revealed by other methods. There is a possibility, therefore, that this complex pattern may apply to other cells. The observations have not been particularly helpful in elucidating the mechanism of division delay but enough information has been obtained to construct a hypothetical model of the effect. This model is highly speculative, but it has been deliberately phrased in as
precise terms as possible; in this way it is to be hoped that future work will soon show its weaknesses. Radiation biologists in general show commendable caution in interpreting their experiments but to the non-specialist entering the field this can be bewildering: he feels the need for precise hypotheses, however speculative, which can be tested. The model presented here does not entirely satisfy this need but certain aspects of it at least are open to test. The development of synchronous cultures in *S. pombe* has made it possible to compare the inhibition of DNA synthesis with the extent of division delay and such an investigation is urgently required. Work is already in progress on the timing of nuclear division and DNA synthesis in various stages of population growth, and it is hoped that this will soon be extended to cells growing at different temperatures. The asynchrony of synthesis in the chromosomes will be difficult to test, but one might at least predict that in organisms with a simpler chromosome structure, e.g. bacteria, the increase in sensitivity and variability during the period of major sensitivity would be much less marked.

For students of the cell cycle this investigation has not been very helpful. It has indicated two points in the cell cycle which are critical for the behaviour of the irradiated cell. These mark the completion of two radiation sensitive preparations for division, but as far as one can see these are nothing new; it has long been recognised that the completion of DNA synthesis and nuclear division are preparations for the
division of the cell. Perhaps the value of this investigation is no more than the contribution it has made to the interplay of ideas within this Department.
Summary

1) The radiation effect which has been investigated is the inhibition of cell division by ultra-violet radiation. The fission yeast *Schizosaccharomyces pombe* was a natural choice of experimental organism because its cell cycle has been extensively investigated in this Department. A short review of the literature on division delay revealed that many of the earlier investigations are inadequate. For various technical reasons *S. pombe* is ideally suited to the investigation of this effect.

2) The normal growth and division behaviour of *S. pombe* was investigated by time-lapse photography. The design of the division delay experiments was based on the results obtained.

3) Ultra-violet induced division delay was investigated by time-lapse photography of irradiated cells. Complex changes in sensitivity during the cell cycle were found. A period of major sensitivity extends from about the time of nuclear division to shortly after the time when cytoplasmic division is completed: at the end of this period there is a distinct critical point at which sensitivity falls dramatically. A period of moderate sensitivity follows which is terminated at a second critical point at about the time of nuclear division. Irradiation later in the cycle than this point produces no delay to the division which immediately follows but the second division following irradiation is greatly delayed. The
results suggest that two radiation sensitive processes are involved in the preparations for cell division. The second critical point corresponds with the most commonly reported critical point at prophase.

4) The changes in sensitivity during the cycle to division delay and lethal mutational killing are very similar and suggest that the two effects are related. A revised analysis of the data of another worker on the distribution of deaths among the progeny of irradiated cells indicated that the period of major sensitivity to killing and division delay is also the period of gene replication. Gene replication may be equivalent to DNA synthesis.

5) Another worker has shown that when stationary phase cells are transferred into fresh growth medium, a burst of DNA synthesis occurs almost immediately. An investigation of division delay in this situation showed that cells become sensitive to division delay at the time of the burst of synthesis; similarly they become resistant when the burst of synthesis is completed. The results lend weight to the hypothesis that the period of major sensitivity is the period of DNA synthesis.

6) An investigation of division delay at various temperatures showed that the two sensitive periods have different temperature dependencies, and that the period of major sensitivity occupies a smaller fraction of the generation time at lower
temperatures. The results support the view that distinct processes are involved in the two sensitive regions and they may be consistent with the suggestion that the major sensitive period is the period of DNA synthesis.

7) The pattern of sensitivity found in *S. pombe* was compared with the data on division delay in other organisms. Although a simpler pattern seems to prevail in some cases there is good evidence that complex changes in sensitivity occur also in the budding yeasts and it is possible that the pattern found in *S. pombe* is typical of all higher cells. No definite conclusions can be drawn about the mechanism of division delay but the suggestion is made that the mechanism underlying the major sensitive period is the inhibition of DNA synthesis and the second sensitive period is explained in terms of interference with nuclear division.
References


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APPENDIX A

A critique of investigations of division delay by indirect methods

An immediate consequence of the inhibition of division in a population of cells is a lowering of the mitotic index. Measurement of the mitotic index following irradiation is therefore one way of approaching the problem of division delay in tissues and tissue cultures of higher plants and animals. It may indeed be the only way because of the difficulties of direct observation in these materials. The usual finding in this type of experiment is that immediately after irradiation the mitotic index falls; at some later time it rises again often to a value which exceeds the normal mitotic index; after further fluctuations it levels off to a value characteristic of unirradiated cells.

Lea (1955) has discussed this type of experiment and has reviewed much of the earlier literature. Briefly his interpretation is that at some stage just prior to prophase cells are delayed following irradiation, the mitotic index therefore immediately falls. Cells which were at an earlier stage at the time of irradiation are not delayed and consequently they come into division at the same time as the delayed cells from just before prophase. This results in an overshoot of the normal mitotic index.

This interpretation is probably basically correct but in
view of the complex pattern of sensitivity found in \textit{S. pombe} one is bound to consider the possibility that it may be an oversimplification. It can be quite easily shown that if the complex pattern found in the yeast occurred also in higher cells it could not be detected by experiments based on the mitotic index method. Figure 29 shows the behaviour of the cell plate index in \textit{S. pombe} following irradiation with 62 ergs/mm\(^2\). The conventional markers for mitosis are not visible in \textit{S. pombe} but in the present context the cell plate is an acceptable substitute. The values for the cell plate index were obtained not by direct counting but by calculation from the recorded time to division of irradiated cells. Thus at any point the cell plate index is a measure of the number of cells which came into division in the previous 20 minutes (the data come from photographs taken at 20 minute intervals). The first two divisions following irradiation were observed directly but as very few observations of the third post-irradiation division were made the data have been supplemented by assuming that the third cycle was the same length as the normal cycle. In fact some variability has been introduced into the third cycle by distributing the generation times in the ratio 120:140:160 mins. - 2:3:2. This is a little less than the normal variability.

In Figure 29 the cell plate index (relative to a control) is shown above, and below it the contribution made to this index by the 1st, 2nd and 3rd divisions. The behaviour of the cell plate index following irradiation is very similar to
Figure 29. The behaviour of the cell plate index following irradiation with 62 ergs/mm² (above) and below, reading downwards, the contribution made to the cell plate index by the 1st, 2nd and 3rd post-irradiation divisions.
typical mitotic index data. The wave of division at about 120 - 140 minutes bears out Lea's interpretation very well: it is composed of cells from quite early in the cell cycle which were not delayed or delayed only slightly coming into division at the same time as cells from just before the critical point which were moderately delayed. However, the cells from near the beginning of the cycle which were greatly delayed, dividing between 200 and 700 minutes after irradiation, make no impression on the cell plate index. By the time these cells come into division most cells are undergoing their 2nd, 3rd or 4th division and the delayed cells are therefore greatly outnumbered.

It is clear that the mitotic index method cannot detect changes in sensitivity of the type reported for *S. pombe*. The possibility that the pattern of sensitivity found in *S. pombe* applies also to higher cells cannot therefore be excluded by this type of experiment. In recent years though several refinements of the mitotic index method have been proposed which in principle should be more reliable, but these have not revealed changes in sensitivity of the type reported for the yeast. It is intended now to show that there are good reasons for believing that the conclusions drawn from these experiments may be unjustified, and that there is therefore a strong possibility that the pattern of sensitivity in *S. pombe* may be of general application. In order to show this, two typical papers will be discussed in detail.

A common procedure in recent years has been to label cells
with $^3$H-thymidine for a short period before irradiation. By distinguishing between labelled and unlabelled cells in subsequent mitotic counts the relative delay of S phase and $G_1$ and $G_2$ cells can be estimated. The most sophisticated example of this technique is the recent work of Bootsma (1965). He employed a double labelling technique. Human kidney cells in culture were labelled for 20 minutes prior to irradiation with $^{14}$C-thymidine. After irradiation they were transferred to a medium containing $^3$H-thymidine. In this way cells in the $G_1$, S and $G_2$ periods at the time of irradiation could be distinguished on autoradiographs; $G_2$ cells would be unlabelled, S phase cells would be double labelled and $G_1$ cells would be labelled only with $^3$H. He worked with two strains of cells but for the sake of illustration only his results on the more sensitive strain will be considered; this had a generation time of 28 hours.

In control unirradiated cultures the unlabelled $G_2$ cells came into mitosis between 0 and 6 hours following irradiation. In irradiated cultures they appeared between 4 and 30 hours. The conclusion that $G_2$ cells suffer a mean delay of 10 - 12 hours seems justified but it must be realised that this statement has only limited meaning. No conclusions may be drawn about any sensitivity changes which may occur during the $G_2$. This criticism applies also to cells at other stages but particularly to S phase cells. In this case the populations of control and irradiated cells overlap considerably (control dividing between 2 and 16 hours, irradiated 6 - 30 hours).
There is a difference between the means of only about 5 hours, but to conclude from this that S phase cells are delayed 5 hours may be quite wrong. It could well be that cells in the last part of the S period were delayed very little or not at all while the cells from early S were delayed perhaps 20 hours or more. Indeed if any importance can be attached to the shape of the curves this seems quite likely. One final point that applies to cells at all stages: it is possible that some of the irradiated cells may not have appeared in mitosis during the limited period of observation. Such greatly delayed cells could probably not be detected by extending the period of observation because by this time the other cells would be dividing for the second time. If a large number of such cells were present their absence might be noticed but in experiments like these the possibility that there is a small population of highly sensitive cells cannot be excluded.

The second refinement of the mitotic index method which will be considered is in some ways based on the recognition of this fact and has been used to study division delay in root meristems of *Vicia faba* (Neary, Evans and Tonkinson, 1959; Evans, Neary and Tonkinson, 1959). These workers treated irradiated meristems with colchicine for two hours before sampling at various times after irradiation. During the colchicine treatment all the cells coming into mitosis accumulate in metaphase so that from a metaphase count at the end of the treatment an estimate can be made of the rate at which cells are arriving in metaphase. By integrating these rates
an estimate can be made of the proportion of cells which have entered mitosis in the interval between irradiation and any selected time. In an irradiated root it will take longer for a given proportion of cells to come into mitosis than in an unirradiated root; the difference in these times is defined as the division delay. The stage in the cell cycle of the delayed cells can be estimated from the proportion of cells which have entered division before them, for these workers have shown that in an unirradiated asynchronous cell population the time any given cell will take to reach division bears a known relation to the proportion of cells which enter division before it. A basic assumption of the method is that this relation is unaffected by irradiation, in practice the assumption is that irradiation will not affect the order in which cells arrive in division. The authors consider that this is unlikely in *Vicia faba*.

The pattern of sensitivity to division delay induced by $^{60}$Co-gamma radiation which this method reveals is comparatively simple. Sensitivity is highest in early interphase and declines fairly gradually through interphase until shortly before prophase when it falls rapidly to zero. With a dose of 188 rads in air the maximum delay is about 6 hours relative to a normal generation time of about 25 hours. This is a much simpler pattern than the one found in *S. pombe*. On theoretical grounds there is no reason to suppose that this method could not detect changes in sensitivity of the more complex type; the validity of the method can in fact be demonstrated
practically by treating the *S. pombe* data on the lines described. It has already been pointed out that the values for cell plate index in Figure 29 are more properly a measure of the rate at which cells are coming into division and these values are therefore roughly equivalent to the values of metaphase accumulation rate in *Vicia faba*. This cell plate data is not strictly comparable to the data from *Vicia* but one feels that the differences are probably of minor importance. The division delay curve obtained by this method (Figure 30) is certainly strikingly similar to the one obtained by direct observation.

The validity of the method is therefore clear and one is tempted to conclude that division delays in *Vicia faba* and *S. pombe* are quite different. This may not be so. The division delay curve is derived from the behaviour of the metaphase accumulation rate; the method by which this is done need not concern us here except to say that in *S. pombe* the peak of sensitivity at the beginning of the cycle results from the fact that the cell plate accumulation rate is falling at 140 minutes after irradiation and continues to fall for some time thereafter. It is unfortunate that in the work on *Vicia faba* observations were only made at 12 hour intervals in a comparable range of times, i.e. 24 hours or 1 generation time after irradiation. In some of the metaphase accumulation rate curves for *Vicia* the rate is clearly falling at 24 hours (e.g. 188 rads in air, Evans, Neary and Tonkinson, 1959). By 36 hours the rate has risen again and the authors draw a
Figure 30. Division delay in *S. pombe* (UV dose 62 ergs/mm²).
The curve represents the sensitivity during the cell cycle and
was derived by an approximation to the method of Neary, Evans
and Tonkinson (1959).
straight line between the two points. The consequence of this straight line is that after 24 hours the rate immediately begins to rise. This seems unlikely and it would surely have been preferable to link the two points by a curve in which the rate continued to fall for some time after 24 hours. The authors point out that their points for 36 hours and 48 hours may be inaccurate because by this time some cells will be dividing for the second time; in this case it seems almost certain that the metaphase accumulation rate for cells coming into their first division would continue to fall after 24 hours. This would make a dramatic difference to the division delay curve; in fact a curve constructed on this assumption very closely resembles the curve for *S. pombe* with a delay at the first peak of over 20 hours and a delay at the second peak of 6 hours.

If for a moment this revised curve for *Vicia faba* is accepted, there are obviously a number of difficulties which must be faced. The curve would apparently confirm the pattern of sensitivity found in *S. pombe* with major sensitivity at the beginning of the cell cycle. In *S. pombe*, however, this period of sensitivity is also the period of DNA synthesis; synthesis in *Vicia*, on the other hand, occurs much later in the cycle. The work of Bootsma on kidney cells showed convincingly that there is little or no delay to G₁ cells but there could be a considerable delay to S phase cells. If there is a general pattern which applies to all cells one is bound to consider the possibility that the major delay in *Vicia* is in the S period.
If this were the case the division delay curve would still indicate a major delay at the beginning of the cell cycle, for the basic assumption that the order in which cells arrive in mitosis is unaffected by radiation would now no longer be justified.

The criticisms which have just been presented involve many assumptions and may in time turn out to have been unjustified. It is clear though that in the interpretation of these experiments on higher cells there are large fields of uncertainty, and consequently the possibility that the complex pattern of sensitivity found in *S. pombe* applies to all cells cannot be excluded.
Edinburgh Minimal Medium

The medium has the following constitution.

1000 ml. of medium contain:

<table>
<thead>
<tr>
<th>(A) C-source</th>
<th>Glucose</th>
<th>10 g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B) N-source</td>
<td>NH₄Cl</td>
<td>5 g.</td>
</tr>
<tr>
<td>(C) Salts</td>
<td>Sodium acetate (buffer)</td>
<td>1 g.</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>10 g.</td>
</tr>
<tr>
<td></td>
<td>MgCl₂</td>
<td>0.5 g.</td>
</tr>
<tr>
<td></td>
<td>Na₂H₂PO₄</td>
<td>10 mg.</td>
</tr>
<tr>
<td></td>
<td>Na₂SO₄</td>
<td>10 mg.</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>10 mg.</td>
</tr>
<tr>
<td>(D) Vitamins</td>
<td>Inositol</td>
<td>10 mg.</td>
</tr>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>10 mg.</td>
</tr>
<tr>
<td></td>
<td>Calcium pantothenate</td>
<td>1 mg.</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
<td>10 ug.</td>
</tr>
<tr>
<td>(E) Trace elements</td>
<td>H₃BO₃</td>
<td>500 ug.</td>
</tr>
<tr>
<td></td>
<td>MnSO₄·H₂O</td>
<td>400 ug.</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄·7H₂O</td>
<td>400 ug.</td>
</tr>
<tr>
<td></td>
<td>FeCl₃·6H₂O</td>
<td>200 ug.</td>
</tr>
<tr>
<td></td>
<td>H₂MoO₄·H₂O</td>
<td>160 ug.</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>100 ug.</td>
</tr>
<tr>
<td></td>
<td>CuSO₄·5H₂O</td>
<td>40 ug.</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
<td>1000 ug.</td>
</tr>
</tbody>
</table>
The medium was made up in five stock solutions as indicated above. These were made up in the following strengths.

<table>
<thead>
<tr>
<th>Components</th>
<th>Strength of stock solutions</th>
<th>Quantity of stock solutions to make 1000 ml. of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) C-source</td>
<td>50x (50 g. made up to 100 ml.)</td>
<td>20 ml.</td>
</tr>
<tr>
<td>(B) N-source</td>
<td>10x (5 g. made up to 100 ml.)</td>
<td>100 ml.</td>
</tr>
<tr>
<td>(C) Salts</td>
<td>10x</td>
<td>100 ml.</td>
</tr>
<tr>
<td>(D) Vitamins</td>
<td>1000x</td>
<td>1 ml.</td>
</tr>
<tr>
<td>(E) Trace elements</td>
<td>1000x</td>
<td>1 ml.</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>778 ml.</td>
</tr>
</tbody>
</table>

The stock solutions were stored in a cold room at 4°C. After making up, the medium was autoclaved for 15 minutes at 15 lb./sq. in. The pH of the medium is about 5.25.
It is probably not necessary to use a perfusion chamber in the study of division delay: Swann (1962), for example, studied radiation effects in *S. pombe* over about six generations in a system which did not involve perfusion. In preliminary observations of division delay, however, it was observed that some highly delayed cells ceased growth before dividing. It is likely that this was a delayed expression of lethality but it seemed possible that the cessation of growth could be due to the depletion of the supply of nutrients by the rapidly dividing non-delayed cells. To guard against this possibility a simple perfusion system was employed.

The structure of the chamber is shown in the diagram. The cells lie on top of an agar pad in the well of the chamber and are compressed beneath a quartz coverslip. The supply of nutrients is renewed by diffusion through the agar. This is not a very efficient system, but it was felt that by selecting cells for observation near to the edge of the agar pad an adequate supply of nutrients would be maintained. The 2" x 1" microscope slide was sealed to the underside of the chamber with nail varnish and the quartz coverslip was sealed to the upper side with a mixture of vaseline and paraffin wax.

The prototype chamber was milled from 1/4" acrylic sheet but this proved to be very difficult to clean so a second model was
THE PERFUSION CHAMBER

COVERSLIP

WELL

SLIDE

0.4"

0.35" - 0.125"

0.5"

1.125"

2.1"
constructed in stainless steel. This was cleaned by scrubbing with detergent followed by perfusion with tap water for 24 hours and finally six rinses in distilled water.
APPENDIX D

The Time-Lapse Apparatus

The time-lapse apparatus was built around the short
interval switch manufactured by Sangamo-Weston (Model S 277).
This switch is designed to 'make' a circuit for periods from
five to seventy-five seconds, at intervals of 5, 10, 15, 20,
30 or 60 minutes. The switch was wired to a solenoid, which
operated the camera shutter through a cable release, and to
the microscope lamp. The camera used was a 35 mm. 'Robot'
Industrial Model. This camera incorporates a clockwork
driven frame advance mechanism which automatically advances
the film one frame after each exposure. The whole apparatus
was controlled by a master time switch (Venner PT3). This
could be set to start or stop filming at any selected time up
to 24 hours, thus when necessary filming could be started
during the night without inconvenience.
APPENDIX E

The Analysis of Bi-modal Distributions

For a detailed description of this method reference should be made to the paper by Harding (1949). The principle of the method is very simple. When the cumulative frequencies in a normal distribution are plotted on probability paper the points lie on a straight line. The position of this line is determined by the mean of the distribution and its slope by the standard deviation. A bi-modal distribution plotted in this way results in a sigmoid curve which is a resultant of the two straight lines which would be given by the two separate distributions. Essentially the method consists of finding the two lines which give a resultant which closely fits the observations.

An alternative to the use of probability paper is to plot the probit values (standard tables are available) of the cumulative frequencies on ordinary graph paper. In Figure 31 some of the division delay data is plotted in this way. This is data on the division delay at the first post-irradiation division of cells grouped around stage 0.53 and irradiated with 62 ergs/mm². The crosses mark the observed cumulative frequencies. The two lines AB and CD have been found which give the resultant curve marked by open circles. This curve fits the observations quite well. The mean of the two distributions is read off where the lines cut the probit value of 5.0 (cumulative frequency 50%) and the range of one standard deviation on
Figure 31. Bi-modal analysis of cells grouped around stage 0.53 irradiated with 62 ergs/mm² UV. The cumulative frequencies of the division delays are plotted as probits (crosses). The two sloping lines represent 76% and 24% of the population respectively and give a resultant, marked by open circles, which is a good fit to the observations.
either side of the mean is given by the intersections at probit value 4.0 and 6.0 (cumulative frequencies 15.87% and 84.13%). The values obtained are: delayed population mean, 72 minutes, S.D., 20 minutes, non-delayed population mean, -4 minutes, S.D., 11 minutes.

The method by which the two lines are derived is described fully by Harding but briefly it consists of estimating the proportion of the whole distribution which lies in the two sub-distributions. This is given by the point of inflexion of the sigmoid curve. For example, in the data illustrated the point of inflexion is at about probit value 5.7; this corresponds to a cumulative frequency of 76%. Thus 76% of the population is in the delayed group and 24% in the non-delayed group. The cells which suffered a delay of 80 minutes and 100 minutes can be unequivocally identified as belonging to the delayed population, the cumulative frequencies for these cells can therefore be adjusted so that they express their cumulative frequency in the delayed population. Thus the cells delayed 100 minutes had a cumulative frequency of 5.7% (probit value 3.42). This is adjusted by a factor of 100/76 = 7.5% (probit value 3.56).

This gives the first point on the straight line defining the delayed population. A few other points treated in this way define the two lines. In practice some further adjustment of the lines may be necessary before a good fit is obtained.

The method is clearly very tedious when large numbers of distributions have to be analysed and like all graphical methods its accuracy is limited. Providing that the two
populations do not overlap excessively it does, however, yield convincing results and it is probably the best method which is available at the moment.
Regression Analysis of the Major Peak

The bi-modal analysis described in the previous appendix gives values for the mean and standard deviation of the two populations. It is not possible to assign any particular observation to one population or the other so a full least squares regression analysis of the delayed populations is not possible. An alternative would be to fit a least squares regression line through the means but this would be to ignore two important features of the data: a) the variability of each group is different: the greater the mean, the greater is the variability; b) the number of observations in each group varies. The method which has been employed is one described by Quenouille (1952). The means are weighted by a factor of \( \frac{n}{\text{variance}} \). The number of observations, \( n \) in this case, is taken as the number of observations within the range of the mean plus or minus one standard deviation. It is a reasonable assumption that this will not include any observations which strictly belong to the other group. For illustration the analysis of the major peak of the data on 62 ergs/mm\(^2\) at 32\(^\circ\)C is given.

Regression Analysis of the Major Peak at 32\(^\circ\)C

The data were transformed as follows:
Division Delay
Zero taken as delay = 110 minutes.
10 minutes of delay = 1 unit.

Stage in the Cell Cycle
Zero taken as stage 0.4.
Stage increase of 0.15 = 1 unit.
<table>
<thead>
<tr>
<th>Weighting coefficient (w) = n/variance</th>
<th>Y</th>
<th>wY</th>
<th>(Y-\bar{Y})</th>
<th>(Y-\bar{Y})^2 w</th>
<th>X</th>
<th>wX</th>
<th>(X-\bar{X})</th>
<th>(X-\bar{X})^2 w</th>
<th>(X-\bar{X})(Y-\bar{Y})w</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.49</td>
<td>1.5</td>
<td>0.74</td>
<td>-6.4</td>
<td>20.1</td>
<td>1</td>
<td>0.49</td>
<td>-2.26</td>
<td>2.50</td>
<td>7.10</td>
</tr>
<tr>
<td>1.39</td>
<td>5.0</td>
<td>6.75</td>
<td>-2.9</td>
<td>11.7</td>
<td>2</td>
<td>2.78</td>
<td>-1.26</td>
<td>2.18</td>
<td>5.08</td>
</tr>
<tr>
<td>0.45</td>
<td>4.5</td>
<td>2.02</td>
<td>-3.4</td>
<td>5.2</td>
<td>3</td>
<td>1.35</td>
<td>-0.26</td>
<td>0.03</td>
<td>0.40</td>
</tr>
<tr>
<td>0.57</td>
<td>14.0</td>
<td>7.98</td>
<td>6.1</td>
<td>21.2</td>
<td>4</td>
<td>2.28</td>
<td>0.74</td>
<td>0.31</td>
<td>2.57</td>
</tr>
<tr>
<td>0.32</td>
<td>16.0</td>
<td>5.12</td>
<td>8.1</td>
<td>21.0</td>
<td>5</td>
<td>1.60</td>
<td>1.74</td>
<td>0.96</td>
<td>4.51</td>
</tr>
<tr>
<td>0.74</td>
<td>11.5</td>
<td>8.50</td>
<td>3.6</td>
<td>9.6</td>
<td>6</td>
<td>4.44</td>
<td>2.74</td>
<td>5.54</td>
<td>7.30</td>
</tr>
<tr>
<td>3.96</td>
<td>31.11</td>
<td>88.8</td>
<td>12.94</td>
<td>11.52</td>
<td>26.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ \bar{Y} = \frac{\sum wY}{\sum w} = \frac{31.11}{3.96} = 7.9 \]

\[ \bar{X} = \frac{\sum wX}{\sum w} = \frac{12.94}{3.96} = 3.26 \]

\[ \text{Slope } r = \frac{(X-\bar{X})(Y-\bar{Y})}{(X-\bar{X})^2} = \frac{26.96/11.52}{3.26} = 2.34 \]
Equation of regression line

\[ Y - 7.9 = 2.34 (X - 3.26) \]

Analysis of Variance

<table>
<thead>
<tr>
<th>Variation</th>
<th>Degrees of freedom</th>
<th>Sums of squares</th>
<th>Est. Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascribable to X</td>
<td>1</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Unaccountable</td>
<td>4</td>
<td>25.8</td>
<td>6.45</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>88.8</td>
<td></td>
</tr>
</tbody>
</table>

Variance Ratio 9.78 with 1 and 4 D.F. Significant at the 5% level.