A study of induced delay in the division of the yeast

Schizosaccharomyces pombe

by

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CONTENTS

INTRODUCTION.

It is only comparatively recently, since the advent of methods for preparing synchronous cultures, that cell biologists have been able to explore biochemical changes in cells that occur with time as the cell traverses its cycle. With the widespread application of the various methods now available for producing synchrony a large body of data has accrued and this data may be broadly described as descriptive; the major fact to emerge has been that many of the cells' activities are periodic. Hence the cell has taken on a new complexity, a complexity in time.

As yet the phenomenon of periodic synthesis of cell constituents remains almost totally unexplained and without a much more comprehensive knowledge of the elements of molecular control mechanisms it is difficult to construct testable hypotheses. Two such hypotheses have been forwarded and will be discussed in outline below but it is quite certain that the study of periodic syntheses will occupy an important position in the field of cell biology for some time to come.

Two major cell constituents have been found to be synthesized in a periodic manner, namely DNA and some enzymes, whilst other enzymes have been shown to be continuously synthesized but to display periodic increases in the rate of synthesis. Periodicity of nuclear DNA synthesis has been found to be a universal feature of higher cells and their cell cycle is quite commonly subdivided into phases determined by the amount of DNA in the nucleus.

The periodic synthesis of enzymes has also proved to be a universal phenomenon and this field has recently been reviewed by Mitchison ('69a) who has described the two major hypotheses

current in the field.

The first of these hypotheses Mitchison has called oscillatory repression and it was first put forward by Masters and Donachie and expanded by Goodwin. Briefly the theory postulates that the periodic rises in enzyme activity observed in synchronous cultures represents the free or possibly entrained oscillation of a control system which incorporated a negative feedback loop. The entrainment idea arose as a necessity of explaining the fact that the oscillations were of a once per cycle nature and the suggestion is that the entraining event is the doubling of the relevant gene which changes the parameters of the system. The theory was propounded to account for results obtained from the study of synchronous cultures of bacteria and is an extension of the theory of enzyme synthesis control put forward by Jacob and Monod but there is no inherent objection to the extension of the theory to higher cells.

The other theory was proposed by Halvorson and his group to account for their observations of periodic fluctuations of enzyme synthesis in synchronous cultures of budding yeast and has been called by Mitchison the linear reading theory. This theory simply asserts that the enzyme 'steps' are due to a wave of transcription along each chromosome in such a way that each chromosome is completely transcribed once per cycle. The theory suggests that reading of the chromosome is from one end and passes through the centromere to the other end of the chromosome.

At the present both of these theories suffer from the drawback that they are both rather difficult to test. They are important, however, because they represent some of the first fruits

of an approach to the investigation of the cell cycle which is experimental as well as being descriptive.

Recently Mitchison has framed a general approach to the study of periodicity in the cell cycle which is both experimental and descriptive which he calls 'marker analysis' (Mitchison '69b). In short this method entails mapping the cell cycle in terms of periodic events at all levels using morphological criteria as well as biochemical ones and then comparing the map sequence of cell cycles measured under a range of different experimental conditions. The purpose of making such comparisons is to try to divine relationships between periodic events and to establish 'causal sequences' of inter-related processes. Clearly the success of this method depends both upon the investigation in depth of a few convenient types of cell and upon the development of methods of modifying the normal cell cycle. It was to try to develop a finely controllable method for distorting the cell cycle of S.pombe that this work was initiated.

The cell of interest in this study is the fission yeast Schizosaccaromyces pombe which has been the subject of interest in this laboratory and has been studied for many years both by the observation of single cells and, with the development of the density gradient technique for producing synchronous cultures (Mitchison and Vincent), by biochemical methods. When this project was begun all of the major cell constituents had been investigated to some extent and DNA and several enzymes had been found to be synthesized periodically in the cell cycle (Bostock et al).

At the outset it was decided that the most profound way in

which the cycle could be distorted was to specifically inhibit
the division of the cells with some agent or treatment which would
produce as little inhibition of the growth of the cell as possible.
It was known that this effect could in fact be produced since the
occurrence of giant cells with a normal rate of growth but an
incapacity to divide had already been noted by two workers. Gill
had produced this effect by exposing the cells to low doses of UV
irradiation, a method which does not lend itself to biochemical
investigation since it is very difficult to treat cells in bulk
in this way, and Schopfer had produced giant cells by chemical
inhibition with an analogue of inositol.

The initial problem was thus the choice of agents to
test although some information was already available as a result of
a previous project (A. Herring). Briefly, three classes of
division inhibitor were known at that time and were considered
for use; these are the alkylating agents, the standard inhibitors
of DNA synthesis and those substances which attack the mitotic
apparatus, the colchicine group. Since the last of these was not
thought to have an effect upon yeasts the choice was limited to
the first two groups.

Two findings suggested that certain of the alkylating
agents might be of use. Firstly, Mitomycin C (henceforth MC) had
been shown by Williamson and Scopes ('62a) to be capable of
inhibiting the division of S. cerevisiae with no measurable effect
on growth and this finding had been extended to S. pombe (Herring).
Secondly, the production of giant cells of S. pombe by Schopfer
referred to above was suspected to be due to the action of the
inhibitor used as an alkylating agent. This effect was found

during an investigation of the effects of inositol analogues upon
the metabolism of S.pombe and more recent work has confirmed
that the inositol analogue in question, methylenepentahydroxycyclo-
hexane oxide, does in fact behave as an alkylating agent (see
Deshusses et al).

There were, however, difficulties attached to the use of
alkylating agents. The inositol analogue was not commercially
available neither was it easily synthesized. Mitomycin C was
available but the levels of drug needed to affect the division of
yeasts was some hundred times as high as those levels normally
used for bacteria or mammalian cells, and this made experiments
of the scale needed for biochemical assays prohibitively expensive
if the drug was to be used by simple addition to a growing culture.
Preliminary experiments upon two other alkylating agents,
nitrogen mustard and ethyl methanesulphonate, did not give giant
cells and it is probable that both these agents affect growth as
well as division.

Thus attention was also directed towards those inhibi-
tors which specifically block DNA synthesis and affect division
by arresting the cycle in the G_1 phase. The following substances
were tested in preliminary experiments in which division and
growth were measured in the presence of the drug: hydroxyurea (HU),
2'deoxyadenosine (AdR), nalidixic acid, 5'fluorodeoxyuridine
(FUdR) and cytosine arabinoside. All of these drugs were found
to have some effect on the division or growth of S.pombe with the
exception of cytosine arabinoside. They will henceforward be
referred to by the abreviations shown in brackets.

The most interesting of this group of inhibitors turned

out to be HU and AdR and my preliminary experiments were con-
tinued by Mitchison and Creanor at the same time as the work
described in this project. I shall return to this subject later
as some of the experiments in this project were performed with
these drugs. The results of the preliminary experiments are
not presented as, for all the inhibitors in the list above, my
work has been superseded by Mitchison and Creanor ('71a) and
many of their findings have since been published.

At this point the discovery was made which led to the
experiments described in the following pages. MC was by far the
best specific inhibitor of division which I had found in my pre-
liminary tests, with no measurable affect on growth as judged by
simple criteria. It was found that it was not necessary to use
the drug continuously and that a short exposure to MC produced a
dramatic division delay. This discovery meant that the cost of
MC experiments became reasonable, and the exploitation of this
technique soon showed that the relationship between induced delay
and the age of the treated cell was quite complex. If MC was to
be a useful probe for the investigation of the cell cycle ob-
viously its action had to be well understood and thus the experi-
ments which are described below were inaugurated.

When this project was begun there was only one experiment
performed which had utilised an inhibitory treatment to investi-
gate periodic synthesis in a synchronous culture. This was an
experiment performed by Masters and Donachie using a synchronous
culture of Bacillus subtilis. They inhibited DNA synthesis with
FUdR and were able to show that periodic steps occurred in the
activity of the enzyme ornithine transcarbamylase for two cycles

in the absence of DNA synthesis and cell division. This clearly demonstrated that steps in enzyme activity could not be accounted for by a gene dosage effect.

However, since this project was started there have been several reports of periodicity being maintained after normal progression through the cycle has been blocked in some way. Eckstein Paduch and Hilz used synchronous cultures of Saccharomyces cerevisiae and followed the changes in the activity of DNA polymerase; they found that this enzyme follows the 'peak' pattern in the cell cycle with the maximum of enzyme activity occuring just before DNA synthesis. X-irradiation of their synchronous cultures caused a long delay in DNA synthesis but the peaks of polymerase activity continued to appear and were quite unaffected by the irradiation.

Still more recently the same approach has been extended by Hartwell and Culotti* who have isolated temperature sensitive mutants of S.cerevisiae which are unable to perform either DNA synthesis or nuclear division at the restrictive temperature. One of these mutants which is blocked in DNA synthesis has proved particularly interesting, as growing this mutant at the restrictive temperature does not prevent bud initiation and the result is a many budded chimera. This mutant clearly demonstrates that initiation of DNA synthesis and bud initiation are independent and form what Mitchison has called a 'non-causal fixed sequence' in the normal cycle. However, another mutant shows that the relationship between DNA synthesis and bud initiation must be complex since this mutant, which can initiate but not maintain DNA synthesis at the restrictive temperature, does not display continued bud initiation.

* See both Hartwell and Culotti and Hartwell

To account for these results Hartwell has proposed that
the cell possesses a 'cellular clock' which controls periodic
events in the cycle, in this case bud initiation. Halverson's
model predicts that this 'clock' is the process of sequential
transcription but the results outlined above impose a new con-
dition, that the transcription process must be able to re-
initiate itself in the absence of the whole process of chromosome
replication. The majority of Hartwell's mutants do not show
continued bud initiation and presumably have stopped the 'clock',
but there is little evidence in yeasts that either DNA synthesis
or nuclear division have an effect upon transcription. Possibly
this system might provide a partial test of the sequential
transcription hypothesis.

I have discussed the work above to try to illustrate the
usefulness of inhibitors or treatments which distort the cell
cycle, as the original aim of this project was to develop a tool
for the dissection of the S.pombe cell cycle. At the present
time three such inhibitors are available, MC, AdR, and HU, and
of these three, AdR and HU act in a way which is fairly simple
and relatively well understood. MC, however, is an inhibitor
with a more complex mode of action which resembles the action of
UV irradiation and most of this thesis is concerned with the
investigation of the effects produced by MC.

MATERIALS AND METHODS.

The organism used for the whole of this study was the
fission yeast Schizosaccharomyces pombe. It is as well to mention
at this point that a comprehensive review of the methods used to
study S.pombe has recently been written by J.M.Mitchison ('70)
and thus I shall restrict myself to a brief description of the
basic techniques and will comment at length only when the pro-
cedures used were not described in the article mentioned above.

Four strains of S.pombe have been used, those being N.C.Y.C.
132 and three other strains designated 972h$^-$, 975h$^+$ and uvsl 1
(derived from 972h$^-$). The majority of the work was performed
with strain 132 and it may be assumed that this strain was used
unless it is specifically stated to the contrary. The cells were
grown for all the experiments in Edinburgh Minimal Medium number
II, the composition for which is given in Appendix I, except for
a small number of experiments which specifically required the
original medium EMM I which differs only in the Phosphate con-
centration being 30 times lower (also Appendix 1).

Stock cultures of the above strains were maintained in
10 mls of medium in Universal bottles and subcultured at intervals
not exceeding 14 days. The temperature of growth was always 32°,
and at this temperature the generation time was typically 150 mins
$\pm$ 10 mins.

Synchronous cultures were made by the method of Mitchison
and Vincent which separates young cells from a logarithmic phase
population by rate zonal centrifugation followed by the selection
of slowest sedimenting cells from the gradient. A sucrose or
glucose gradient was employed.

Growth of the large sub-cultures used for making the synchronous cultures was monitored by following the optical density at 595 mμ which provides a convenient and quick measure of culture growth. Cultures were harvested at optical densities which indicated cell concentrations of between 2.0 to 4.0 x 10^6 cells/ml. The volumes of the fractions removed from an 80 ml 10-40% sucrose gradient to make the culture varied from 0.5 mls to 6.0 mls according to the size of culture required and the appearance of the gradient.

After inoculation of the selected cells culture growth was estimated by optical density measurements and cell division was measured by two separate methods. The direct method of measuring cell number increase was to use a Model B Coulter Counter fitted with a 70 μ orifice tube as described by Mitchison ('70). However, the method most commonly employed was to calculate the cell plate index (henceforward CPI) which is an estimate of the percentage of cells in the culture which are undergoing division and thus possess a cell plate. The CPI is the direct equivalent of the mitotic index in higher cells.

The CPI was estimated either from slides prepared from samples taken from the cultures at intervals and stained with crystal violet as described in Mitchison ('70), or by direct observation of the live cells using dark ground illumination with a total magnification of about X400. The latter method is by far the best as it is very quick and it is possible to tell without doubt whether a cell has a cell plate, which is not always possible in stained preparations. Most of the data presented in this thesis were collected by direct observation. Estimates of the CPI are based on counts of 200-400 cells. Figure 1. shows the typical fluctuation in CPI with time for a synchronous culture; there is a

peak at about 2 hrs which is followed by a second peak at about 4 hrs. It is these peaks in the CPI that have been used to calculate mean generation time of the cells and the mean cycle position at any given time,and the length of induced delays. A convention has been adopted to pin-point the so called 'cell plate peak' (henceforward CPP); it is defined as the time at half the breadth of the peak of the CPI measured at half its height.

When this value was calculated (using both my own data and that of Mitchison and Creanor) for nine synchronous cultures on which both CPI and Coulter Counter measurements had been made the CPP was found, on average, to precede the mid-point of doubling of cell numbers by 15 minutes. Hence, using the fission to fission convention to define the cell cycle, the cycle was held to begin, for the purposes of calculation, 15 minutes after the CPP.

The mean generation time of a culture is thus the time from the first CPP to the second CPP and other cycle positions were calculated from this data. The length of the 1st cycle was considered to be the same as the 2nd and the cycle positions of events before the first cell plate peak were calculated backwards from the estimated end of the first cycle. Where only one CPP was measured an average value for the mean generation time of 150 minutes was assumed; this value was derived from an average of 12 mean generation times.

Since great reliance has been placed upon measurements of delays calculated from the displacement in time of the CPP by various treatments, I shall briefly consider the errors affecting the estimate of the CPP. Figure 1 shows some typical data from a fairly good synchronous culture; the vertical bars represent the 95% confidence intervals for each estimate of the CPI (calculated

FIGURE 1. - An experiment to investigate the errors involved
in the estimation of the time of the 1st cell
plate peak of a synchronous culture.

The open circles represent the raw data, whilst the bars
are 95% confidence intervals (see text). The dotted and dashed
peaks were constructed to arrive at the earliest and latest
estimates of the cell plate peak using the extremes of the 95%
confidence limits. The two arrows above the abscissa represent
the mid-point of their extreme estimates whilst the arrow below
the abscissa represents the mid-point as measured from the raw
data.

The ordinate is the percentage of cells showing cell plates;
the cell plate index (CPI).
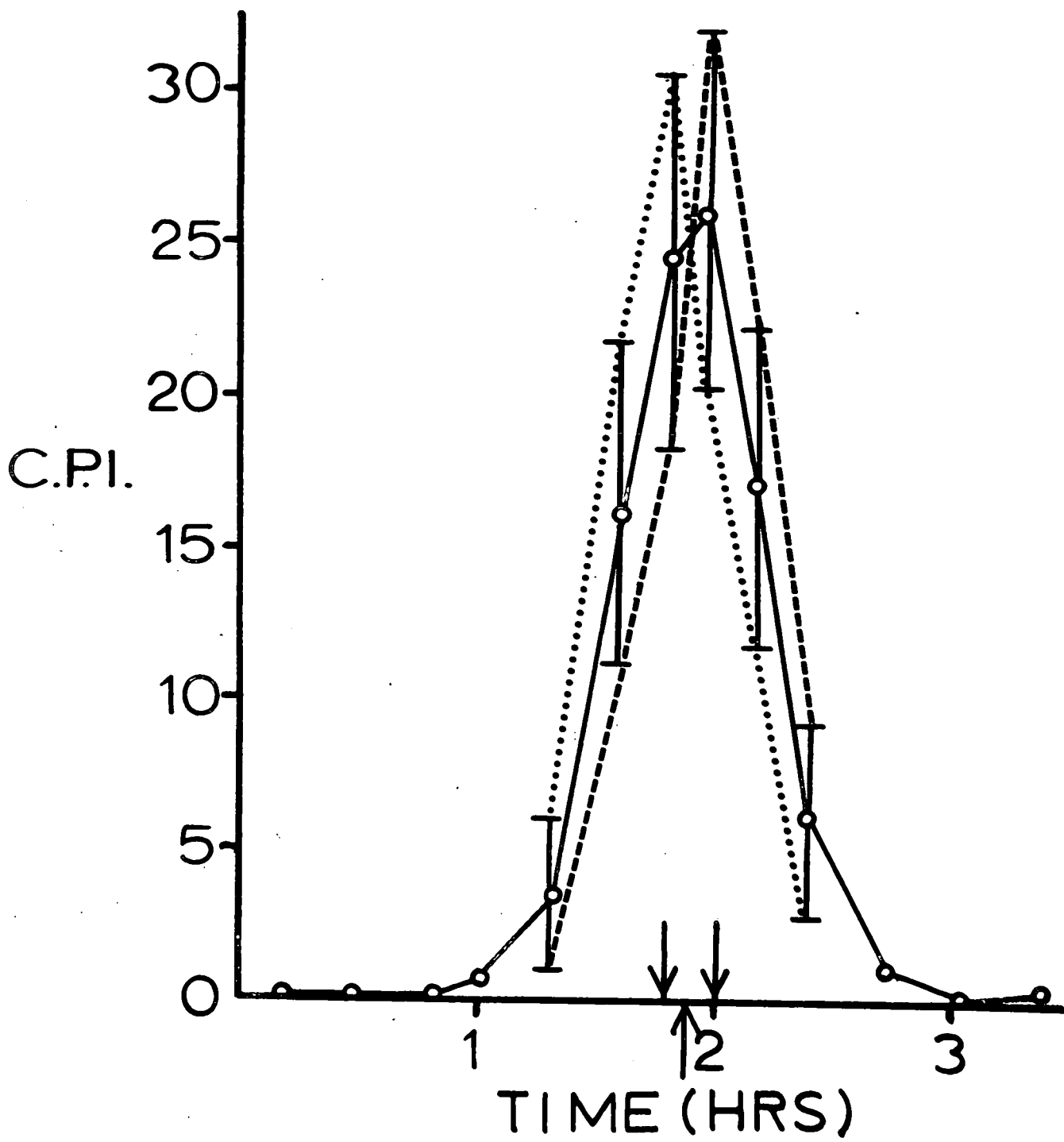
according to the formula given in Bailey for measurements follow-
ing a binomial distribution). The dotted lines are drawn through
the extremes of the intervals so as to give the extreme early and
late estimates of the CPP. These estimates are separated by only
13 minutes; moreover, they represent very extreme estimates because
they are calculated by combining already extreme events. As 13
minutes represents only 0.085 of a cycle I conclude that counting
error alone is unlikely to give rise to large inaccuracies. This
view is further supported by the data in Figure 2; since it is
unlikely that the treatment of the three pulsed cultures affected
the division of the cells at all these data may be treated as
replicate estimates and as such they are quite consistent, all the
CPP's falling within an interval of 5 minutes.

Unfortunately although random counting error is thus
quite small in its effect on the estimation of any one CPP, this
error will affect the calculation of the mean cycle position of
the cells three times, twice in the calculation of the mean genera-
tion time and once in the calculation of the end of the cycle.
Thus when a cycle position is given to two places of decimals
the second place of decimals is only relevant when comparing cycle
positions within the same experiment where the time between two
events, for example pulse treatments, is accurately known.

An alternative way of producing rather poor synchronous
cultures was to use the reinoculation system described by Bostock, ('68).
This system has only a low degree of synchrony but its advantages
are that the cells are in a different phase of their growth cycle,
the lag phase, and that there is a sharp burst of DNA synthesis
which occurs just after reinoculation.

Harvesting of the cells was by filtration exactly as

described by Mitchison ('70). Precautions were taken when harvesting the cells for transfer into or out of medium containing inhibitor. Oxoid membrane filters were used exclusively for this purpose and care was taken to see that not all the medium was removed from above the cells during the last stages of filtration; the cells were never allowed to dry on the filters. The use of Oxoid filters is important since other filters may affect the growth of cells (Mitchison and Creanor, personal communication). A 2.1 cm. filter was used for volumes of culture up to 80 mls. and above this volume a 6 cm. filter was used.

Inhibitors were added to the cultures dissolved in a small volume of medium to give the desired concentration, and in most experiments were removed again by the above procedure to effect a pulse treatment. However, this procedure could not be adopted in the case of Mitomycin C due to the fact that high concentrations of the drug are needed to affect S. pombe and the price of Mitomycin C is such that it would be quite uneconomic to treat the cells in this way. Thus the Mitomycin was prepared at required concentration in a small volume of medium at $32^{\circ}C$ and the cells were resuspended in this reduced volume. Normally the cells were concentrated no more than x 10 by this procedure, but in some experiments the cells were concentrated eightyfold in order to treat with the drug. Tests with Glucostat for exhaustion of the medium during this concentration showed that the cells did not exhaust the glucose in the medium during the period of concentration (details of the Glucostat test will be given later) and it is glucose that limits growth in EM II (Mitchison, personal communication). It is notable that this concentration of the cells is far less severe than they normally suffer during the normal procedure

FIGURE 2. - Four estimates of the cell plate peak
made upon a synchronous culture treated
by the concentrating procedure used to
pulse the cells with MC.

Top graph:- open circles - control; closed circles -
culture concentrated for 15 minutes
beginning at an estimated cycle position
of 0.13.

Bottom graph:- open triangles and closed circles -
culture treated at cycle position
0.33 and 0.53 respectively.
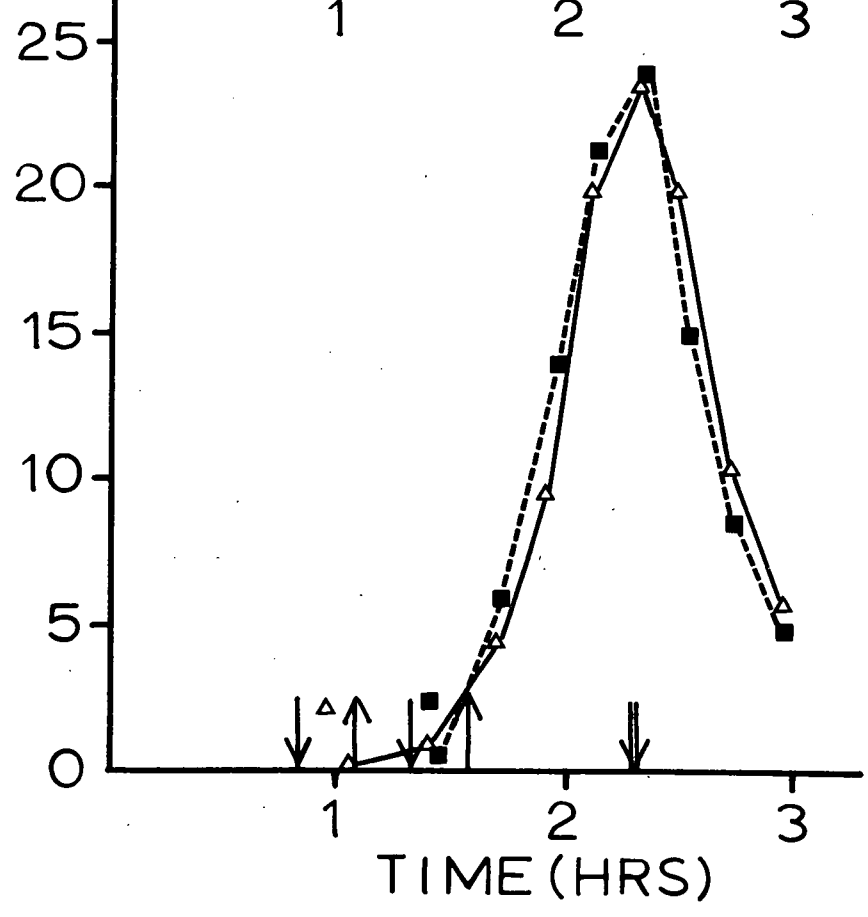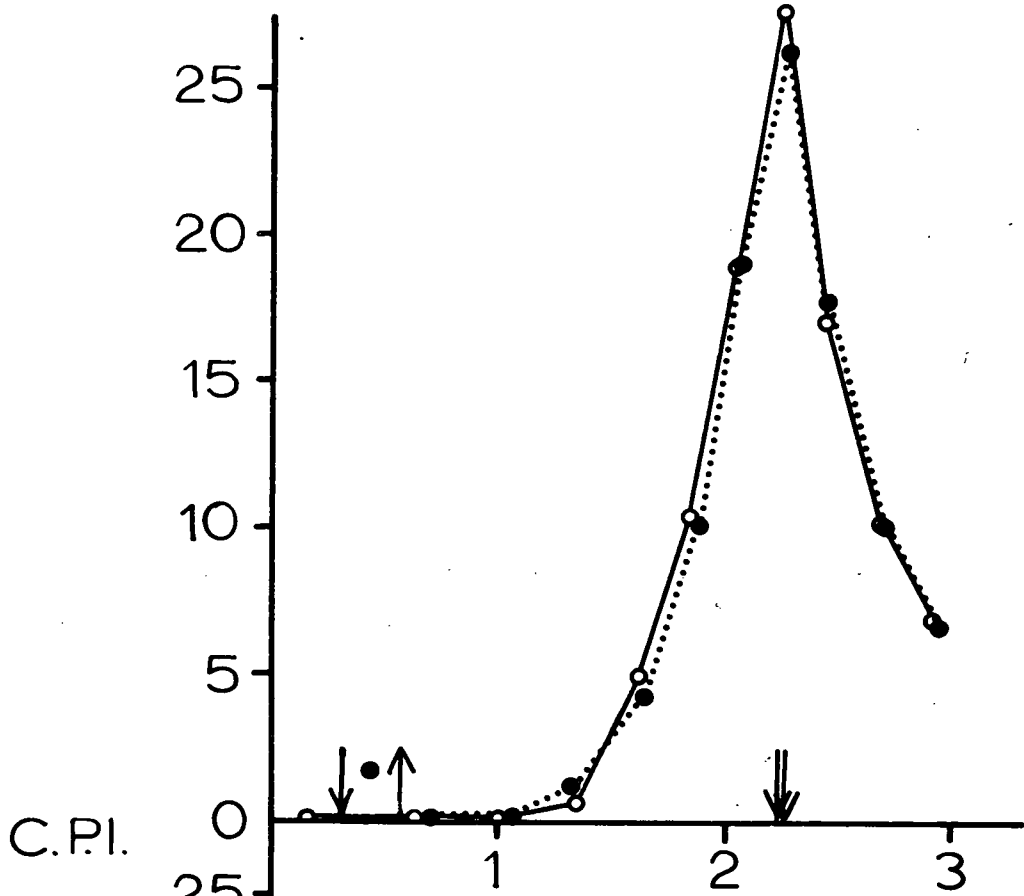
for producing the synchronous culture although it usually lasts a little longer. Before synchronisation the cells are concentrated to a thin paste (an eight hundredfold concentration) for some five minutes; the normal duration of the pulse treatment was fifteen minutes. Finally as shown in Figure 2, control experiments in which the cells were concentrated without the drug showed that the manipulation and concentration of the cells had no detectable effect on division. Where the duration of the pulse was longer than fifteen minutes cells were concentrated by factors smaller than x 10.

The inhibitors were removed as is described above and at this point the cells were washed free of the inhibitor by thorough washing with fresh warm medium; the volume of the wash was usually approximately equal to the volume in which the cells had formally been suspended, and in the case of cultures that had been concentrated for Mitomycin C pulse treatments considerably greater.

The inhibitors used in this study were Mitomycin C (MC), Hydroxyurea (HU), 2' Deoxyadenosine (AdR) and Cycloheximide (CHX). The sources of supply and a note regarding obtaining Mitomycin C are in Appendix IV. Solutions of the drugs were made up immediately prior to the experiments except in the case of MC which was dissolved in medium as soon as possible before use. The latter precaution was taken since the medium has a pH of 5.2 which is outside the stable range of MC and thus MC cannot be considered to be stable for long periods in EMM, and indeed there is a clear change in the absorbance spectrum of MC left overnight in EMM (D.Sutherland, personal communication). MC is also subject to degradation by visible light (Wakaki et al). However, the time-course of this degradation is slow and it is not expected that

this would contribute greatly to breakdown of the drug during the experimental period; some small precaution was taken by simply keeping the solutions out of direct light and strong sunlight.

Nuclear division was estimated in synchronous cultures by observing the proportion of binucleate cells found in fixed and stained cells taken from the culture at intervals. The method used to stain the cells was slightly different from the procedure for Giemsa staining given in the Appendix by C.F. Robinson to Mitchison's review ('70), but it has been found to be very satis-factory when it is simply desired to display a nucleus (or nuclei) in every cell. Samples were taken at intervals from the cultures and dried down on to pre-warmed slides. Afterwards the slides were washed free of medium in tap water and redried, and the cells fixed with Carnoy's fixative (absolute alcohol, chloroform, and glacial acetic acid in the proportions 6/3/1) for 15 minutes, and washed thoroughly in distilled water. They were then extracted with ribonuclease A (Sigma crystalline pancreatic RNase) at a concentration of 100 μg/ml in phosphate buffer pH 7.0 for some three hours at a temperature of $30-40^{\circ}$C. The enzyme solution was heated to 100°C for 10 minutes before use to denature any DNase present. The slides were then washed for exactly 10 minutes with running tap water and stained in 4% Giemsa solution (Gurr's R66) made up in phosphate buffer pH 7.0 for $4\frac{1}{2}$ hours whereupon they were removed for inspection. If they were adequately stained they were then mounted in stain highly diluted with the same pH 7.0 buffer, most of the liquid was removed from underneath the cover-slip and the coverslip was then pressed down firmly to compress the cells. The mounts were then sealed with clear nail varnish. Nuclear structure was not revealed by this method but the nucleus

is strongly stained and clearly visible and it was easy to distinguish cells with two nuclei. Proportions of binucleate cells were estimated by scoring not less than 400 cells.

Cell lengths were measured by examining slides of cells stained with crystal violet and negatively stained with an Indian ink solution (see Mitchison '70) with a microscope fitted with a graticule eyepiece. At least 50 cells were measured and scoring was usually restricted to those cells with cell plates. Comparison of the lengths of treated cells with those of the control culture was made by using Student's 't' test as modified by Bailey for use in cases where the variances are unequal.

The method used to estimate DNA was that developed by C.J. Bostock ('68) and involves the DNA extraction of Schneider as modified by Hutchison and Munroe and the standard Burton diphenylamine assay or the modified Burton assay described by Giles and Myers. Samples of at least 80×10^6 cells were removed from the culture and harvested by collection on Millipore filters; in some early experiments Oxoid membrane filters were used but these were found to give rather variable results compared to Millipore filters. The filters were placed in disposable polystyrene tubes and stored in the deep freeze over night. Next day the samples were resuspended in 0.50 M perchloric acid (PCA) at $0^{o}C.$, and the filters were removed from the tubes. After 30 minutes the cells were spun down and the supernatant removed with a pasteur pipette redrawn to give a very fine tip using a filter pump adjusted to give a low suction. The cells were then washed in 0.50 M PCA and once more the supernatant was discarded. Great care was taken to remove all the PCA with as small a loss of cells as possible. 0.25 mls of 0.50 M was added to each tube and the tubes were

stoppered and placed in a 70°C water bath and incubated for 20 minutes with agitation by vortex mixer every 5 minutes. The tubes were then cooled to 0°C and the cells spun down, 0.2 mls of the supernatent was removed from each tube using a capillary pipette and assayed for purine bound deoxyribose by the diphenylamine method.

The diphenylamine (DPA) reagent used was either that of Burton (2% DPA, 1.5% concentrated Analar H_2SO_4, and 0.1% aqueous acetaldehyde (Analar made up at 16 mg/ml) in Analar glacial acetic acid) or in later experiments that of Giles and Myers (4.0% DPA in glacial acetic acid with 0.1% aqueous acetaldehyde) 0.4 mls of reagent was added to each tube which was then stoppered and incubated for 16 to 20 hours at 32°C after which the samples were spun to remove any turbidity that had developed and then the absorbance at 595 mμ and at 700 mμ was measured in a Hitashi Perkin Elmer spectrophotometer. Deoxyadenosine in 0.5M PCA was used as a standard and calibration curves were linear up to concentrations of 20 μg/ml; separate calibration curves were made for each assay. The DNA content of the samples was calculated by subtracting the $O.D_{700}$ from the $O.D_{595}$ and comparing this value with the calibration curve ($O.D_{595} - O.D_{700}$ plotted against concentration of deoxyadenosine) and assuming that if in DNA the purine to pyrimidine ratio is one then one unit weight of deoxyadenosine will produce the same absorbtion as 2.60 units weight of DNA. Controls to check that this procedure does in fact measure only DNA were performed by C.J. Bostock ('68) who found that all the diphenylamine positive material was sensitive to deoxyribonuclease.

Protein estimations were by the method of Lowry et al. Cells were first fractionated by a slightly modified Schmidt-

Thannhauser fractionation (described N.Stebbing, '69 and '71); the procedure was as follows. Samples of between 10 and 40 x 10^6 cells were taken and harvested on Whatman GFA glass fibre filters and washed with at least 100 mls of distilled water. Both the choice of filter and the thorough washing are important since GFA filters give a very low background absorbtion in controls and the medium gives a Lowry positive reaction. The filters were placed in disposable polystyrene tubes and 1.5 ml of 1.0M NaOH was added to each of the tubes including a control containing only a filter. The PCA pool extraction was avoided. The tubes were then incubated for 3 hours at $32^{\circ}C$ after which the digest was agitated to disrupt the filter and a 1.0 ml aliquot was removed from each tube to another 10 ml Sterilin tube containing 1.0 ml of 1.0N PCA at $0^{\circ}C$. The tubes were kept in an ice bath for a few minutes to ensure the complete precipitation of protein and then the tubes were spun and the supernatant was removed and retained for the estimation of RNA. . The precipitate was then raised in 1.0ml of 0.2M NaOH containing 2% deoxycholate and left overnight at a temperature of $32^{\circ}C$ to re-dissolve completely. The addition of deoxycholate is the modification of N.Stebbing ('69).It was found by him to aid the dissolution of the protein precipitate and to reduce the standard error of the estimations. However, he also found that it effected the stability of the colour developed in the assay. The colour was found to reach a maximum after 8 minutes and to decline after 30 minutes after the addition of the reagent, thus the samples were always read in the spectrophotometer before 30 minutes had elapsed. The protein estimation was made using the following reagents.

A.   0.10 M sodium hydroxide

B.   4% sodium carbonate

C.   2% sodium potassium tartrate

D.   1% copper sulphate

E.   Folin-Ciocalteu reagent (obtained from Hopkin and Williams Ltd. Folin-Ciocalteu for phosphatase, diluted threefold before use).

The copper alkali solution was prepared by mixing 100 mls of A. with 100 mls of B. and adding 2 mls of C. and 2 mls of D. in that order, and 5 mls of this solution was added to each 1.0 ml sample with an Arnold Horwell automatic pipette to ensure good mixing. After 10 minutes 0.5 mls of Folin-Ciocalteu reagent was added to the tubes whilst they were being mixed with a vortex mixer to ensure that the reagent was dispersed immediately. The tubes were then set aside and read as mentioned above between 8 and 30 minutes after the addition of the reagent. The absorbance at 740 mμ was read in a Hitashi Perkin Elmer spectrophotometer against a filter and reagent blank. The standards used to calibrate the results were Bovine serum albumin solutions made up freshly in 0.2 M NaOH containing 2% deoxycholate at concentrations of 100, 200, 300 and 400 μg/ml BSA , which were read against a reagent blank. The calibration curve is not quite linear as noted in O.H.Lowry et al.

RNA was estimated by measuring the absorbance of the supernatant left after the precipitation of protein by cold PCA. from the 1.0 M NaOH digest at 260 mμ and assuming that the absorbance readings were directly proportional to the amount of RNA present. This assumption is reasonable since DNA in S.pombe represents only about 1% of the total nucleic acid (Mitchison and Lark '62; Bostock '69) and the pool of nucleotides during the exponential phase of growth may be expected to contribute only 2% of the absorbance (N.Stebbing '69).

The sucrase derepression experiments were performed in
the manner described by J.M.Mitchison and J.Creanor ('69). 2$\frac{1}{2}$ ml
aliquots were taken at intervals from synchronous cultures and
diluted x 20 with fresh warm EMM II made up without glucose. From
these derepressed sub-cultures 3 or 4 10 ml samples were taken at
intervals and the cells were harvested on Millipore filters and
given eight washes of distilled water to remove all trace of glu-
cose; the filters were then removed to disposable polystyrene
tubes (Luckhams,LP3).

To assay the sucrase activity in the samples the cells
were resuspended in 1.0 ml of 5% sucrose mixed with two volumes of
0.067M Sorensens phosphate buffer, pH 6.8 containing 0.01M
ethylenediaminetetra-acetate (sodium salt) and incubated, leaving
the filter in the tube for about two hours. At the end of this
incubation the filters are removed and 0.5 mls of Glucostat
special reagent (Worthington) was added to each tube at intervals
of 10 seconds in order. When sufficient colour had developed as
judged by eye the reaction was stopped by the addition of 1 ml of
0.25M HCl in the same order as the reagent at 10 second intervals
to ensure a constant incubation time with the reagent. The cells
were then spun to the bottom of the tube and the supernatant was
measured for absorbance at 400 mμ against filter and a reagent
blank in a Hitachi Perkin Elmer spectrophotometer.

Another technique used to investigate both the division
and the growth of the cells was time lapse photography of single
cells growing on a small pad of agar; this technique was develop-
ed by Swann and has been described by him. The apparatus is a
Carl Zeiss Photomicroscope enclosed in a perspex enclosure kept at

constant temperature with an electrical fan assisted heating unit.
The photomicroscope camera is controlled by a time switch which
may be adjusted to control both the interval at which exposures
are taken and the duration of the exposure. The agar mount is
prepared by placing a few drops of molten agar (1.5% Oxoid Ionagar
No.1 in EMM II) on a clean slide such that it spreads to give a
small flat pool about 1 mm deep. When this has set it is trimmed
to give a small pad roughly 1 cm square. A sample of about 5 mls
is removed from the culture and is concentrated by centrifugation
roughly ten times using a bench centrifuge at full speed for three
minutes. Cells from this concentrated culture are then placed on
to the pad using a sterile loop, several loopfuls are necessary,
and the pad is covered with a clean coverslip. The slide is then
placed on the stage of an old microscope the objective of which has
been replaced with a special tool. This tool is merely a solid cy-
linder of brass about the same size as a normal objective which
has been threaded at one end to allow it to be screwed into the
nose of the microscope, the other end of which is quite flat. The
microscope is racked down until this flat end is in contact with
the coverslip and then, using the fine focus, the block of agar is
very slightly compressed and the coverslip is fixed in position
with four drops of hard wax. This procedure ensures that the
coverslip lies in a parallel plane to the slide so that all the
cells under the coverslip can be accommodated in the plane of focus
of the objective which will later be used to observe them. Next a
small capillary tube, drawn down to about 250-500 microns, into
which a small drop of liquid paraffin has been drawn is placed on
the slide with one end under the coverslip and near the agar pad.
The edges of the coverslip are then sealed with soft wax so that

the agar pad lies in a sealed chamber. The purpose of the capillary tube is to allow the pressure within this chamber to equilibrate with the outside when the preparation is transferred to the constant temperature enclosure.

The preparation must be closely inspected for any air leaks as these can lead to dessication and shrinkage of the agar. The agar mount can be made in a very small space of time, some two minutes being ample. The preparation is then observed under the photomicroscope and a suitable field of cells is chosen for observation; in a typical experiment designed to observe only division this would constitute a sample of about 150 cells, and the photomicroscope is adjusted to begin taking exposures at the desired time lapse. Thereafter it is only necessary to check that the microscope stays in focus as there is a tendency during the first two hours of the experiment for the focus to slip, probably due to temperature equilibration of the mount. The growth temperature in all experiments in this study was $32^{o}C$. Dark ground illumination was used and the camera was loaded with Kodak Panatomic x 35 mm film. Illumination was the maximum obtainable and the exposure time was 40 seconds.

The resulting negatives were not printed but were analysed by projection using a Robot 35 mm film projector; this is a very convenient device which throws an image on to a semi-opaque screen which can then be marked with a water soluble marker (Pentel, Sign-pen) making it easy to follow the development of individual cells.

CHAPTER 1.

The action of pulses of Mitomycin C on synchronous cultures.

As I have previously mentioned in the introduction it was known from the outset of this work that the division of S.pombe could be inhibited by high concentrations of MC (400 μg/ml) and that, since there was little effect on growth, giant cells were produced. However, no satisfactory experiments had been performed to test the reversibility of this inhibition and thus the effect of limited exposure to the drug was unknown.

The result of such a treatment (henceforward referred to simply as a pulse of the drug in question) may be seen in Figure 3. and in Plate 1. Figure 3. shows two preliminary experiments in which asynchronous cultures were used. After a pulse of the drug division continues for about 40 minutes, ceases for a period and then resumes in a 'parasynchronous' wave of division. These results immediately suggested two points, that there was a transition point at the end of the cycle after which the cells were resistant to delay and that for the majority of the cycle the pulse treatment caused a division delay which probably varied with cycle position. This last inference was made because if a constant delay had been induced then one would not expect division to restart in this partially synchronous manner.

Plates 1 and 2 simply illustrate the production of giant cells by a pulse treatment with MC.

This indication of differential delay through the cell cycle suggested the use of synchronous cultures and at this point a standard pulse of 500 μg for 15 minutes was decided upon as a dose that was likely, judging from preliminary results, to produce

PLATE I   -   An asynchronous culture of S.pombe showing

cells at all stages of the cell cycle.

PLATE II   -   An asynchronous culture some hours after

treatment with 500 μg/ml mitomycin C

for 15 minutes, showing highly elongated

cells.

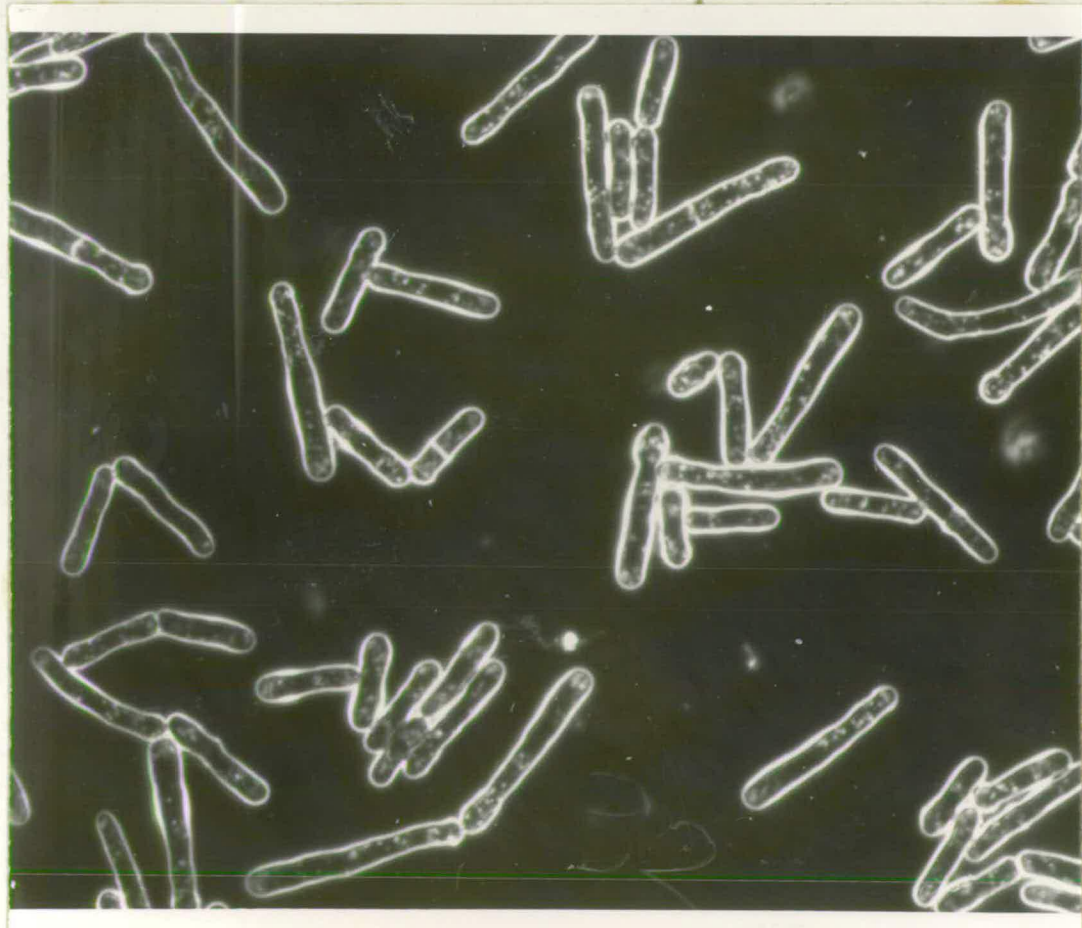
(In both Plates I and II, the magnification

is the same).

FIGURE 3. – Two experiments to show the effect of a
pulse of MC on the division of an
asynchronous culture.

Graph A – The effect of a pulse of 500 µg/ml MC
from 0-15 minutes upon division measured
by the effect on the CPI.
Open circles - control CPI, closed circles -
treated culture CPI.

Graph B – The effect of a pulse of 400 µg/ml MC of
30 minutes duration (ending at 0 minutes)
upon division measured by the direct
cell counts.
Closed circles - cell density in the
control culture.
Open triangles - cell density in the
treated culture.
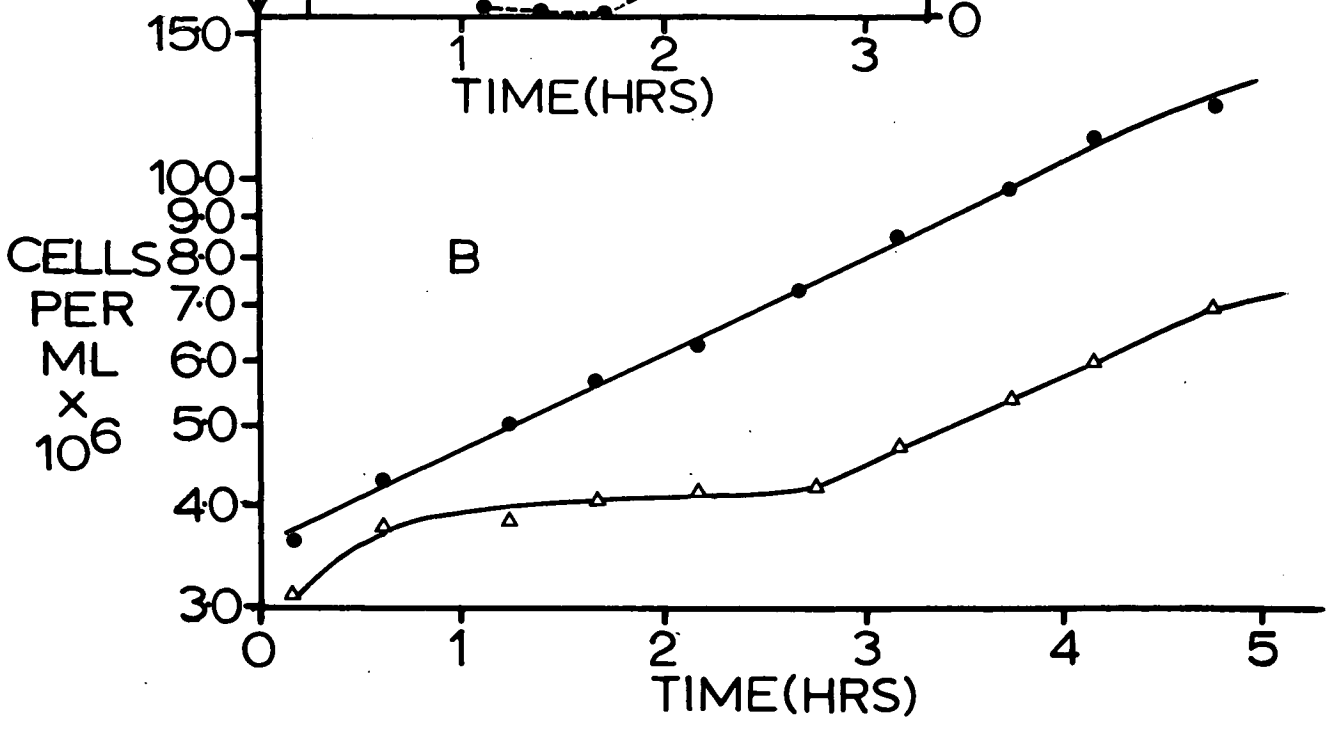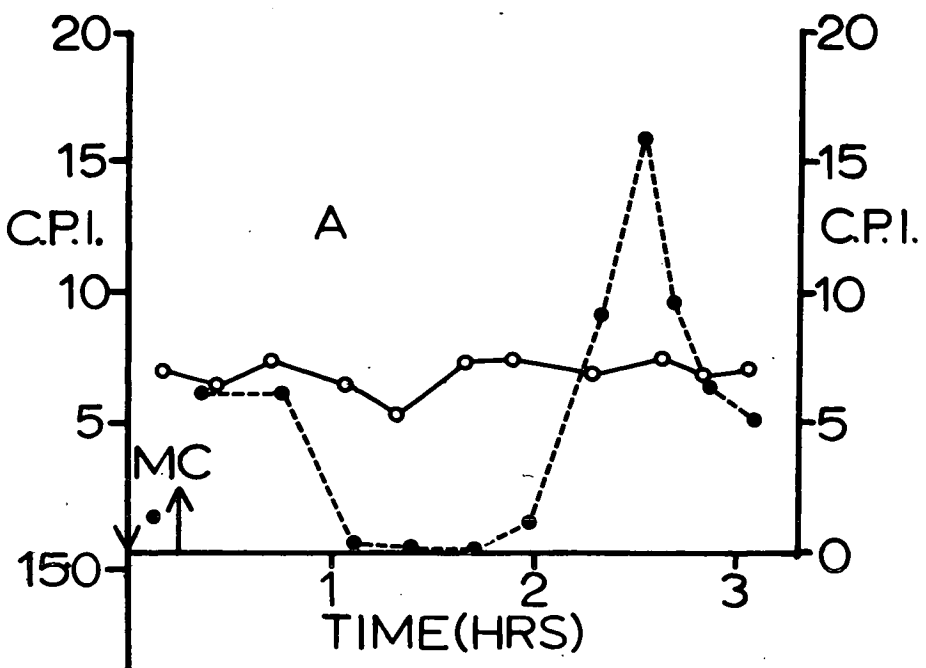
a workable delay. Since MC has been found in later experiments to be a fast acting inhibitor inasmuch as short pulses will cause delays, all pulse positions in the cell cycle are calculated from the beginning of the pulse.

Figures 4, 5 and 6. show the results of three experiments in which synchronous cultures were pulsed at different points in the cell cycle. Figure 4 shows the results of pulses that fall in the first part of the cycle; the forthcoming division is delayed in these cells and as the position of the pulse is moved later in the cell cycle the delay is seen to increase. The two latest pulses show that a small number of cells are not delayed and that there are two small 'escape' peaks in the CPI. These escape peaks can be seen even more clearly in Figure 5 which shows a series of pulses placed in the beginning and around the middle of the cycle of a culture of 972h⁻.

This failure of the cells to be delayed if they are treated after a certain point in the cycle is a feature of many inhibitory treatments and will be referred to as a transition point. Several experiments showed that this transition point occurred regularly in synchronous cultures at the same point in the cycle.

Figure 6 shows the result of treating the cells at the end of their cycle after they have passed their transition point. In both cases the first division is completed normally but the subsequent division fails to occur and is subject to a very long inhibition which for many cells exceeds the duration of this experiment. Thus the cells, whilst they are completely insensitive to first division delay, are extremely sensitive to second division delay.

FIGURE 4.   -   The effect of a "standard" pulse of MC

applied at early stages of the cell

cycle upon the division of a synchronous

culture.

The estimated pulse positions in the cell cycle were 0.19,

0.27, 0.35 and 0.44. The long arrows on the abscissa

represent the estimates of the cell plate peak. The two

short arrows labelled MC represent the position of the

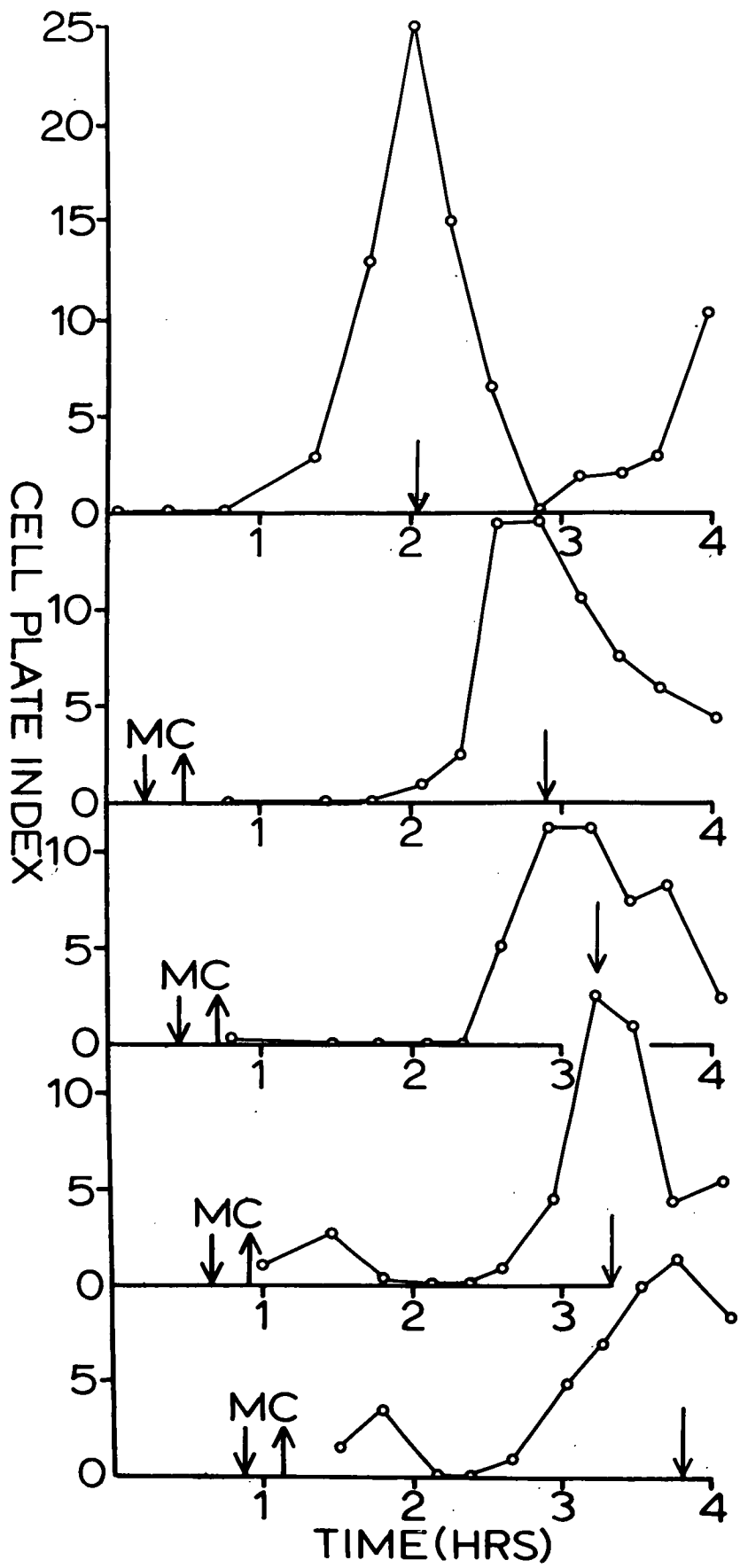pulse. (This will be standard notation in subsequent

figures).

FIGURE 5.   -   The effect of four MC pulses upon a syn-
               chronous culture of 972h⁻ which illustrate
               the transition to resistance to first
               division delay during the first cycle.

Top graph   :-   Open circles - control, closed circles -
                  MC pulse at 0.12.

Middle graph:-   Open squares - MC pulse at 0.26.
                  closed squares - MC pulse at 0.59.
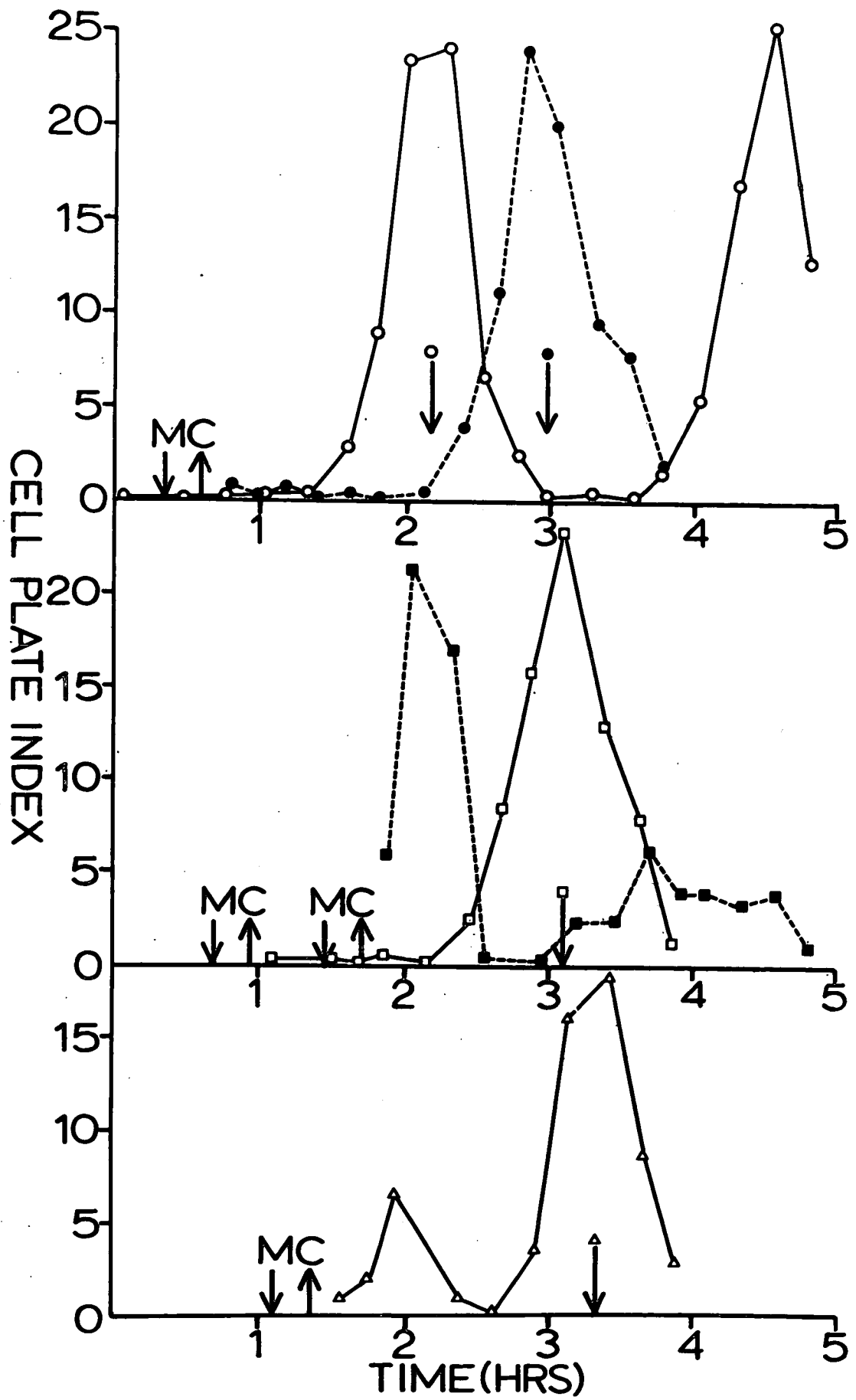
Bottom graph:-   Open triangles - MC pulse 0.45.

FIGURE 6.   -   The effect of two "standard" pulses of

MC into in the cell cycle.

Top graph   :- Round circles - control culture.

Solid squares - treated culture.

Pulse position was 0.77.

Bottom graph:- Second treated culture. The pulse
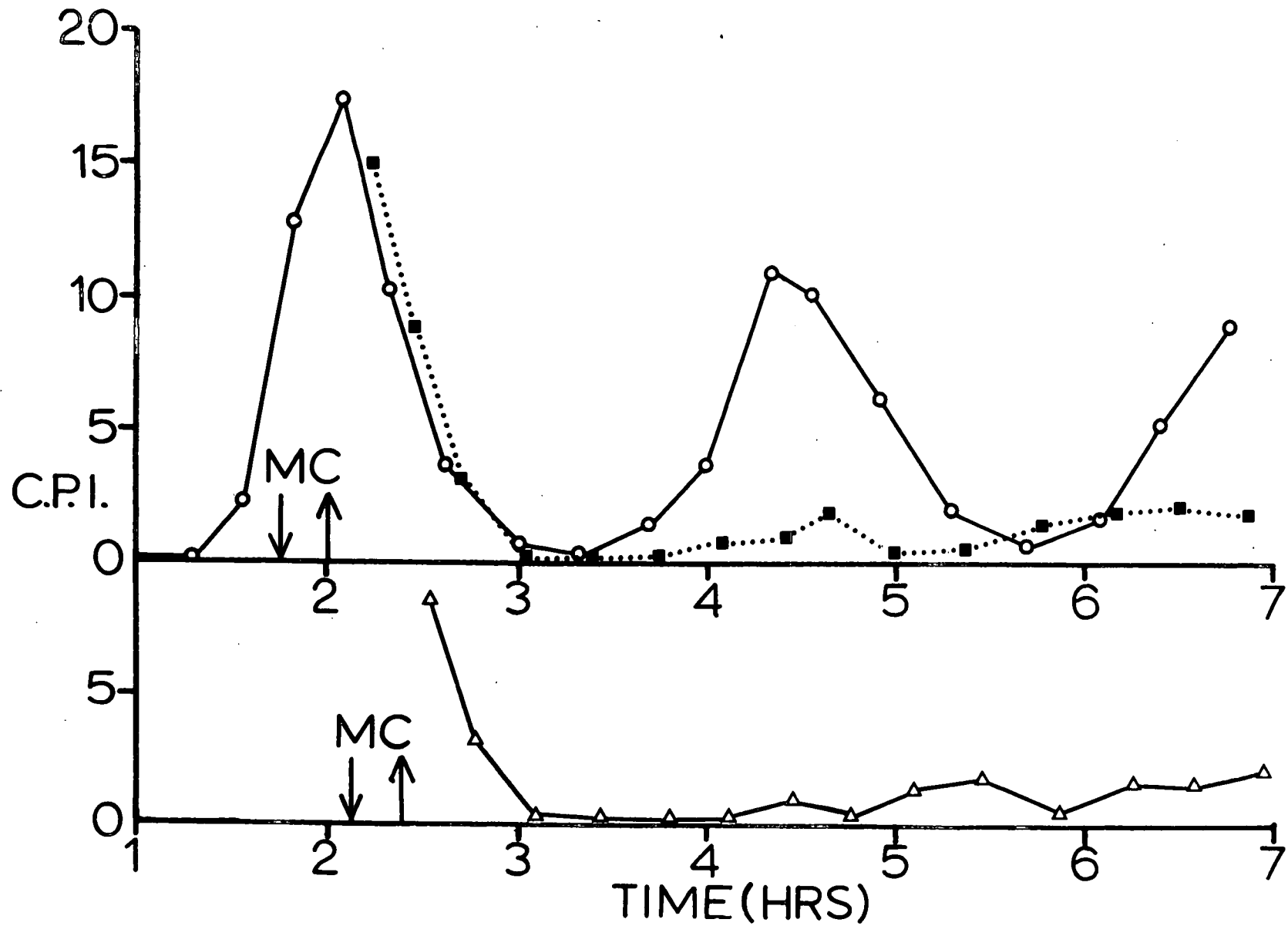
position was 0.93.

FIGURE 7.   —   Four MC pulse treatments which illustrate
the transition to resistance to division
delay.

Top graph   :—   Open circles — control

                                      Closed circles — MC pulse at 0.88.

Upper middle graph :—   MC pulse at 0.95

Lower middle graph :—   MC pulse at 0.03.

Bottom graph   :—   MC pulse at 0.09.
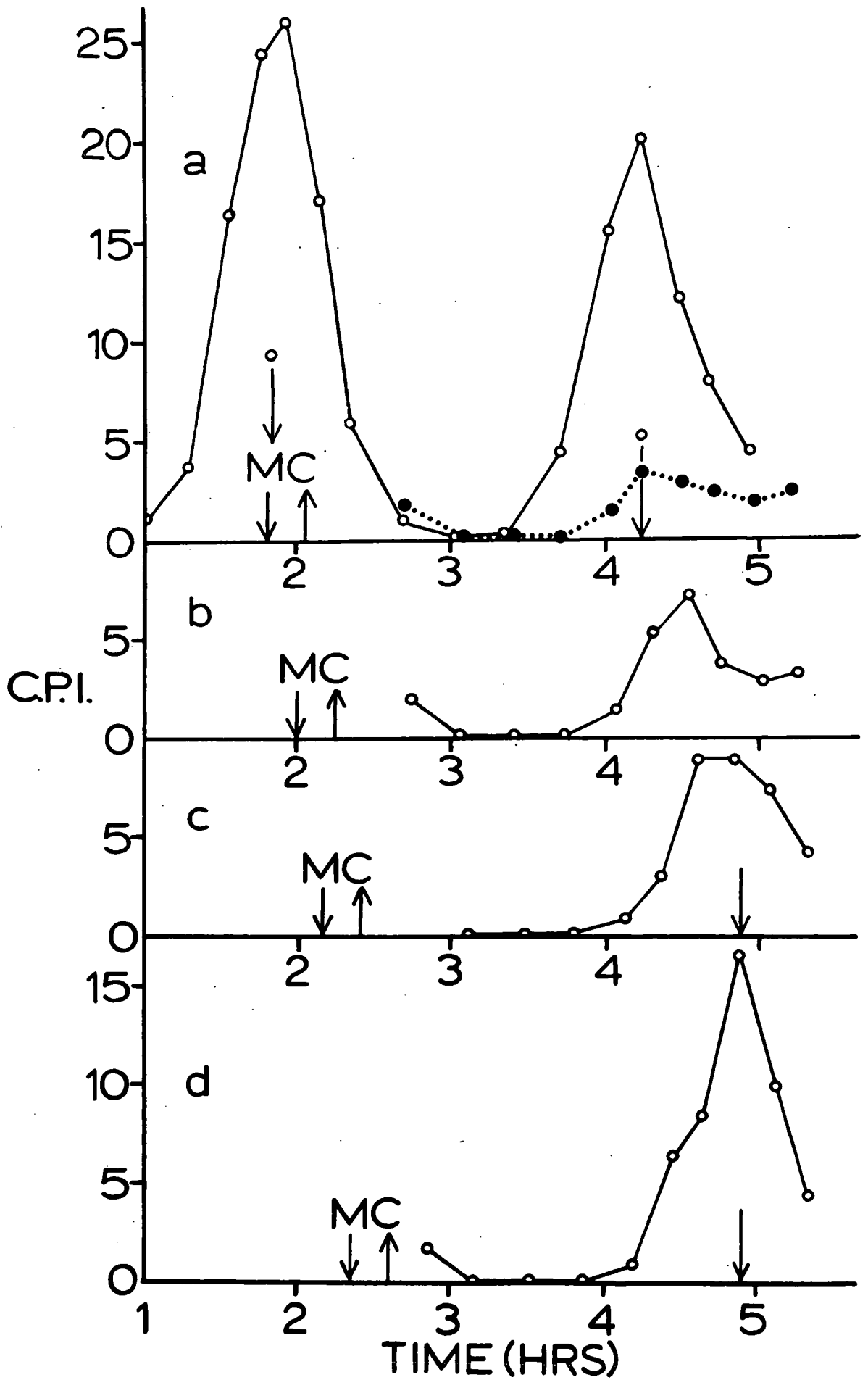
FIGURE 8.   -   Collected data to illustrate the change in
              delay induced by a "standard" MC pulse during
              the minor period of sensitivity as the pulse
              position is changed.

        In both graphs the ordinate shows delay in minutes whilst
the abscissa represents the full cell cycle. The calculated
positions of the transition points are shown by the vertical
solid lines labelled 'TP' whilst the horizontal solid lines
delineate the period of high sensitivity (shown 'HS' on the top
of the ordinate axis).

        The top graph shows the collected data for strain 132
(closed circles).

        The bottom graph shows the data for strain 927h$^-$ (open
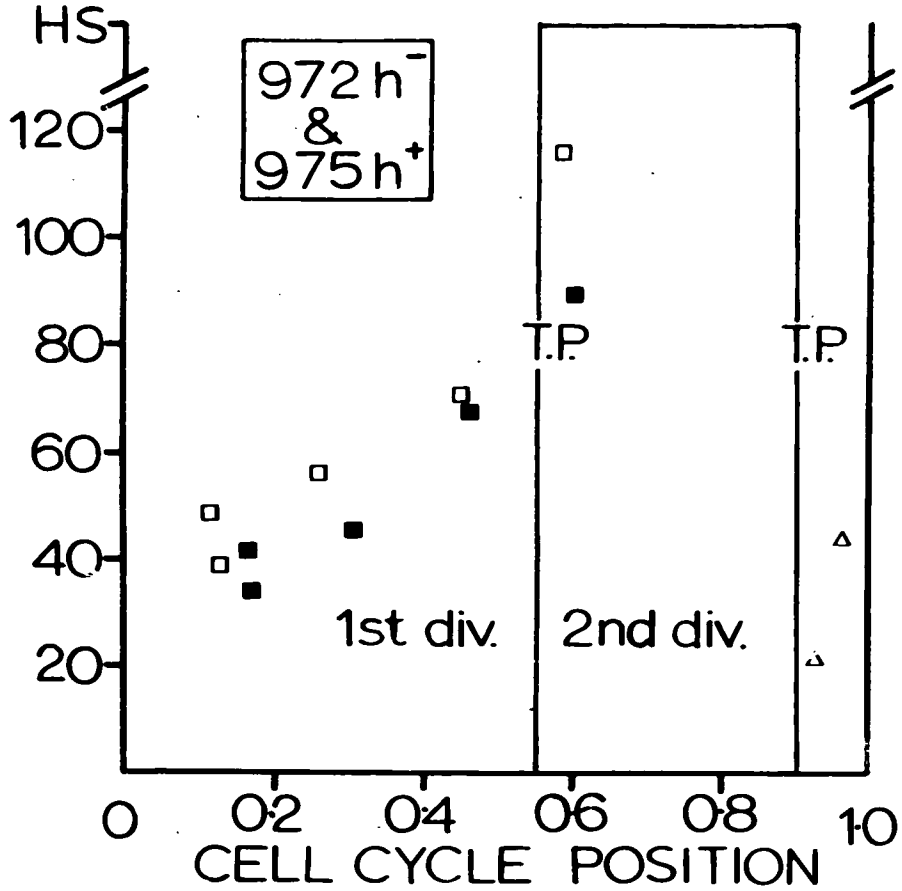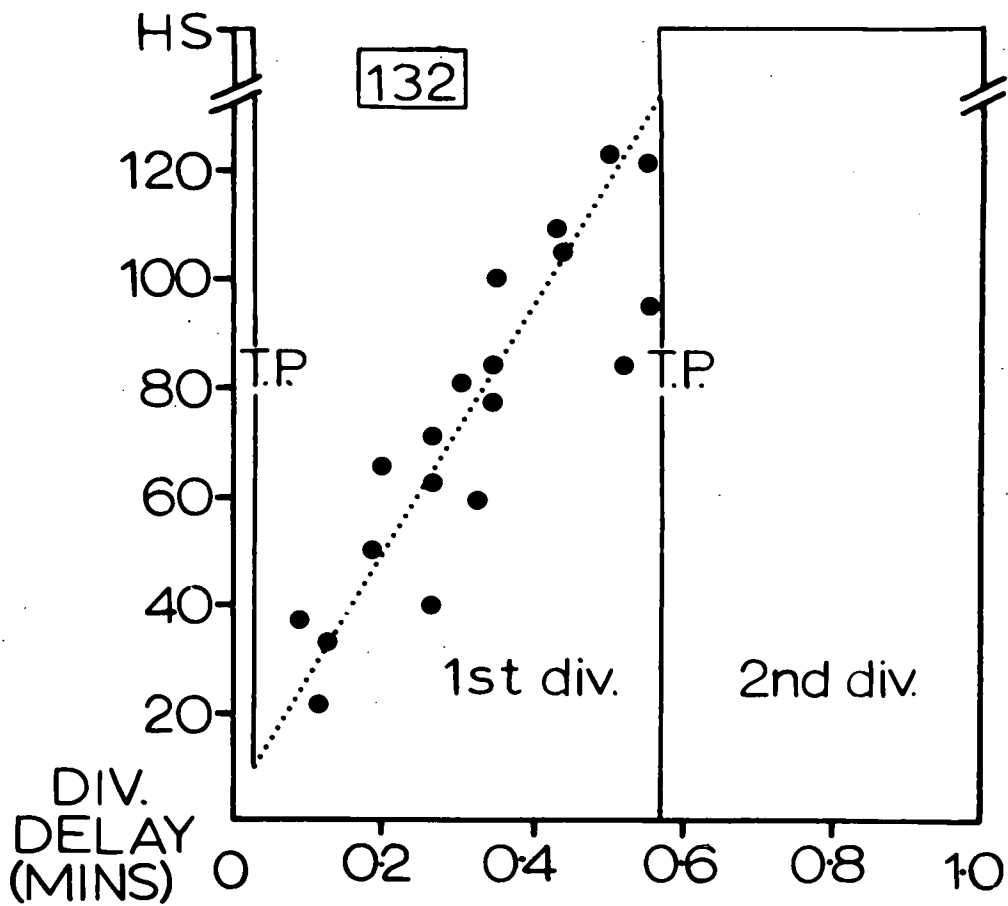squares) and strain 975$^+$ (closed squares).

Figure 7 shows that around fission the cells undergo another transition and revert to the same low level of sensitivity found in the early part of the cycle.

Figure 8 summarises the results of several experiments of the type described above, performed both upon strain 132 and upon strains 972h$^-$ and 975h$^+$. All the results are quite consistent with the following pattern of sensitivity through the cell cycle. At the beginning of the cycle the cells are at their most resistant to induced delay but as they pass through their cycle they become progressively more sensitive to first division delay. This rise in sensitivity is roughly linear but the data is not good enough to allow this to be stated definitely.

Just after mid-cycle the cells suddenly become completely resistant to MC induced delay; they pass through a transition point that I shall term the first transition point (TP-1). If treated in this phase the cells divide normally but they suffer a very severe delay in their second division.

At around fission the cells once more return to their original resistant condition and there is thus a second transition point (TP-2).

When this fairly complex pattern of sensitivity had emerged it was immediately recognised as being almost identical to that found by Gill in his investigation of the sensitivity of S.pombe to division delay induced by low doses of ultra violet irradiation. These results thus furnish another example of the UV radiomimetic effect of MC which have been noted by a number of workers, a point to which I shall return in the discussion.

This similarity to Gill's data will be discussed below; meanwhile I shall adopt one of the terms used by Gill. That part of

the cycle between TP-1 and TP-2 during which the cells are very
sensitive will be referred to as the major period of sensitivity
or simply the major period and that part of the cycle between
TP-2 and TP-1 during which the cells are relatively resistant
will be termed, by extension of Gill's nomenclature, the minor
period.

Delays produced in the minor period were relatively easy
to quantify as such delays may be measured simply by the dis-
placement of the CPP. Figure 8 contains the results of 11
experiments on strain 132 and the rising pattern of delay is quite
clearly shown(as mentioned above the linearity of the rise is only
tentative) Where more than one pulse was placed in the minor
period in a single experiment the results were always consistent
with a rising pattern of delay.

Figure 8 also shows that the other two strains examined
showed the same overall pattern of sensitivity (with possibly a
slightly earlier TP-2); this result was encouraging as it shows
that this complex pattern is more likely to reflect complex
changes in the cells and is not merely an idiosyncrasy of our strain.

It is also noteworthy that the rising pattern of delay
during the minor period explains the 'parasynchronous' wave of
division caused by a pulse upon an asynchronous culture as this
effect will tend to align the division of cells treated in this
period.

Measurement of delays caused by the standard pulse
(500 μg/ml for 15 minutes) during the major period poses a more
formidable problem since the delays are both long and rather
variable. Figure 5 shows that the delays are much too long for
the CPP displacement method to be of any use. The method of choice

FIGURE 9. - The effect of two "standard" pulses upon the

division of a synchronous culture measured by

photomicroscopy.

Open circles - CPI in untreated culture.

The 1st pulse was at 0.96. The closed circles represent the rise

in number produced by the 1st division of observed cells only

(i.e. the 2nd synchronous division) whilst the top plot (- open

circles) shows the total number of cells in the field. The

estimated "mean" delay was 214 minutes.

The 2nd pulse was at 0.22 (- open squares). The estimated mean

delay was 93 minutes.

Three other experiments similar to that presented above gave

the following results :-

Strain.	Cycle position of pulse.	Delay (minutes).
132	0.81	199
132	0.88	250
975h[+]	0.76	greater than 240
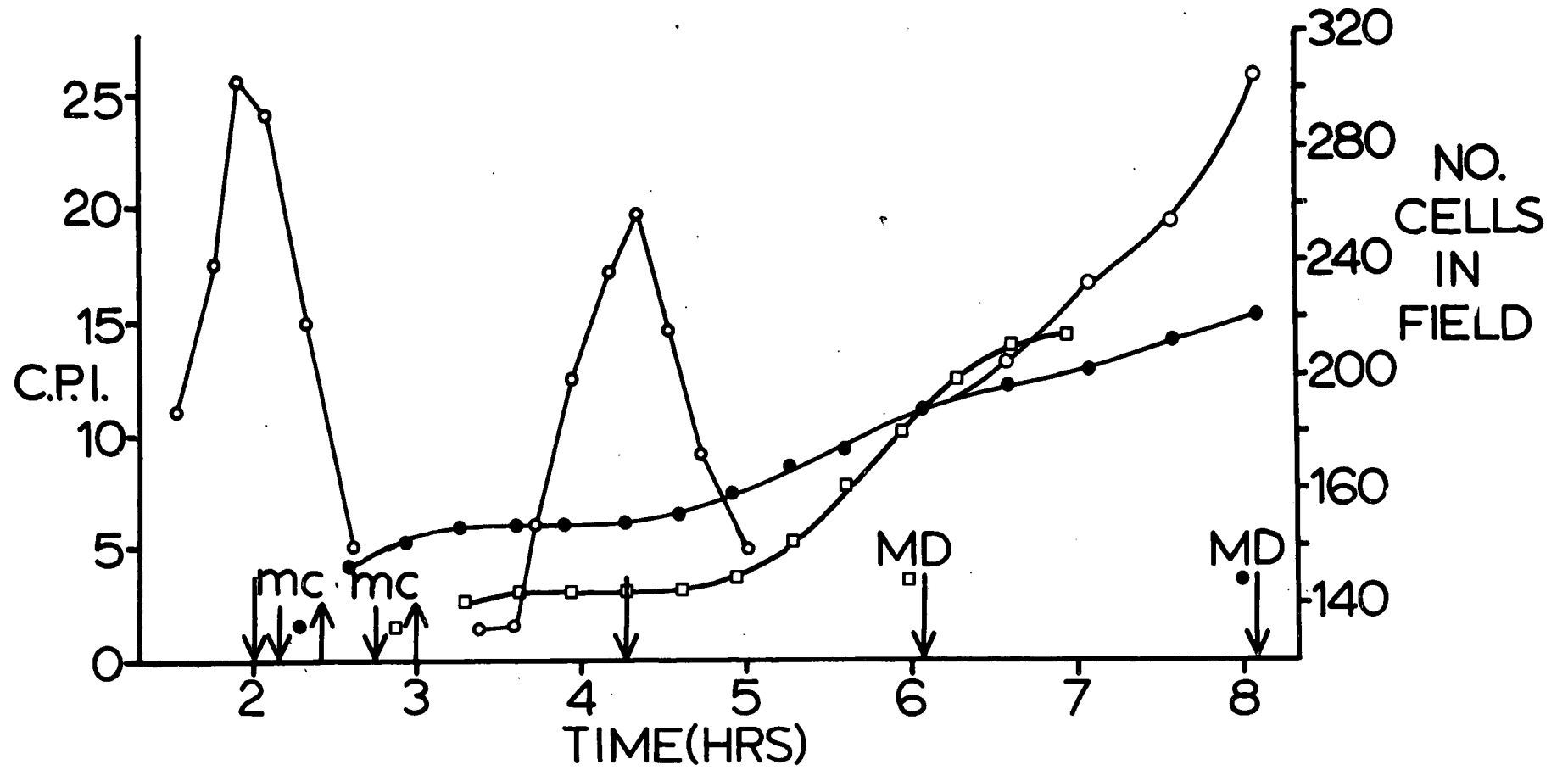
was thus direct cell observation by time lapse photography, the same technique that was used by Gill for his study of UV induced delay with the exception that synchronous cultures were used.

The use of synchronous cultures for these experiments was necessitated by the fact that with a chemical inhibition observation of the cells before the pulse treatment is not possible without the use of a perfusion chamber of the type used by Faed in his study of cell growth. The use of a perfusion chamber brings its own attendant problems of regulating drug dosage. Thus if asynchronous cultures are used the position of each cell in its cycle must be decided by the criterion of length alone. The errors attached to this method have been thoroughly discussed by Gill and he showed that it was specially inaccurate for cells in the last half of the cycle. Since the major period covers this part of the cycle it was decided to use synchronous cultures and to assume that the 100 to 150 cells selected for observation were representative of the whole culture.

The criterion used for measuring the length of the division delay was the time taken for half the cells observed to undergo the affected division and the time at which the control cells underwent that division was calculated from the position of the CPP in the control culture.

Figure 9 shows the result of an experiment performed by observation of a synchronous culture. The culture was treated at an estimated cycle position of 0.96 and a small sample of cells removed to an agar pad as described in the materials and methods section. A field was selected which at time 2 hours and 30 minutes contained 144 cells, most of the cells having undergone their first division before removal to the pad. As may be seen the division of

these cells was greatly retarded and it was not until 214 minutes after the control culture had divided that this culture had achieved a half doubling of cell numbers due to the second synchronous division.

It must be noted that the total number of cells in the observed field rises steeply after 6 hours but most of this increase is due to the division of cells which did not suffer a long delay; in all probability these cells were those cells which were not in their major period at the time of treatment. Fortunately the method allows the fate of every individual cell to be followed and thus the increase due to just the second synchronous division (which is the first observed division of the cells on the pad) may be plotted. However, it is not possible to follow the fate of cells for much more than 8 or 9 hours since the progeny of divided cells rapidly overcrowd the field. It was certain that some of the cells did not undergo their second division at all but it is not known if this was due to MC damage or to lack of nutrients; also a very few cells became refractile and died.

At best this method can only give a rough estimate of the true division delay. Apart from the difficulties mentioned above the method of calculating the average position of the pulse from the parent culture and applying this to a small sample of cells is liable to sampling error. Also the measurement of delay is subject to two serious errors. Firstly that it is based on an observation of only half the cells, the fate of the other half of the population being unknown, and secondly a small but unknown proportion of the cells will not have been in their major period when treated due to the fact that synchrony in the parent culture

FIGURE 10. - The division delay produced by a dose of
62 ergs/mm^2 of UV irradiation at various
times during the cell cycle.

N.B. This data is reproduced by kind permission of B.Gill.

The stage in the cell cycle was calculated according
to the cell plate convention of Swann.

The solid line represents 1st division delay, the
dotted line, 2nd division delay.
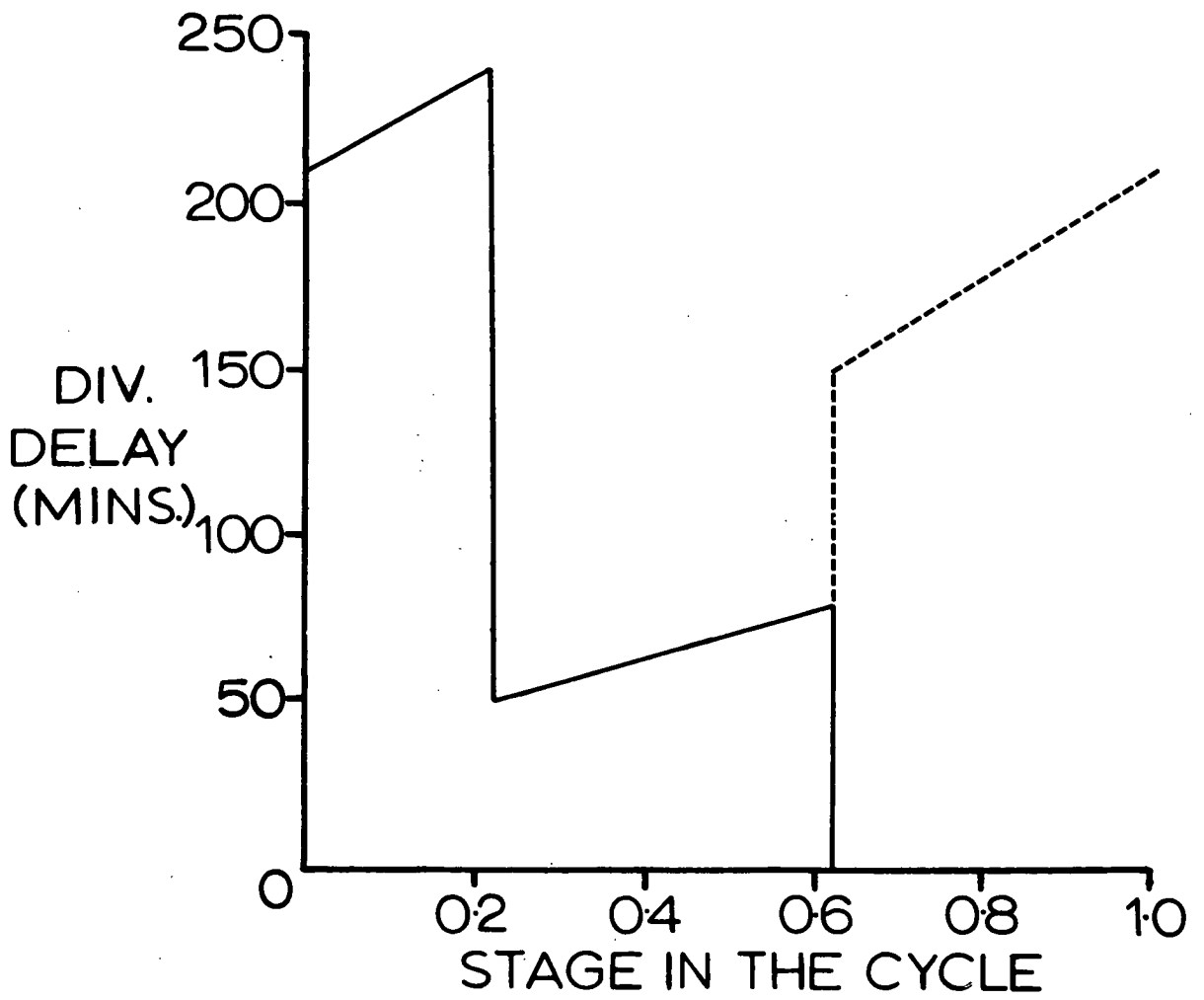
was not perfect.

Nevertheless, the results of four experiments carried out by this method are reasonably consistent and indicate a delay of between 200 and 250 minutes for strain 132 and rather a longer delay for strain 975h$^+$ where only 32% of the cells were seen to have divided 240 minutes after the second division of the parent culture. It is concluded that if MC induced delays are to be accurately measured in this highly sensitive phase of the cell cycle a lower dose of drug must be used.

Now that the basic pattern of sensitivity has been described I shall return to the comparison of MC induced delay with that produced by UV irradiation; to assist this comparison Gill's findings are reproduced in Figure 10 in the form of a simplified graph of delay against cell age at irradiation. The dose of UV used by Gill was 62 ergs/mm^2 and the wavelength was 265 mU. The cell cycle indicated in Figure 10 is calculated using the convention of _Swann_ which regards the end of the cycle as the appearance of the cell plate and to convert this to the fission convention it is necessary to subtract 0.09 from the estimate of cell age.

The broad similarity between the results obtained with UV and MC is immediately obvious although for UV the minor period is shorter and shows less of a range of delay. Another similarity is that Gill notes that there is less variability of delay in the minor period which is certainly true with MC as synchrony is maintained after a pulse; however, this may be a function of the length of delay or be explained by the synchronising effect of a rising delay with cell age.

Good agreement is also found in the position of the transition points for UV and MC. The data used to calculate the two

FIGURE 11.   -   The effect of four MC pulses upon a single
               synchronous culture to estimate the position
               of the first transition point.

Graph a   -   Control.

Graph b   -   Pulse at 0.43

Graph c   -   Pulse at 0.50

Graph d   -   Pulse at 0.57

Graph e   -   Pulse at 0.64
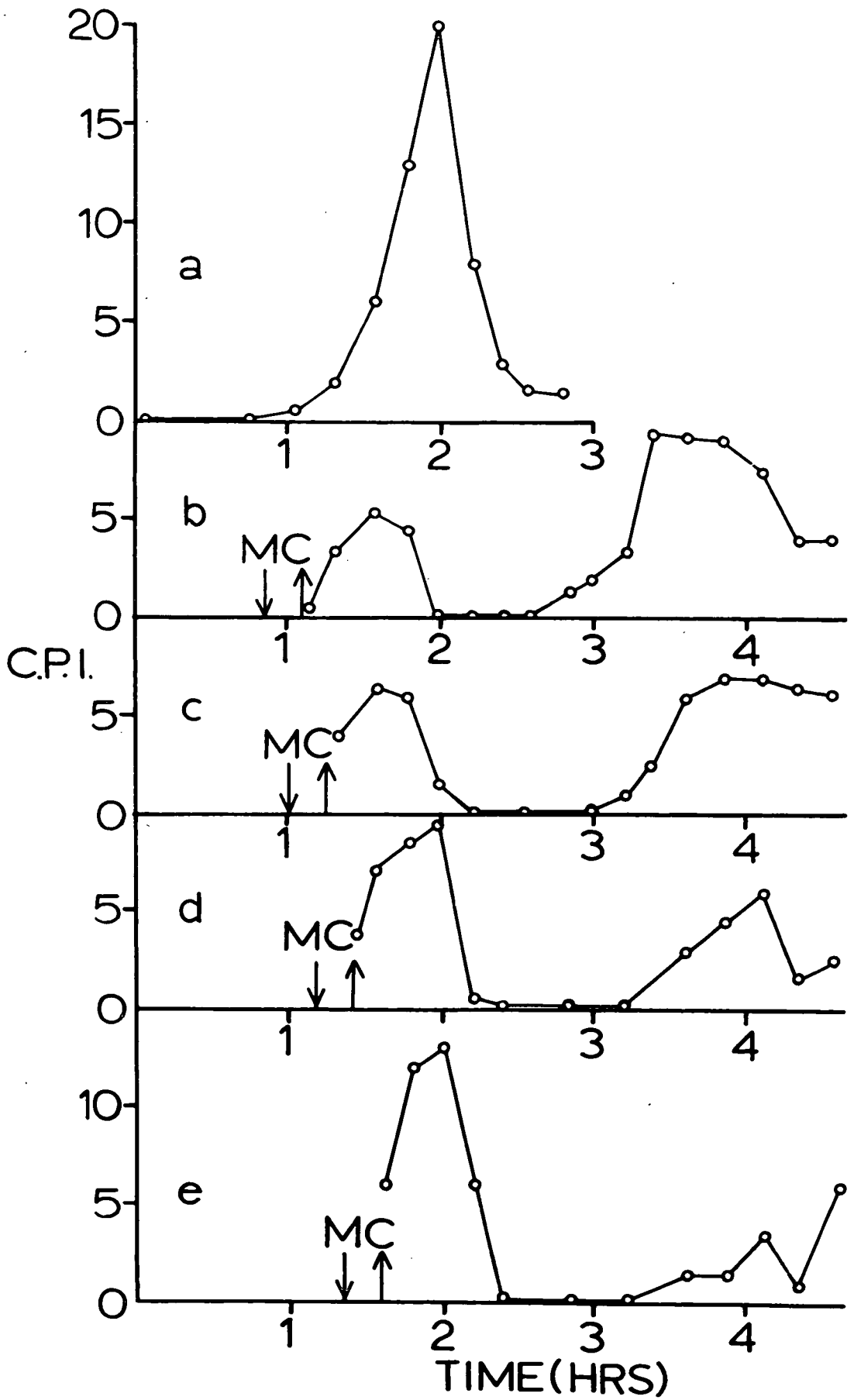
transition points is shown in Figures 12 and 7; the method used to make this calculation was to estimate the mean cycle position at which a pulse treated half the cells in the minor period and half in the major and thus resulted in only a 50% rise in cell number. Increase in cell number was measured either directly with a Coulter Counter or by the numerical integration method devised by N.Stebbing, details of which may be found in Appendix 2. This latter method is not a precise estimate but gives good agreement with data obtained from direct measurements.

The first TP (Figure 11) was estimated by direct measurement to be at 0.57 of a cycle and the second TP (Figure 7) was estimated by numerical integration to be at 0.03 of a cycle. Comparison of these estimates with the results of eight other experiments which located the transition points with less accuracy showed all the results to be in complete agreement. (These experiments provided the data for Figure 8.).

When expressed using the fission convention Gill's transition points are at 0.53 and 0.11 and thus the greatest difference is in the second TP and is only 0.08 of a cycle. Considering that completely different methods were used by Gill and myself to calculate these results and that stocks of strain 132 were different, the disparities in the T.P's are very small and of doubtful significance. That different stocks of the same strain can vary has been shown by measurements of DNA synthesis upon two stocks of strain 132 by Mitchison and Creanor ('71b).

When this pattern of changes of sensitivity had been discovered in strain 132 and the correlation with UV sensitivity noticed two questions arose immediately, was the pattern the same for other strains of S.pombe and could the pattern be altered by mutation? The results which provide the answer to the first of

FIGURE 12. - Four 800 Āg/ml 15 minute MC pulses made at
various points in the cycle of strain uvel 1
cells.

Upper graph:- Open circles - control.
Closed circles, dotted line - pulse at 0.17.
Open squares, dotted line - pulse at 0.01.

Lower graph:- Open triangles, solid line - pulse at 0.45.
Closed squares, dotted line - pulse at 0.74.

The delays produced in this experiment by 800 Āg/ml and in two
other similar experiments are as follows :-

Experiment	Cycle position	Delay: 1st Division	2nd Division
	0.01	36 minutes	-
	0.17	25 "	-
as above	0.45	0 "	29 minutes
	0.74	0 "	31 "
1	0.40	15 minutes	-
	0.66	0 "	26 minutes
	0.92	0 "	0 "
	0.33	35 "	-
2	0.58	0 "	21 minutes
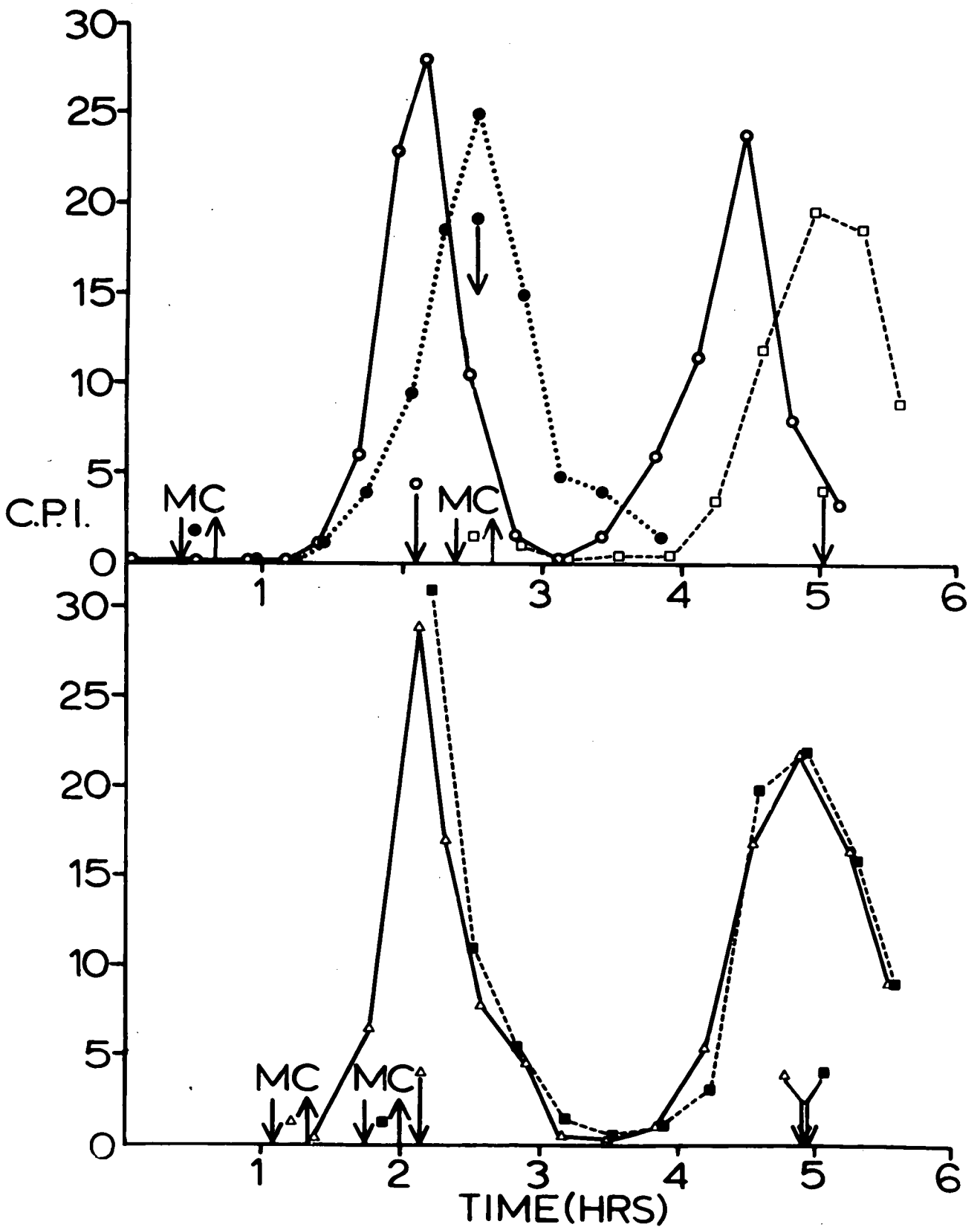
these questions have already been presented; strains 972h⁻ and
975h⁺ both show the same basic pattern of sensitivity as may be
seen in Figures 5 and 8, with the one proviso that TP-2 may be a
little earlier in these two strains.

Consideration was then given to the selection of a MC
sensitive mutant but in view of the high levels needed to affect
S.pombe and the observation made by Swann and Sutherland (personal
communication) that still higher levels were needed if solid medium
was used, this would have been a rather costly procedure. However,
during the course of this investigation a UV sensitive mutant was
kindly made available to me by Dr. B. Kilby. This mutant is
designated uvsl 1 and is derived from strain 972h⁻; it was
selected by its sensitivity to the lethal effects of UV by Dr. M.
Schupbach.

Figure 12 shows the result of pulsing a synchronous
culture of strain uvsl 1 at four different positions in the cell
cycle and this result and the results of two further experiments
are summarised in the legend. One point is immediately clear from
this result which is that uvsl 1 is strikingly resistant to MC
induced delay. The concentration used in the pulse treatments had
to be raised to 800 μg/ml to obtain any measurable effect at all
and even at this concentration the maximum delay produced was only
in the region of 30 minutes. Unfortunately not enough results
were collected to reveal the pattern of sensitivity through the
cycle and make a comparison with those obtained for strain 972h⁻,
but one feature does emerge from the results presented which is
that there appears to be a transition from first to second
division delay at around mid-cycle but no period of high
sensitivity is found.

Since the criterion by which uvsl 1 is deemed sensitive is its susceptibility to UV induced killing and not division delay one preliminary experiment was performed to test whether uvsl 1 was more sensitive to killing by a pulse of MC. Asynchronous cultures of 972h$^-$ and uvsl 1 were treated with a 500 μg/ml MC pulse of 15 minutes, washed and observed by time lapse photography for $13\frac{1}{2}$ hours following treatment. Two classes of cell were scored : those that were refractile and obviously dead and those which simply did not divide in the period of observation. In the control 7% of the observed cells died or gave rise to progeny of which one died and a further 7% failed to divide, in the uvsl 1 sample there was 24% lethality and no cells failed to divide.

These results suggest that in spite of the absence of an effect on division MC might have an effect upon viability. However, after the experiment was performed it was learned that strain uvsl 1 has a reduced viability in normal cultures (M.Schupbach, personal communication) some 6% of the cells being dead at the end of logarithmic growth. Thus the experiment described above was not adequately controlled. Nevertheless, the idea that delay and lethality may be negatively correlated has been proposed by other workers notably by Sinclair who suggests that the inverse dependance of delay and survival may be explained by the fact that a long delay allows time for the completion of repair processes.

Unhappily, time did not allow this particular approach to the problem of MC induced delay to be carried any further, but the results above suggest that a few more experiments could be very profitable. The construction of survival curves for MC killing of uvsl 1 and 972h$^-$ would allow some idea of whether the resistance to MC induced delay is due to reduced permeability or to a more

fundamental effect. It would also be interesting to see if the pattern of UV induced delay is modified in any way in the sensitive strain. Finally, it would be revealing to mate uval 1 with a normal strain and analyse the spore progeny for resistance to UV killing and to MC induced division delay to see if the respective sensitivity and resistance to these two treatments remain linked.

One last point must be made to conclude this section. In demonstrating any periodic event in the cell cycle by the use of synchronous cultures it is highly desirable to show that event occurring in more than one cycle, as the first cycle may be distorted by the synchronisation procedure. Experiments in which the second cycle of a synchronous culture was treated with MC pulses showed that the same pattern repeated itself in this cycle as well. This may also be seen in Figure 7 where after TP-2 the cells showed the same sensitivity as those which had just been removed from the gradient as in Figures 4 and 5.

The following points summarise the findings of this chapter.

1. A pulse treatment of MC is able to cause a delay in division at all points in the cell cycle.

2. There is a periodic change in the sensitivity to MC induced division delay and the cycle may be divided into two periods, the major period and the minor period, the cell undergoing two sharp transitions from one period to the next per cycle.

3. The minor period in strain 132 extends from 0.03 to 0.57. During the minor period sensitivity to first division delay rises but falls abruptly to zero at the transition point.

4. During the major period which extends from 0.57 to 0.03 the cells do not suffer a first division delay but divide normally.

However, the next division is highly delayed. The delay obtained with a standard pulse being of the order of 250 minutes.

5. This pattern of sensitivity through the cycle is very similar to that found by B.Gill for UV induced division delay for low doses which did not affect growth.

6. Four strains of S.pombe have been investigated, these being 132, 972h$^-$, 975h$^+$ and uvsl 1 derived from 972h$^-$. The first three of these strains show the pattern of sensitivity described in 1-4 above whilst uvsl 1 is highly resistant to delay. uvsl 1 does show a transition from first to second division delay around mid-cycle but it lacks the period of high sensitivity completely.

CHAPTER 2.

The relationship of induced delay to the dose of MC used.

The experiments in the preceding section all used the standard rather arbitrarily selected dose of 500 μg/ml for fifteen minutes. The results of this section describe the relationship between the dosage of MC administered and the resulting delay. Clearly the dosage may be varied in two ways, by changing the pulse strength and by changing the pulse length. The effects of both of these changes were investigated.

Figures 13. and 14. show the results of increasing the dose by increasing the pulse strength. Figure 13. shows quite clearly that delays may be elicited by doses which are relatively low compared with the 500 μg/ml used in the previous experiments. A particularly interesting point about this data is that the height of the 'escape peak' in the CPI is greatest in the cultures treated with a lower dose, a result which immediately suggests that the transition point may vary with dose.

Transition points in general have been recently discussed by Mitchison ('71) who has identified two types which he has called event and delay transition points. An event transition point is one which is due to a change in the state of the cell which renders it basically insensitive to the action of the inhibitor whilst a delay transition is due to a delay in the action of the inhibitor which allows the cell to complete its cycle before the inhibition takes effect. Typically this delay may be due to slow penetration of the inhibitor or to the fact that the cell possesses an appreciable pool of the substance upon which the inhibitor acts or just to the reaction of the inhibitor being slow. Since an event transition

FIGURE 13. — The effect of changing the concentration
of MC on the delay caused by a pulse in the
minor period.

Graph A — Open circles — control CPI.
Closed circles and dotted line — treated CPI.
The MC concentration was 31.8 µg/ml.

Graph B — MC pulse of 62.5 µg/ml.

Graph C — Open circles and solid line — MC pulse of 125 µg/ml.
Closed circles and dotted line — MC pulse of 250 µg/ml.

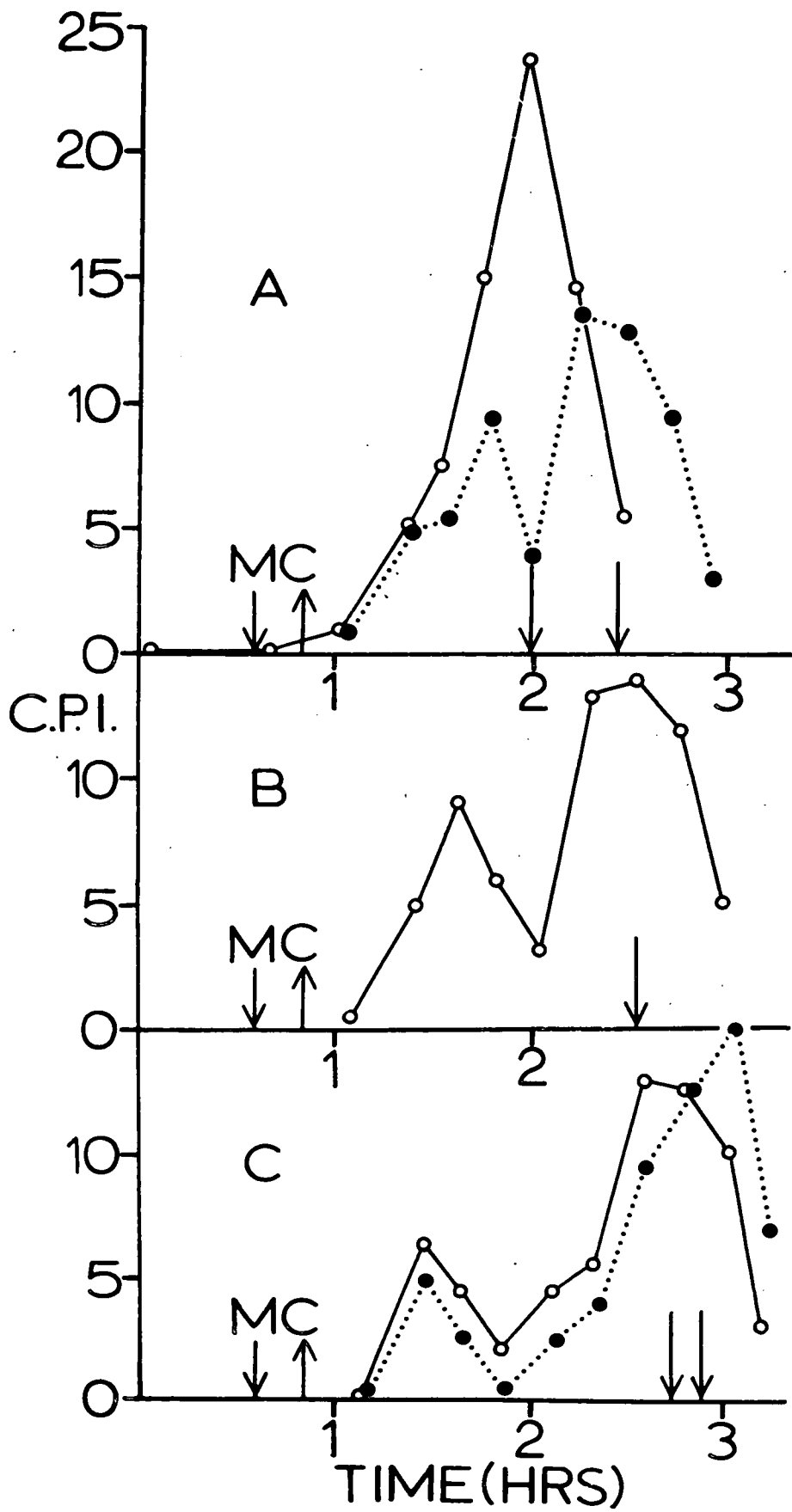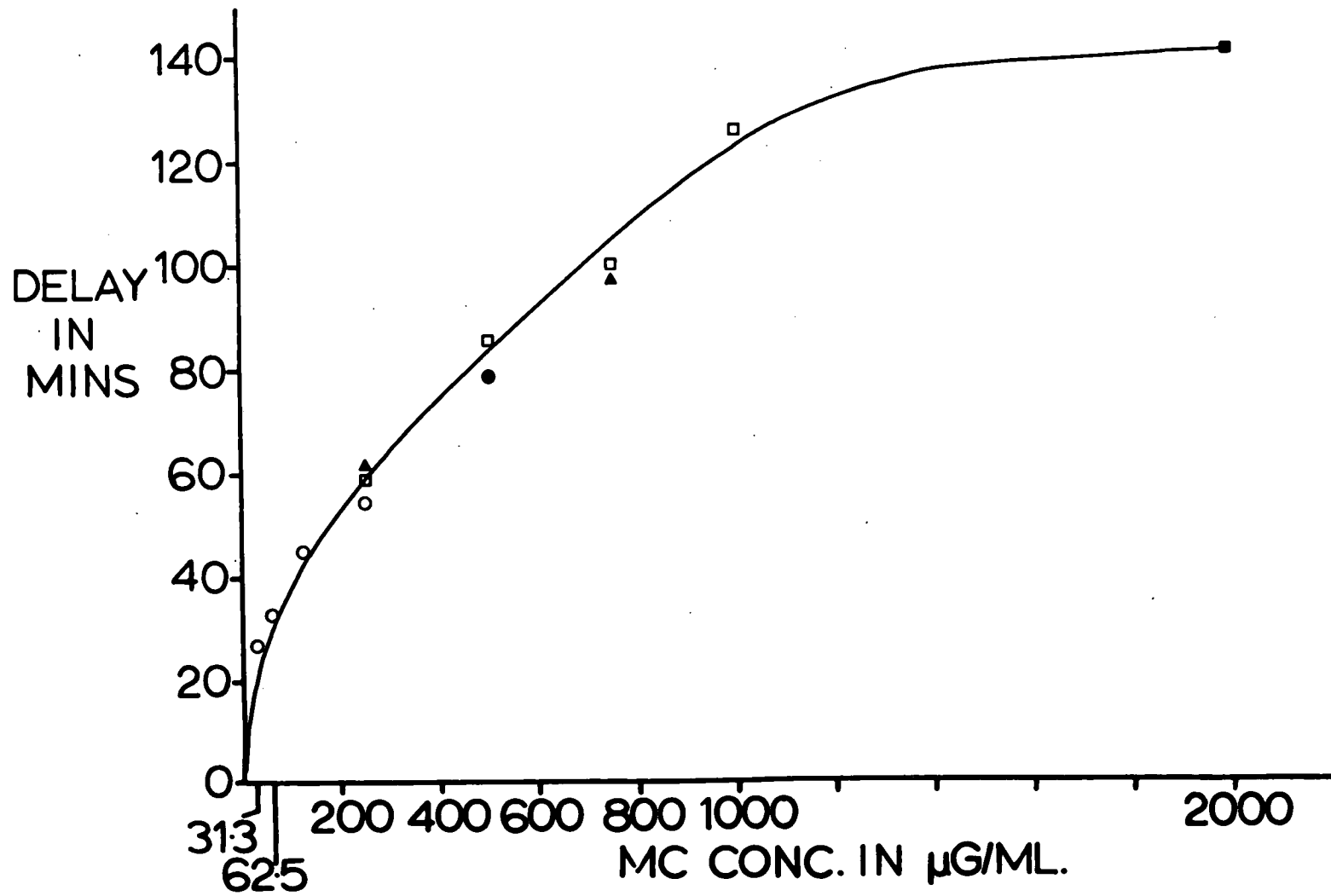N.B. In all the above cases the MC pulse position was 0.35.

FIGURE 14.   -   The dosage : delay relationship for

a pulse of MC at around 0.35 of a cycle.

The different symbols represent separate experiments
and the pulse positions were as follows :-

Open circles   -   0.35

Open squares   -   0.35

Closed circles   -   0.35

Closed triangles   -   0.28

Closed squares   -   0.33

point is due to a change in the cell one would not expect variation
with dose. However, a delay in the action of an inhibitor may
reasonably be expected to vary with dose and thus a dose variable
transition point suggests a delay transition point, especially if
the transition point is earlier at lower doses.

That the first TP in fact appears to be earlier in the
results shown in Figure 13. in those cultures treated with lower
doses thus suggests that the transition point may be of the delay
type. However, there is good evidence which will be presented
later in this work that the second transition point at least is an
event transition point and it seems unlikely that the first
transition point which is only just after mid-cycle is of the
delay type. In addition the similarity of the patterns of sensi-
tivity to delay induced by MC and UV irradiation also argues
strongly for the transition points being of the event type as the
action of UV irradiation is very unlikely to be of a delayed nature.

The answer to these apparently conflicting findings most
probably lies in the observation that very high levels of MC are
required to have an effect on yeast cells; in E.coli for example
a dose of 0.1 µg/ml is quite sufficient to produce a bacteriostatic
effect and 5 µg/ml is decidedly lethal (Suzuki and Kilgore).
Mammalian cells are also sensitive at these relatively low levels.
It thus seems quite likely that at lower concentrations of MC
there is a delay in penetration of the drug due to the low
permeability of the thick cell wall. A delay in penetration may
thus mean that a pulse of drug at a lower concentration is
effectively a later pulse than a pulse of high concentration
administered for the same time. This effect would be especially
marked if there is also a critical concentration of drug which

FIGURE 15. - The effect of increasing the length of a

500 µg/ml MC pulse upon the division in

a synchronous culture.

These data were collected by observing a sample of treated
synchronous cells by time lapse photography. The pulses were
begun at 20 minutes after inoculation. Each graph represents
a separate culture.

        Top graph   :-   Control

        Middle graph   :-   25 minute pulse

        Bottom graph   :-   45 minute pulse

M.D. is the point where a 50% increase in cell number has
been reached.

The open circles represent the rise in cell number due
to the first division and the closed circles represent the rise
due to the second division.
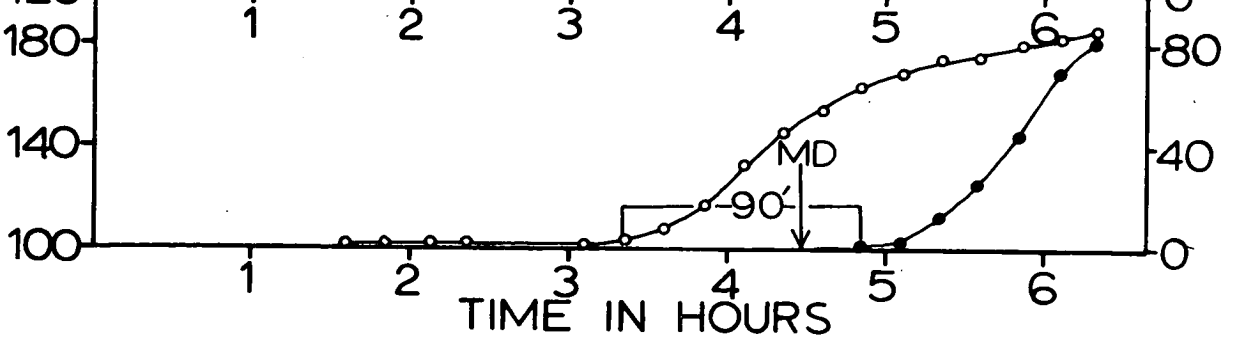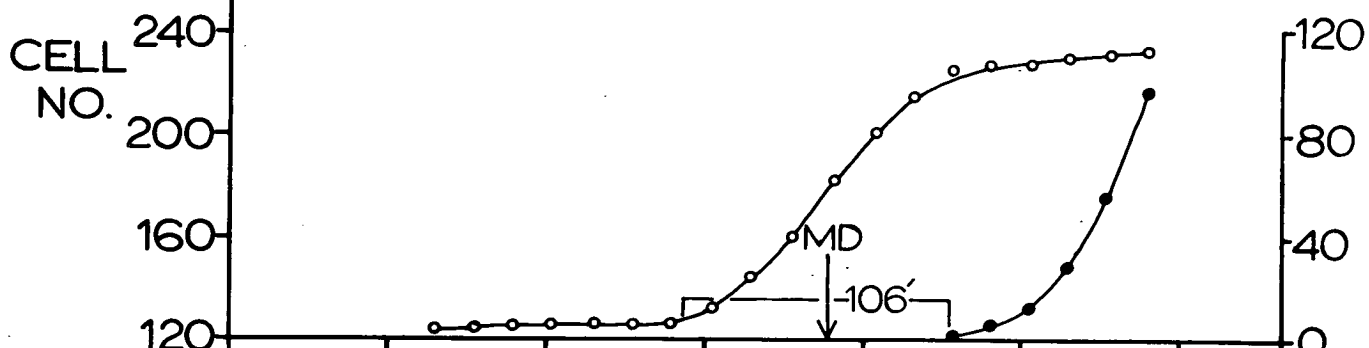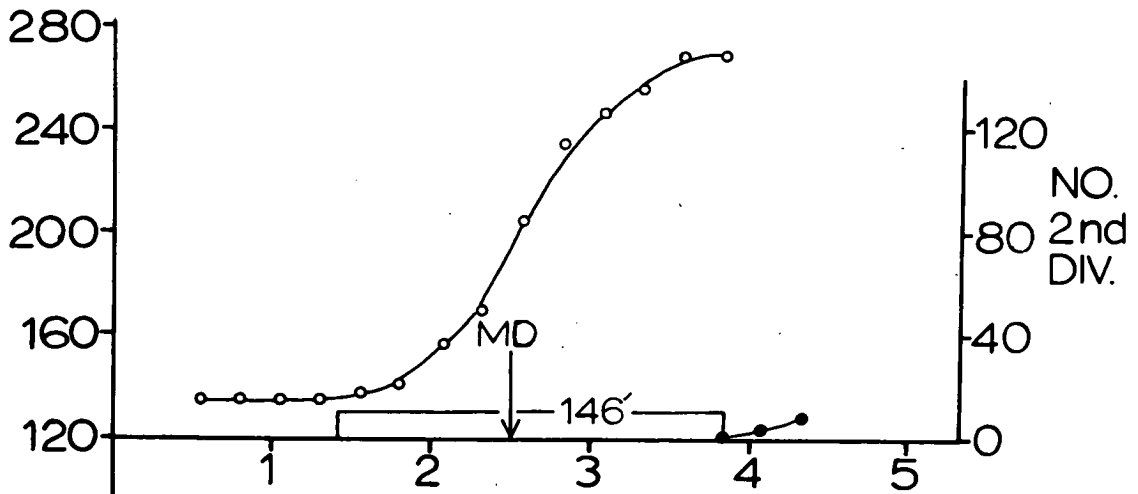
must be reached inside the cell to cause enough damage to produce
a measurable delay.

Figure 14. shows the dosage delay relationship for a pulse
of MC positioned about halfway through the minor period. There
is an initial steep response followed by a roughly linear section
and at very high doses there appears to be a saturation effect.
Interpretation of this data is difficult since the parameter
measured, division delay, is dependent on the interaction of at
least two separate processes namely the process of damaging the
cell and the process of repairing that damage,and both of these
are unknown and probably complex. Certainly this data might be
more easily interpreted if a more direct method could be used to
measure the uptake and binding of the drug caused by pulses of
different concentrations since this would be much more likely to
be proportional to 'damage' than pulse concentration. Radioactive
drug can be prepared and this would be of great use in such a
study. I shall return to this point in the discussion. The con-
clusions from these experiments are thus limited to one simple
finding, that delay increases with increasing dose up to a point
where some form of saturation effect begins to manifest itself.

The effect of increasing the dose by increasing the pulse
length was measured by the time lapse photography method and some
typical data may be seen in Figure 15. This method was chosen
because it was anticipated that very long delays would be found.
However, the use of this method introduces an additional error
since each result was obtained with a different synchronous
culture and a parallel control culture could not be observed. In
each experiment the treatment was started 20 minutes after inocu-
lation which corresponds to 0.23 in an 'average' synchronous culture

FIGURE 16. - The relationship between division delay and

pulse length.

Closed circles   -   Data from photomicroscopy.

Open circles   -   Data from cell plate counting.

Open squares, dotted -   The length of the cell cycle

                          immediately following the delayed

                          division estimated by photo-

                          microscopy.

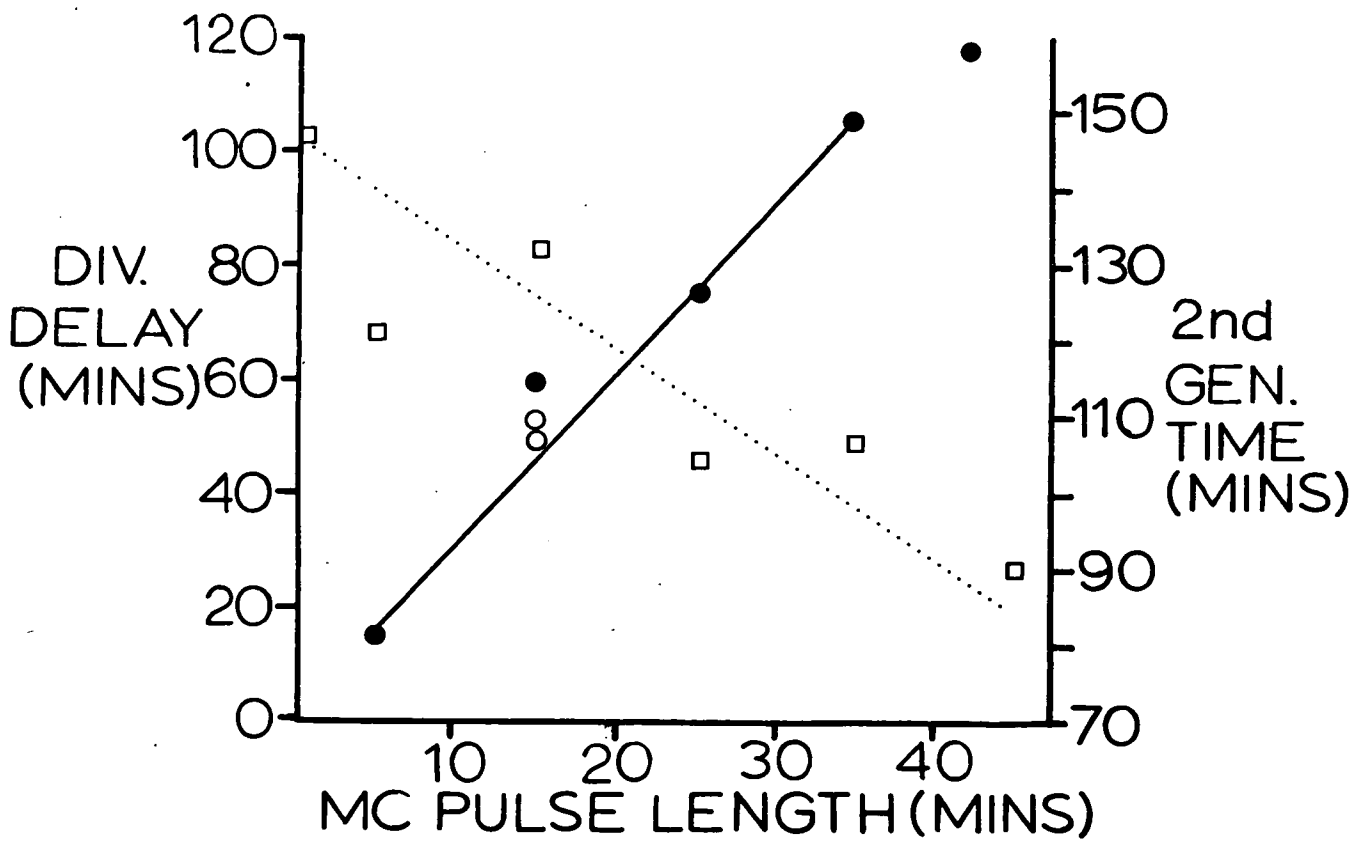FIGURE 17. - The relationship between cell length at division
and the length of the subsequent cycle in MC
delayed cells.

Top graph - a scatter diagram of cycle time against length
at the first division for 50 cells. The data
include sister cells and were taken from both
treated and untreated cells.

Bottom graph - a simplification of the scatter diagram made
by simply averaging the cell length classes.
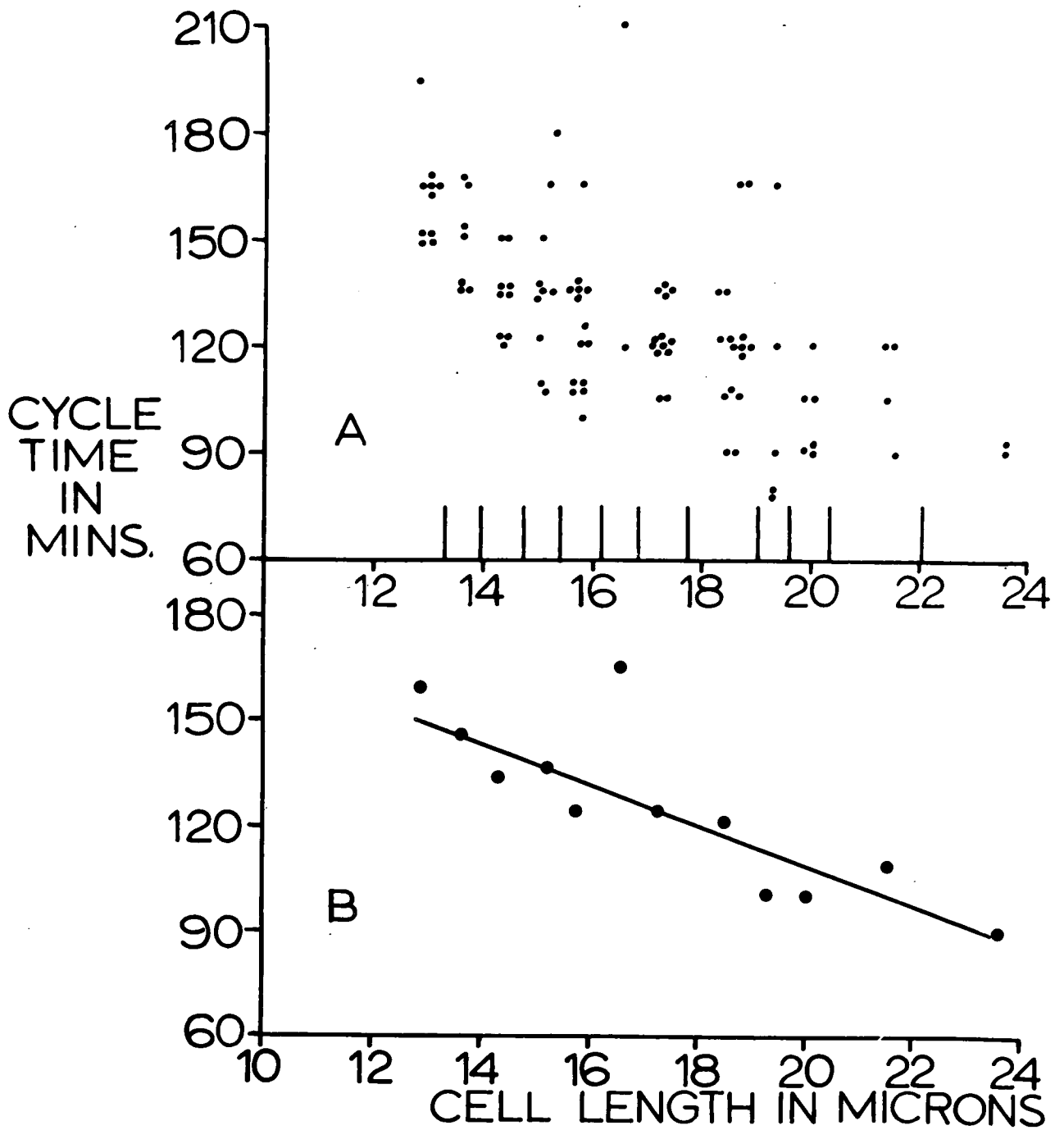
and the delay was calculated by comparison with the results from a separate untreated control culture. Once again the concentration of the pulse was 500 μg/ml.

Figure 16. shows the collected results from five such experiments; measured in this way there appears to be a linear relationship between dosage and delay but once again the result at the highest dosage suggests a saturation effect may be operating. Using the slope of this line and that of the linear portion of Figure 14. an estimate may be made of the 'effectiveness' of MC in terms of minutes delay per μg/ml MC per minute exposure. This gives values of 0.307 and 0.293 minutes per μg/ml per minute exposure respectively; thus effectiveness of MC in producing delay appears to be independent of 'dose rate' over a limited range. This result and the linearity of the dose delay relationship shown in Figure 16. suggests that there may be a simple relationship between dose and the amount of damage produced and between damage and the length of delay produced.

Figure 16. also shows another relationship which was revealed by the analysis of the time lapse photographs used to estimate the division delay, this being the effect of pulse length upon the length of the cycle immediately after the delayed division. A rough estimate was made of this generation time by measuring the interval from the first delayed division scored to the first second division scored. Quite clearly as the pulse length and hence the delay increase there is a definite contraction in the time taken to complete the subsequent cycle. The same phenomenon is illustrated in Figure 17.; here the length of cycle after the delayed cycle is plotted against the length of the cell at the end of the delayed cycle. The data is presented both as a scatter diagram

and as a simple graph constructed by averaging the cycle times within various cell length classes. Again the data shows that there is a shortening of the second cycle in the larger cells.

Clearly the cells are able to regulate their size by shortening the duration of the cycle which follows the artificially long delayed cycle. Exactly the same effect was found by Gill for cells that had become highly elongated due to a UV irradiation induced division delay. He recorded a condensed second cycle as short as 60 minutes and suggested that where the length at division of the delayed cell was equivalent to twice the length of the cell at the beginning of its cycle, the length of the shortened cycle may represent the minimum time in which the cell can prepare itself for another division. That is the time for that synthesis and organisation which is obligatory for division. Certainly this condensed cycle might well repay more careful investigation either by single cell techniques or perhaps by the use of carefully MC treated synchronous cultures. One interesting question is for example, does nuclear division preserve the temporal relationship with the fission process in this shortened cycle or does the end cycle become condensed?

Having dealt with the dosage delay relationship for pulses in the minor period of sensitivity the question remains, what is the situation for pulses in the major period? Sadly only one experiment was performed to answer this question and the result may be seen in Figure 18. The data do not really permit any conclusions as to the division delay caused by these treatments, but one feature of the results is immediately apparent, that the escape peak of cells which do not suffer a long second division delay becomes larger with decreasing dose. Thus once more there is the

FIGURE 18. - The effect of different concentrations of pulse during the major period of a synchronous culture.

The pulse length was the usual 15 minutes and its position was 0.81.
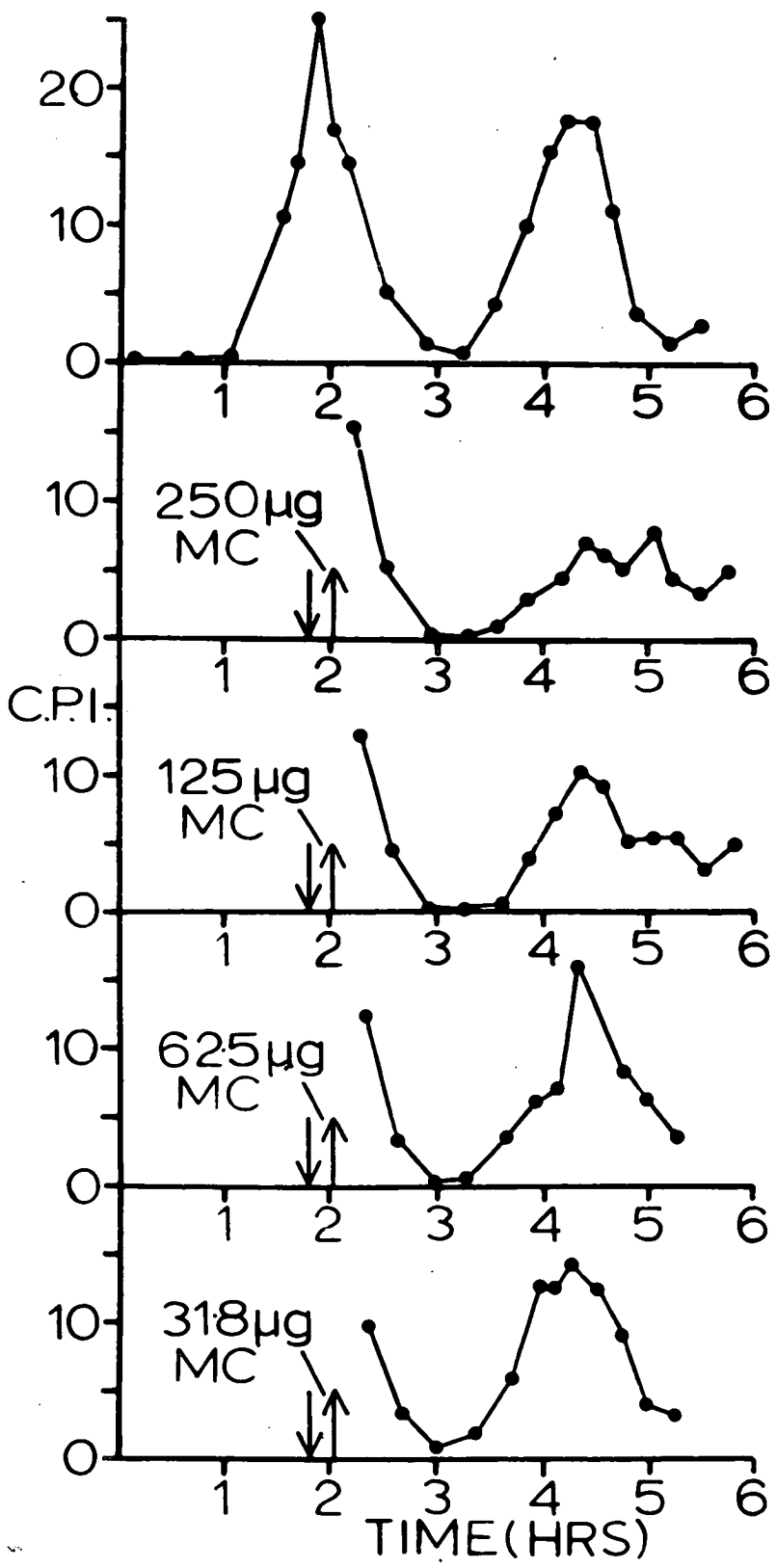
suggestion that the transition point is dose variable. What is
alarming about this result is that the transition point appears to
move by a very considerable fraction of a cell cycle. There is
every reason to consider that this transition point is of the event
type as later results will I hope show, and thus it appears that
the action of MC pulses at low concentration may be highly delayed.
One attraction of this idea is that it may explain the high delays
at low doses seen in Figure 14. since the rising pattern of delay
in this part of the cycle would mean that a delay in action would
lead to the drug acting in a more sensitive part of the cycle.
Two types of experiments could be performed to try and resolve this
difficulty. Firstly the whole cell cycle pattern of sensitivity
should be determined for another lower dose and, assuming that the
pattern resembles that for 500 μg/ml, the position of the tran-
sition points calculated. Secondly, the delay caused by varying
dosages should be measured upon synchronous cultures treated with
deoxyadenosine which causes the G_1 period of the cycle to be ex-
tended and thus avoids the difficulty caused by lack of complete
synchrony in the G_1 phase in normal synchronous cultures.

The major findings of this section may be summarised thus.
1. Delay in the minor period rises as the dose of MC administered
rises whether the dose is increased by raising the length or
concentration of the pulse.
2. When, for a dose of 500 μg/ml, the dose is increased by ex-
tending the pulse duration the dose delay relationship is roughly
linear; increasing pulse concentration leads to an initial high
response followed by a linear rise. In both cases the results
suggest a saturation effect at high doses.

3. The results suggest that for both major and minor periods the transition points are dose variable becoming earlier with lower doses.

4. After the cells have suffered a delay they are elongated by the consequent period of unbalanced growth but cell size appears to be regulated by a foreshortening of the following cycle.

CHAPTER 3.

The action of pulses of Mitomycin C upon nuclear division and macromolecular synthesis.

The contents of this section describe experiments which attempt to answer questions which arise naturally from the results reported in section 1.

The first obvious question which was raised by the finding that pulses of MC could produce sizable division delays was, is the effect upon nuclear division or upon cytokinesis ? In the yeasts the division of the nucleus and the cytokinetic event, in this case the formation of the cell plate, are not always as closely associated as they are in most mammalian and plant cells. In S.pombe the division of the nucleus is separated from cell plate formation by approximately a quarter of a cell cycle. Hence the fact that the first TP was found to be at 0.57 (for strain 132) and that division delay was calculated by the observation of the cytokinetic event made it possible for either event to be sensitive.

At the time that this work was performed there had been two estimates made of the timing of nuclear division in the cell cycle of S.pombe, both Mitchison ('70) using time lapse phase contrast microscopy and Bostock ('68), who based his estimate upon the proportion of binucleate cells in an asynchronous culture, concluded that nuclear division took place at 0.75 of a cycle. However, since neither of these estimates was made on the stock of 132 used at the time of this work and neither of these estimates was made upon synchronous cultures, it was decided to make another estimate of nuclear division based on the observation of synchronous cultures.

The method by which the position of nuclear division was

PLATE III - Cells of a synchronous culture of S.pombe
stained with Giemsa stain. The plate shows
three binucleate cells which were the class
of cells scored on a percentage of the
total to derive the nuclear division index
(NDI).

FIGURE 19. -   The calculation of the mid-point of nuclear
                division in a synchronous culture.

Top graph   :-   Left hand ordinate: closed squares - CPI.
                   open squares, dotted line - corrected CPI.
                   Right hand ordinate: closed circles - percentage
                   of cells having undergone fission. Open
                   circles - percentage of cells having entered
                   (or passed through) the cell plate stage.

Bottom graph:-   Left hand ordinate: closed squares - NDI.
                   open squares and dotted line - corrected NDI.
                   Right hand ordinate: closed circles - percentage
                   of cells having entered the cell plate stage.
                   Open circles - percentage of cells having
                   entered the binucleate phase (or passed through
                   it).

Thus, the arrow to the abscissa in the top graph shows the time
when 50% of the culture had divided and similarly the arrow in the
bottom graph shows the mid-point of nuclear division (0.81).

N.B. Consult the text for further explanation.

C.P.I.
&
N.D.I.

%AGE.

TIME (HRS)
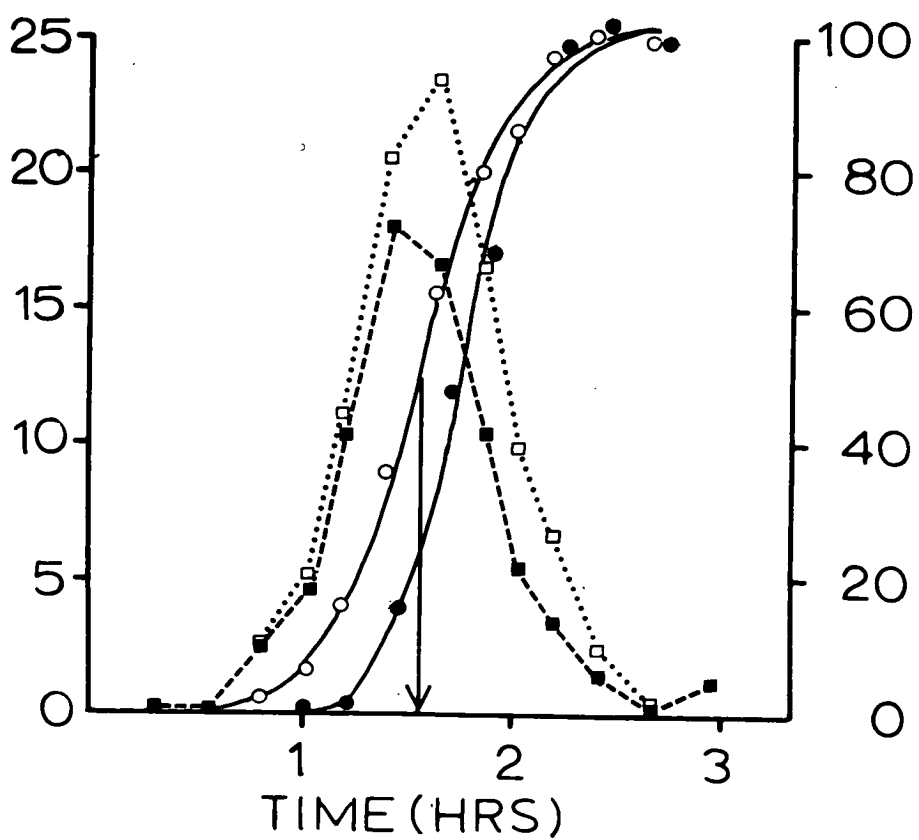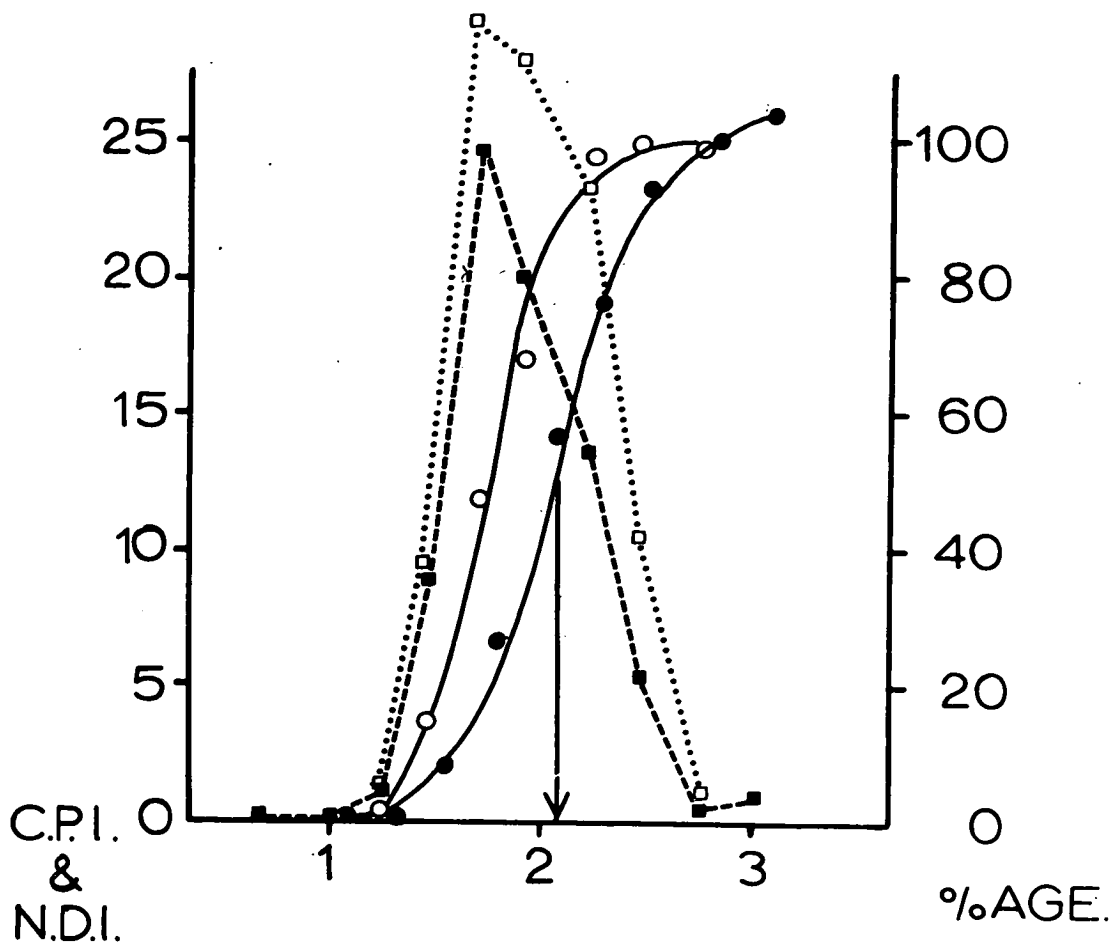
calculated is as follows. Slides were made at intervals from a synchronous culture and stained with Giemsa as described in the Materials and Methods section. The appearance of the stained cells may be seen in Plate 3.;nuclear structure is not revealed in the cells but the nucleus is unequivocally demonstrated in all cells in a good preparation. The slides are used to calculate the nuclear division index (NDI) which is simply the percentage of cells which are in the binucleate phase. Three of the class of cell scored as binucleate may be seen in Plate 3; they are cells which have a clearly divided nucleus but had not initiated cell plate formation. Occasional cells were seen which had nuclei which had assumed an hour-glass shape and these were presumed to be cells which were in the process of nuclear division. These cells were not scored as binucleate.

Now in order to use the normal criterion for calculating the position of an event in the cell cycle which is the point in time at which half the cells have undergone the relevant event, it was necessary to make an estimate of the point at which half the cells had undergone nuclear division. The results used in this estimation may be seen in Figure 19. The data on which this estimate is based are the NDI the CPI and the cell counts obtained by the use of a Coulter counter and expressed as a percentage of the original cell count.

The first step in the calculation is to calculate what will be referred to as the corrected cell plate index (CCPI) which is defined as the proportion of cells with cell plates expressed as a percentage of the original number of cells in the culture. For example, if the division of 100 cells in a synchronous culture is considered at a point in time at which 20 of the cells have cell

plates and 30 of the cells have undergone cell division the CPI will

be $\dfrac{20}{100+30}$ x 100 = 15.4% but the CCPI is just the proportion of 100

cells with cell plates i.e. 20%. The CCPI is thus corrected for the

dilution effect that fission has upon the CPI. The method used to

calculate the CCPI from the CPI and cell count data is shown in

Appendix 3.

Having derived the CCPI it is a simple matter to combine

this value with the percentage of cells which have undergone division

and to estimate the proportion of cells which have entered or passed

through the cell plate phase. The top graph in Figure 19. shows the

CPI, the CCPI percentage increase in cell number and the proportion

of cells which have entered the cell plate phase, all plotted on the

same time axis. The difference between the last two curves at the

50% level is thus an estimate of the duration of the cell plate

period; as can be seen this interval is approximately 15 minutes

which compares very favourably with Mitchison's value of 0.095 of a

cycle (Mitchison '70) for the cell plate duration estimated by low

power microscopy (0.095 of a cycle is 14 minutes).

The bottom graph in Figure 19. shows exactly the same pro-

cess repeated using the proportion of cells which has passed through

the cell plate stage and the NDI to calculate a corrected NDI and

to thus combine the data to derive a graph of the proportion of cells

which have entered or passed through the binucleate stage plotted

against time.

It is notable that it would not have been necessary to obtain

the final curve by this two stage process if the original scoring of

binucleate cells had simply included cells with cell plates in the

binucleate class. However, this would not have allowed the calcu-

FIGURE 20. - The effects of MC pulses in the major and minor
periods on nuclear division.

Top graph (a)   open circles - CPI of control.
                  closed circles and dotted line - CPI of treated
                  culture.
                  open squares and dotted line - NDI of treated
                  culture.

Bottom graph (b) open circles - control CPI.
                  open triangles and dotted line - control NDI.
                  open squares and dotted line - treated CPI.
                  closed squares and dotted line - treated NDI.

The pulse positions were 0.85 for (a) and 0.45 for (b), and the
MC concentrations were 500 and 660 μg/ml respectively.
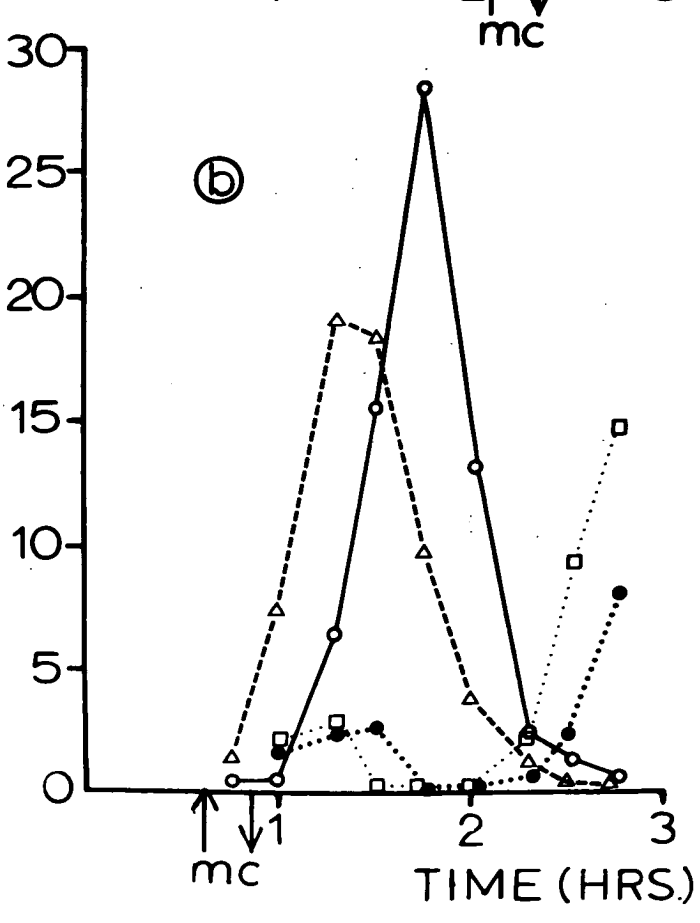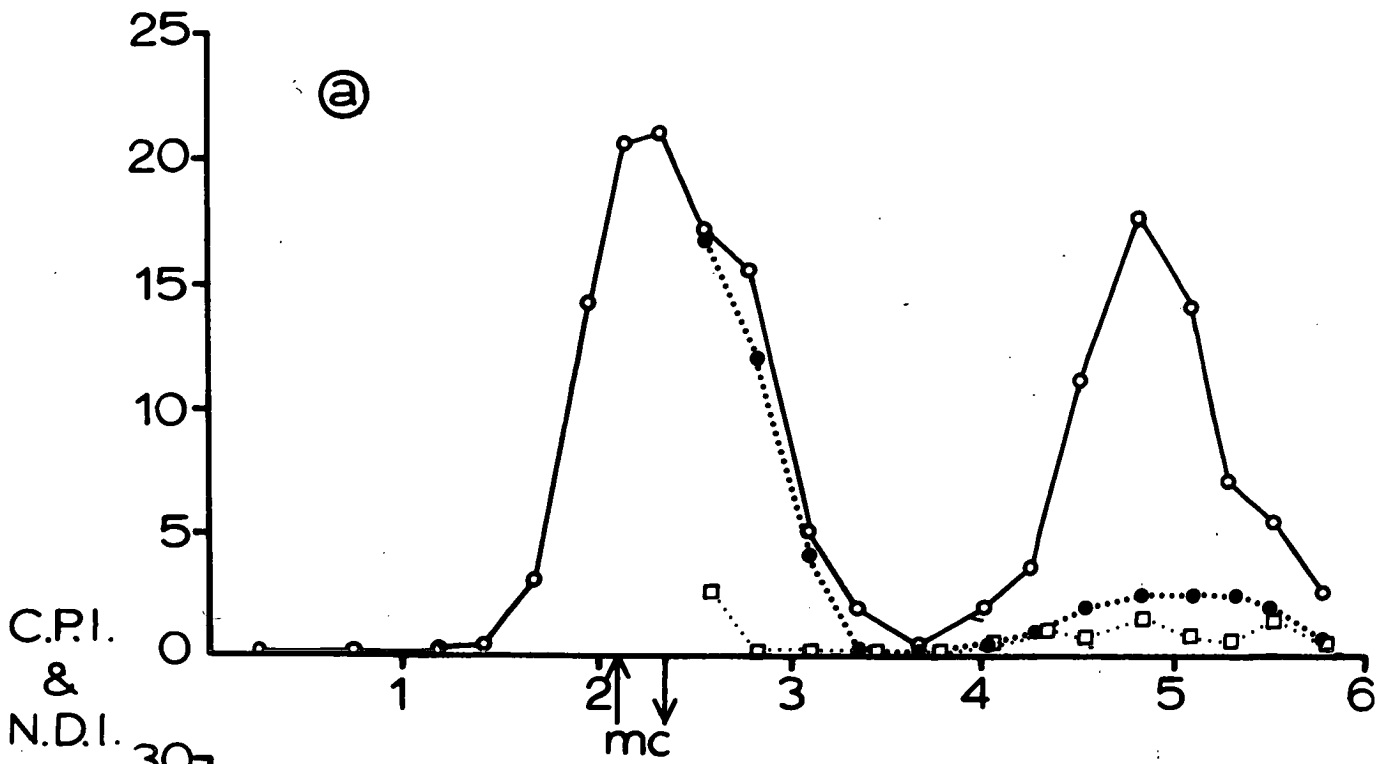
FIGURE 21.   -   The effect of a pulse of MC just after the

first transition point on nuclear division in

a synchronous culture.

Top - control;   bottom - treated culture;   solid lines - CPI;

broken lines - NDI.   The pulse was at 0.63.

     This experiment illustrates that the MC pulse does not

interfere with the process of nuclear division as binucleate cells

continued to appear during and after the pulse. The pulse position

was at 0.63 just after the first transition point.

C.P.I.
(○)

&

N.D.I.
(●)

MC

time in hours.
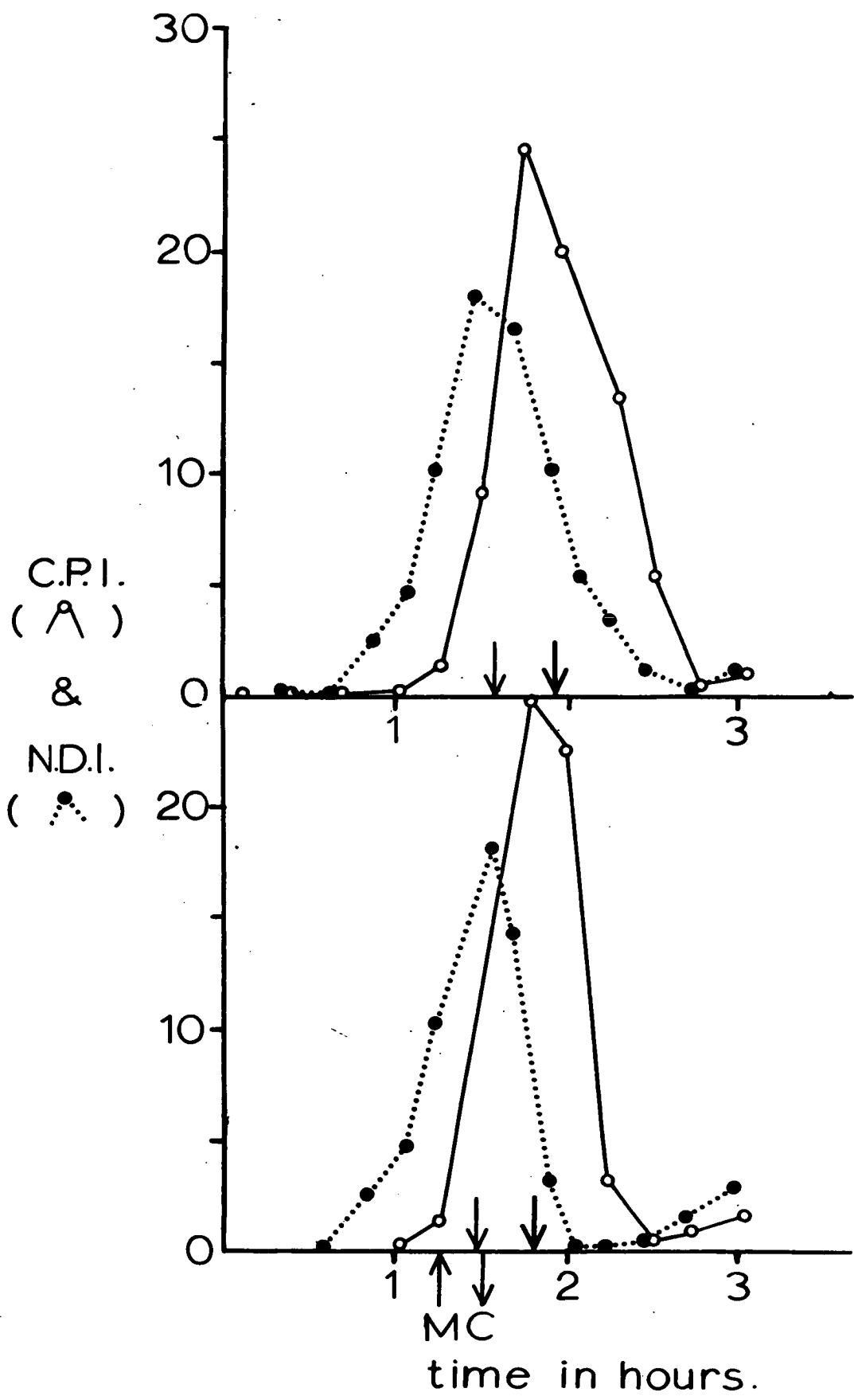
lation of the cell plate duration which provides a useful check on the accuracy of the method.

The result of this rather ponderous arithmetic is to arrive at an estimate for the cell cycle position of the entry into the binucleate phase (that is the completion of nuclear division) of 0.81 of a cycle. This is only 0.06 removed from the previous two estimates and thus it is not considered that nuclear division is affected by the procedure used to produce the synchronous culture.

The effect of MC upon nuclear division in synchronous cultures was investigated by simple comparison of the NDI in treated and untreated cultures, and the results may be seen in Figures 20. and 21. Graph A in Figure 20. shows that a pulse in the major period which causes considerable delay in cell division also prevents the cells undergoing a further division of their nucleus. Graph B illustrates much the same point for a pulse of MC in the minor period of sensitivity; once again nuclear division as well as cell division is delayed. The data shown in Figure 21. shows that, as may be expected with the transition point at 0.57, the last stages of nuclear division are entirely resistant to blockage by a pulse of MC placed at 0.63 of a cycle.

The conclusion drawn from these experiments is that after exposure in either the major or minor period of sensitivity cells are arrested in a stage of their nuclear division cycle which is prior to nuclear division. I shall return to these results later in this chapter.

The second basic question which was asked about the action of MC was, is the effect of the drug limited to an inhibition of division or is there an effect on cell growth as well ? Three

criteria were selected to measure growth, the increase in cell
size, the synthesis of protein and the synthesis of RNA.

Cell size in S.pombe may be conveniently measured by the
measurement of cell length as the length of cell is proportional
to its volume to a good approximation. At the time of this work
the effect of continuous exposure to MC was being studied by
Professor M.M.Swann by time lapse microscopy who kindly made some
of his photographs available to me. These photographs revealed
that, as may be expected from the considerable elongation of the
cells which may be seen in Plate 2., there is little effect on the
increase in size of the cells. Obviously an extremely sensitive
test would have to be used to detect any small effect on growth
that there might be caused by exposure to MC.

The method of comparison chosen was to compare the rate
of increase in cell length in treated and control cultures.
Measurements were made upon time lapse photographs of control cells
and of cells growing in the presence of 500 μg/ml of MC. In these
experiments the agar pad on which the cells were growing also con-
tained MC and fairly high magnifications were used to permit an
accurate measurement of cell length (x 25 objective and x 3.2
Optovar setting). Rate of increase was measured over a period of
100 minutes in the control cells and 200 minutes in the treated
cells. These rates of increase were then considered to be normally
distributed and were compared using Bailey's modification of
student's 't' test (since the variances were unequal, see Bailey).

The result of comparing two samples of 25 cells in this
way was that the rates of increase of cell size were found to be
significantly different at the 0.1% level, the treated cells having
a mean growth rate which was approximately 20% higher than that

of the controls !

Consideration of the manner of growth in size of the cells provides the answer to this somewhat paradoxical result. Volume growth in S.pombe follows an increasing rate curve for the first three quarters of a cycle and then comes the constant volume phase during which the cell plate is layed down (Mitchison '70). Measurements on the control cells were thus restricted to the first two thirds of the cycle to avoid the constant volume period as far as possible. However, the fact that growth rate follows a curve of increasing rate means that a cell is growing faster at the end of its cycle than it is at the beginning; hence, it appears that inhibited cells do not follow the same growth pattern as they do not have a constant volume period and continue to grow either at the maximum rate found at the end of the cycle or possibly they continue for some time to grow at an increasing rate, hence the higher average growth rate. Examination of the pattern of size increase of individual treated cells confirmed that the constant volume period does not occur and suggested that the growth rate might become linear. No attempt was made to try to resolve this question of the pattern of growth of inhibited cells, but it might repay further investigation. The simple conclusion was drawn that there is no measurable effect of MC upon growth as measured by increase in cell size.

The next measurements which were made upon the inhibited cells were upon the synthesis of protein and RNA since the result above does not preclude a situation in which cell volume increases without a corresponding increase in cell constituents, although this was considered most unlikely. Estimates of total protein
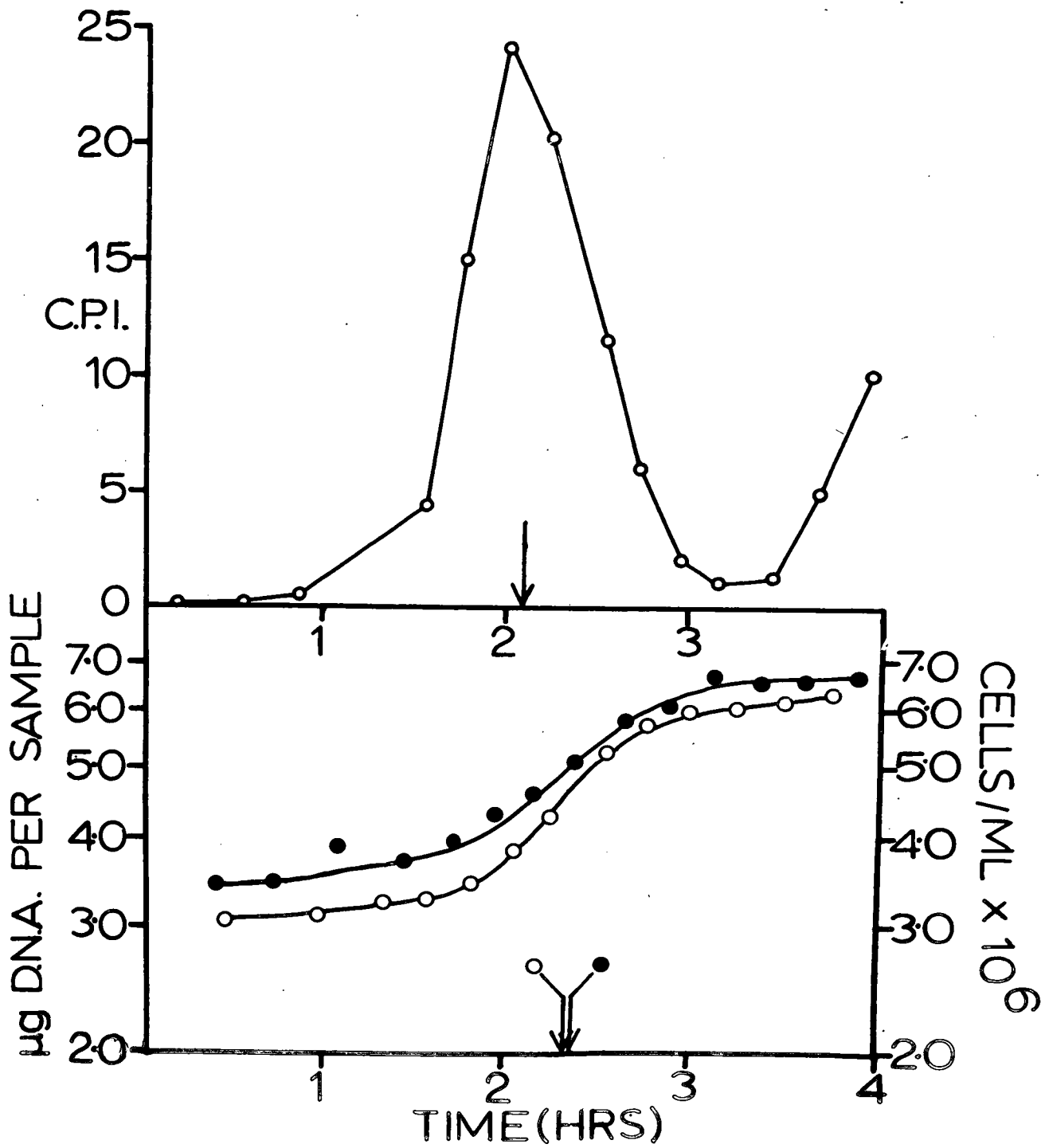
FIGURE 24. — DNA synthesis in a synchronous culture of
975h$^+$.

Top graph :- CPI.

Bottom graph:- Cell numbers (open circles) and μg DNA/sample
(closed circles).

The arrows to the abscissa represent the mid-points of
the rises in cell number and bulk DNA.
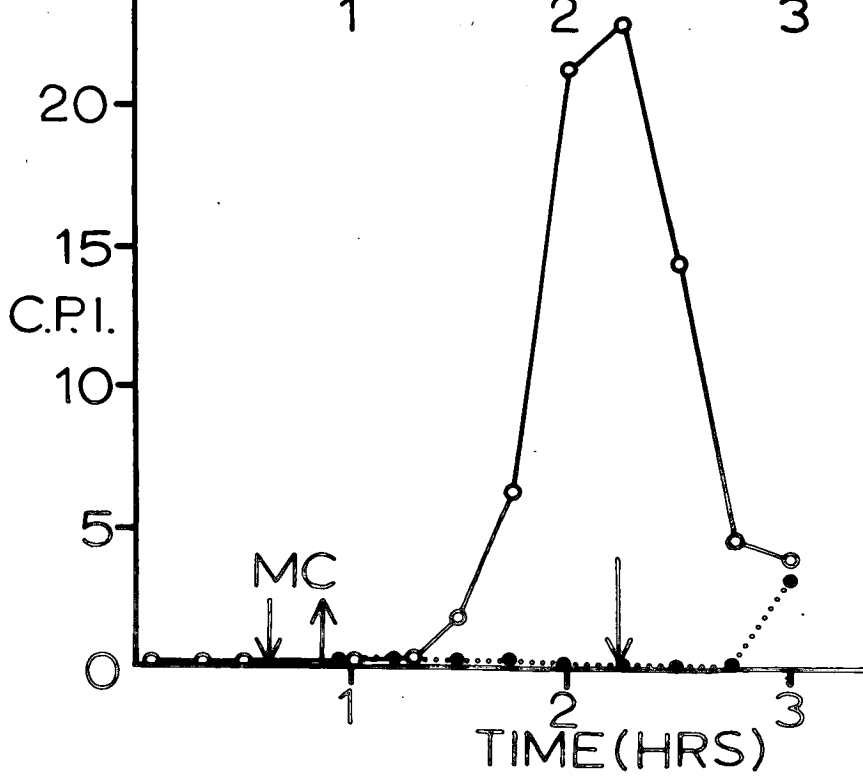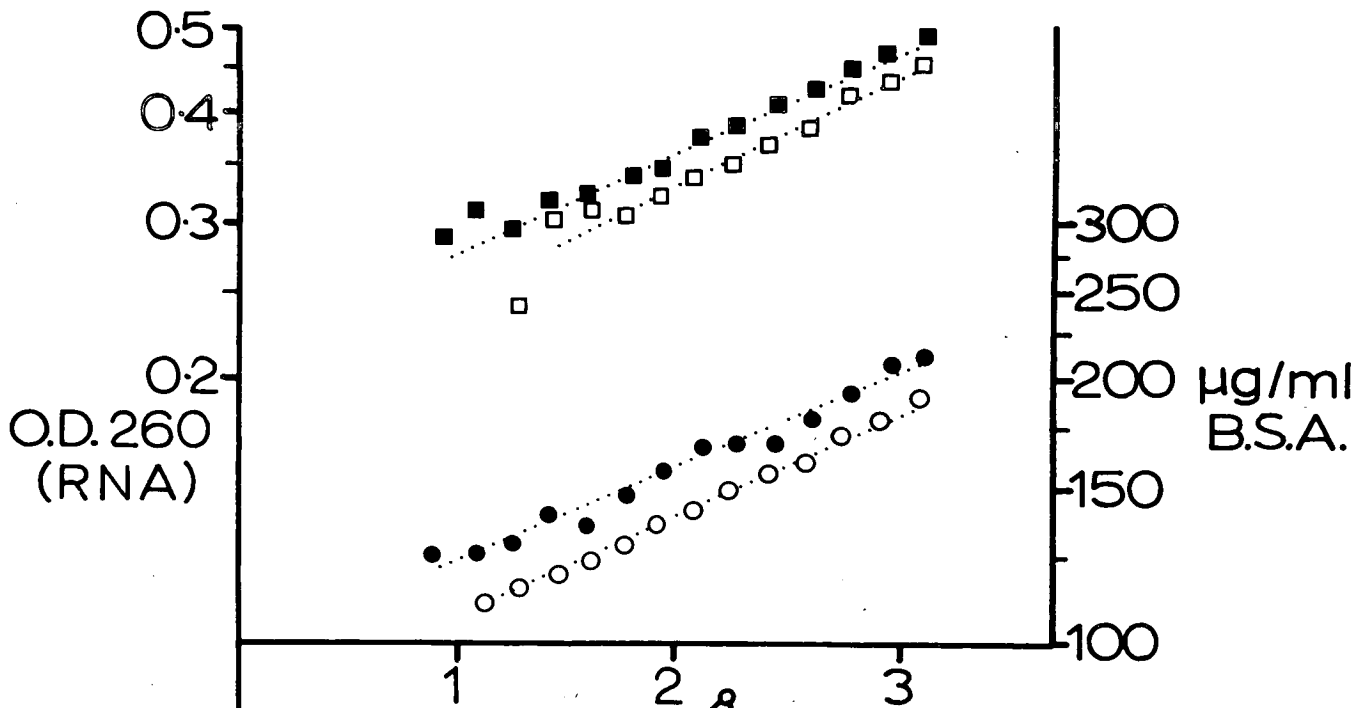
FIGURE 23. -   The effect of an MC pulse in the minor period

upon protein and RNA synthesis.

Bottom graph:- CPI in control (open circles) and treated culture

(closed circles). The MC pulse position was

0.27 and the pulse concentration was 660 μg/ml.

Top graph   :- Left ordinate O.D.260. closed squares - control,

open squares - treated culture.

Right ordinate - Protein per sample -

closed circles - control, open circles -
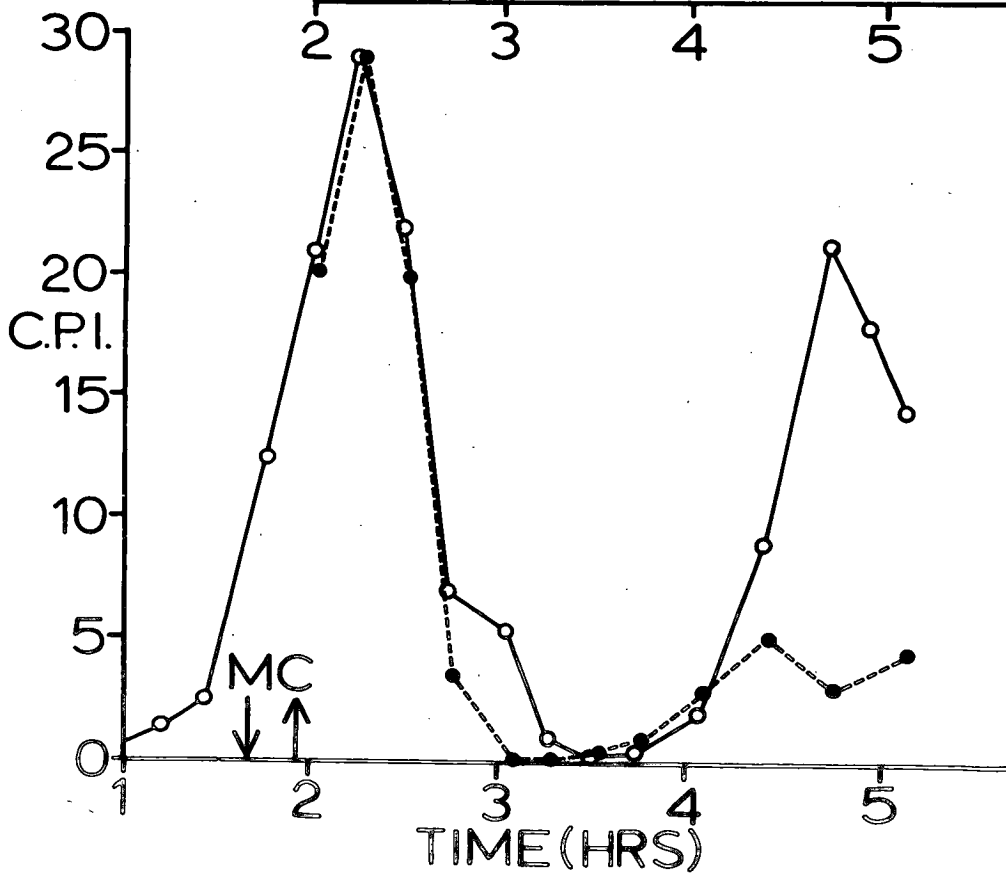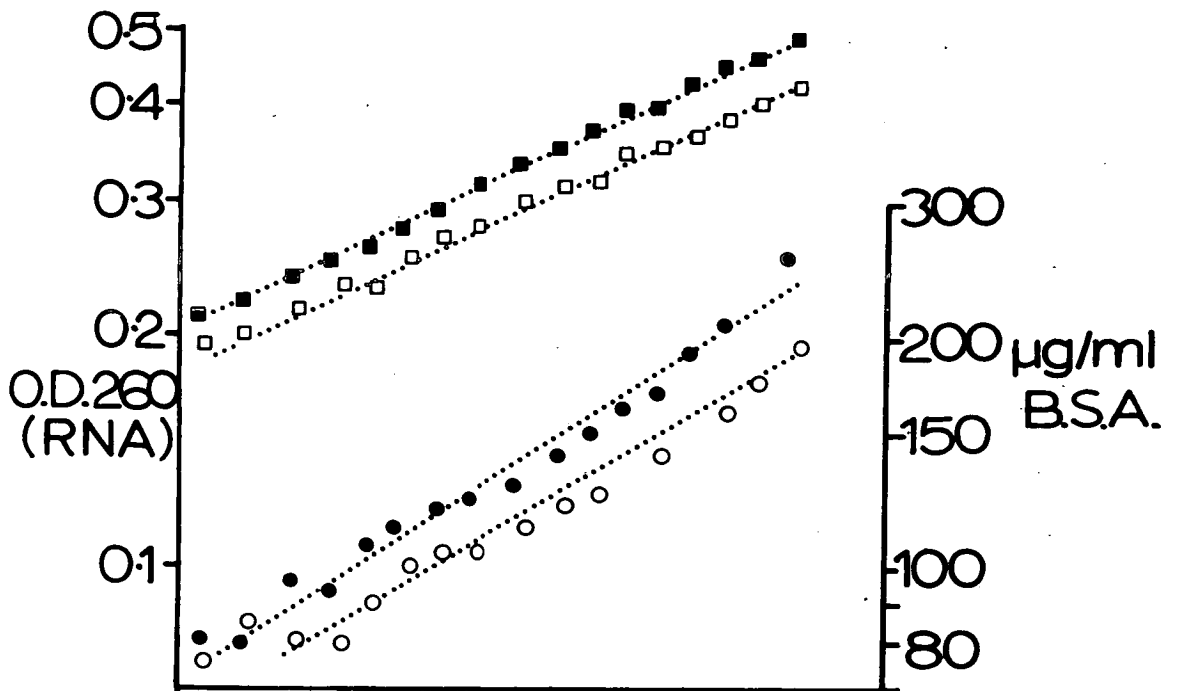
treated culture.

FIGURE 22. - The effect of an MC pulse in the major period
upon protein and RNA synthesis.

Bottom graph:- CPI in control (open circles) and treated culture
(closed circles); MC pulse position was 0.71,
and the pulse concentration was 660 μg/ml.

Top graph :- Left ordinate O.D.260 (equivalent to RNA per
sample).
closed squares - control.
open squares - treated culture.
Right ordinate - bulk protein per sample ex-
pressed as the equivalent concentration of
Bovine serum albumin ("Lowry" assay).
closed circles - control.
open circles - treated culture.

and RNA were made by the method used by N.Stebbing ('69 and '71) which employs the Schmidt- Thannhauser fractionation procedure and which allows both estimates to be made upon the same samples. The results of assaying total protein and RNA synthesis for a period of 2-3 hours after the MC pulse may be seen in Figures 22. and 23. The pulses are in the major and minor periods of sensitivity respectively. Both of these experiments were repeated with essentially identical results.

As may be seen the results support the conclusion that a pulse of MC has no detectable effect upon growth and are in good agreement with the data of Williamson and Scopes ('62a) who found no effect of a continuous treatment of 400 μg/ml of MC on the synthesis of protein and RNA in S. cerevisiae.

Having thus shown that the nuclear division cycle is sensitive to MC induced delay and that cell growth is not implicated in this inhibition quite clearly the one cellular activity which was of paramount interest was DNA synthesis. This process had been made the subject of an extensive investigation by Bostock ('68) and ('70). He had found DNA synthesis to be periodic, to occur at the very end of the cell cycle at 0.86 and that the S period had a rather short duration somewhere in the region of 10 minutes. However, further measurements on synchronous cultures with the new stock of 132 cells and upon other strains by Mitchison, Creanor and myself have shown that in these cells the position of the S period is at 1.0 of a cycle (see Mitchison and Creanor '71b). Figure 24. shows one such result, DNA synthesis measured in a culture of 975h+, the result is identical with those obtained for strain 132.

The effect of placing a pulse of MC in the minor period

FIGURE 25. - The effect of a pulse of MC in the minor
period upon DNA synthesis in a synchronous
culture.

Top graph   :- DNA synthesis.

Bottom graph:- CPI.
open circles - control.
closed circles and dotted line - treated culture.

The pulse position was 0.35 and the MC concentration was 660 μg/ml.
The arrow to the abscissa in the top graph is the point at which
the bulk culture DNA had increased by 50%.

FIGURE 26.   -   The effect of a pulse of MC at the beginning
                of the major period upon DNA synthesis in
                a synchronous culture.

Top graph   :-   DNA synthesis.

Bottom graph:-   CPI - open circles - control culture,
                  closed circles - treated culture.

        The MC pulse position was at 0.77 and the concentration
was 660 µg/ml.

        The arrow to the abscissa in the top graph indicates
the mid-point of the rise in DNA per sample.

N.B. At time 2 hours 48 minutes after inoculation it was
estimated by inspection that 24% of the cells had failed to
divide in the treated culture.

of sensitivity upon DNA synthesis may be seen in Figure 25.; clearly the S period has been delayed(with division)by about 90 minutes,occurring at around the time that the cells divide. Figure 26. gives the effect of a pulse in the major period; in this case there appears to be only a slight delay in the S period. These two results are from a series of seven experiments on DNA synthesis in synchronous cultures treated with HC, three involving minor period treatments and four involving major period treatments. In all cases the results were consistent with those shown, the basic finding being that minor period treatment delays DNA synthesis whilst major period treatment produces only a very short delay and that this is of doubtful significance.

Whether or not there is a delay produced by treatment in the major period is, in fact, a very difficult question to answer for the following reasons. Firstly, the delay in the position of the S period in Figure 26. is just 30 minutes, about 0.2 of a cycle, but this is only 0.1 of a cycle later than the most extreme position of the S period found by Mitchison and Creanor('71b). The natural experiment to detect a delay performed by splitting a culture and treating only one half was unfortunately impossible as the size of culture needed to follow DNA synthesis and position the S period was the maximum obtainable at the time. Thus the only way to decide if the observed delays are significant is by much arduous repetition of the experiment. Additionally, if the delay is significant it may well be explained by procedure used to treat the cells with the drug since these cultures were concentrated approximately 100 fold during the pulse due to the large volumes involved. Bostock ('68) investigated the effect of keeping
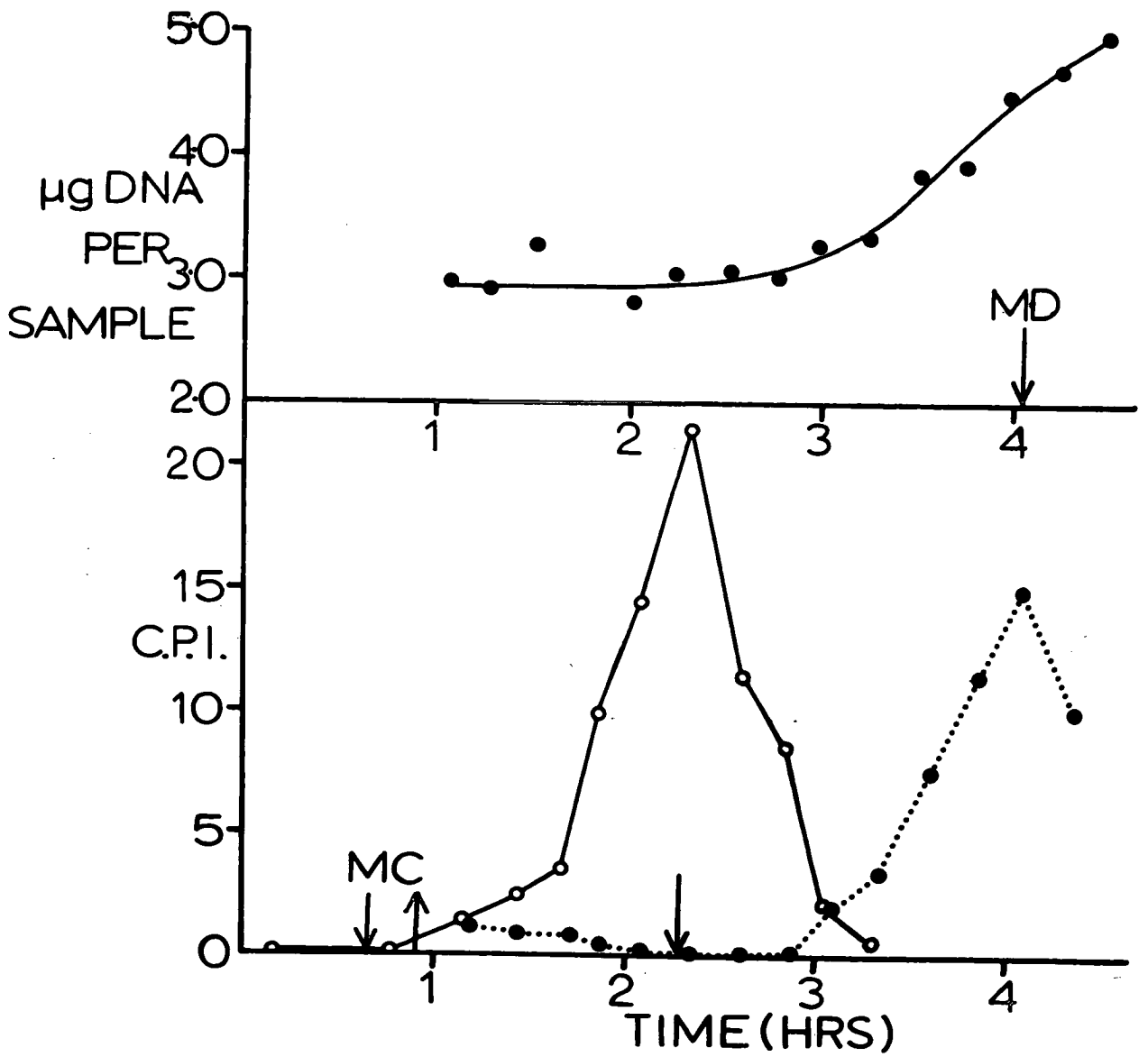
FIGURE 27. - The effect of an MC pulse at the end of the

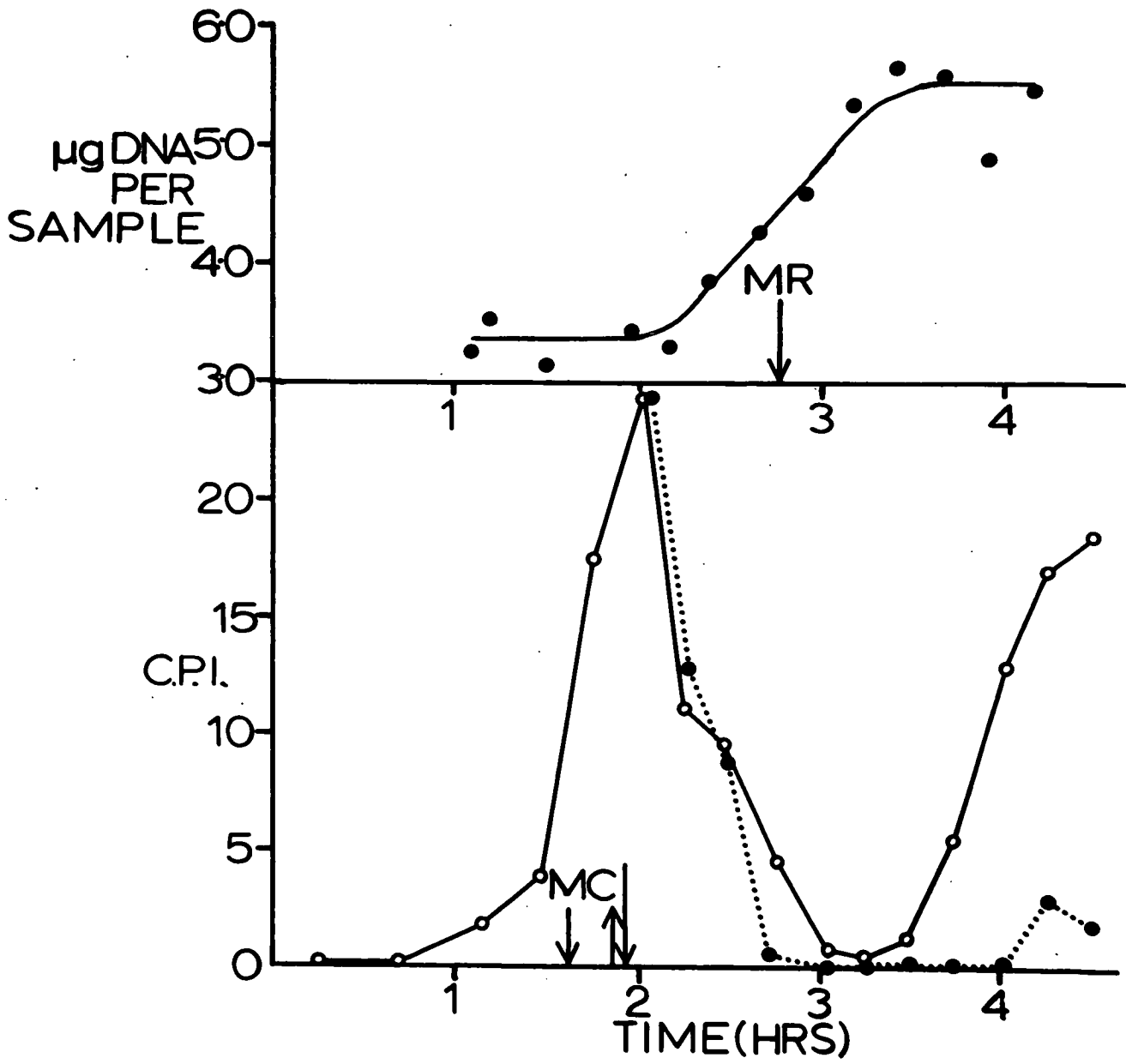major period upon DNA synthesis in a

synchronous culture.

Top graph   :- DNA synthesis.

Bottom graph:- CPI - open circles - control culture, closed

circles - treated culture.

The MC pulse position was 0.85 and the pulse concentration
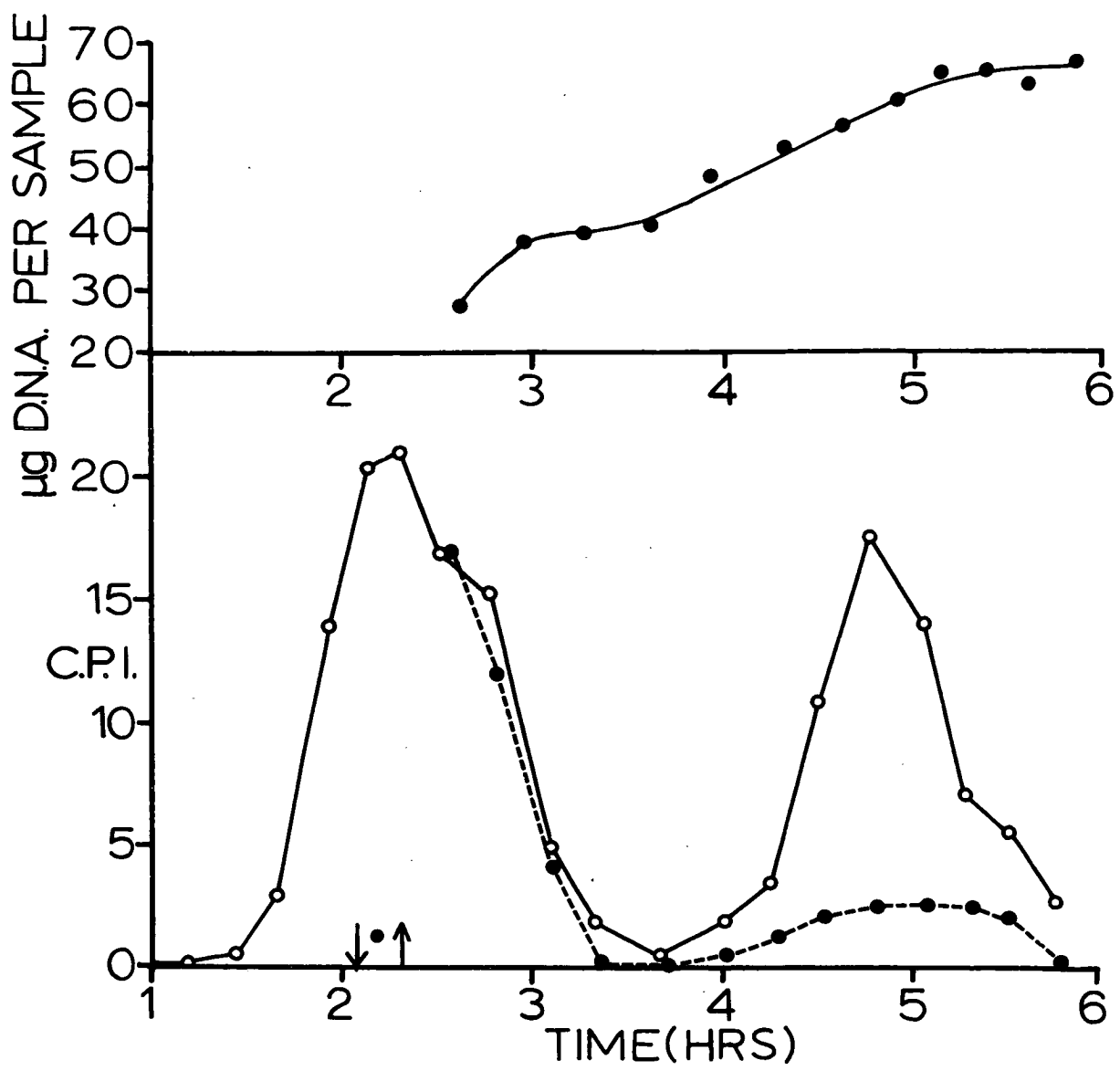was 500 µg/ml.

FIGURE 28. -   The effect of an MC pulse late in the major
period upon cell division in a synchronous
culture.

Open circles -   CPI - control.

Closed circles and dotted line - CPI treated culture.

Closed circles and solid line -   cell numbers in the treated
culture.

The MC position was 0.80 and the concentration 500 Hg/ml.
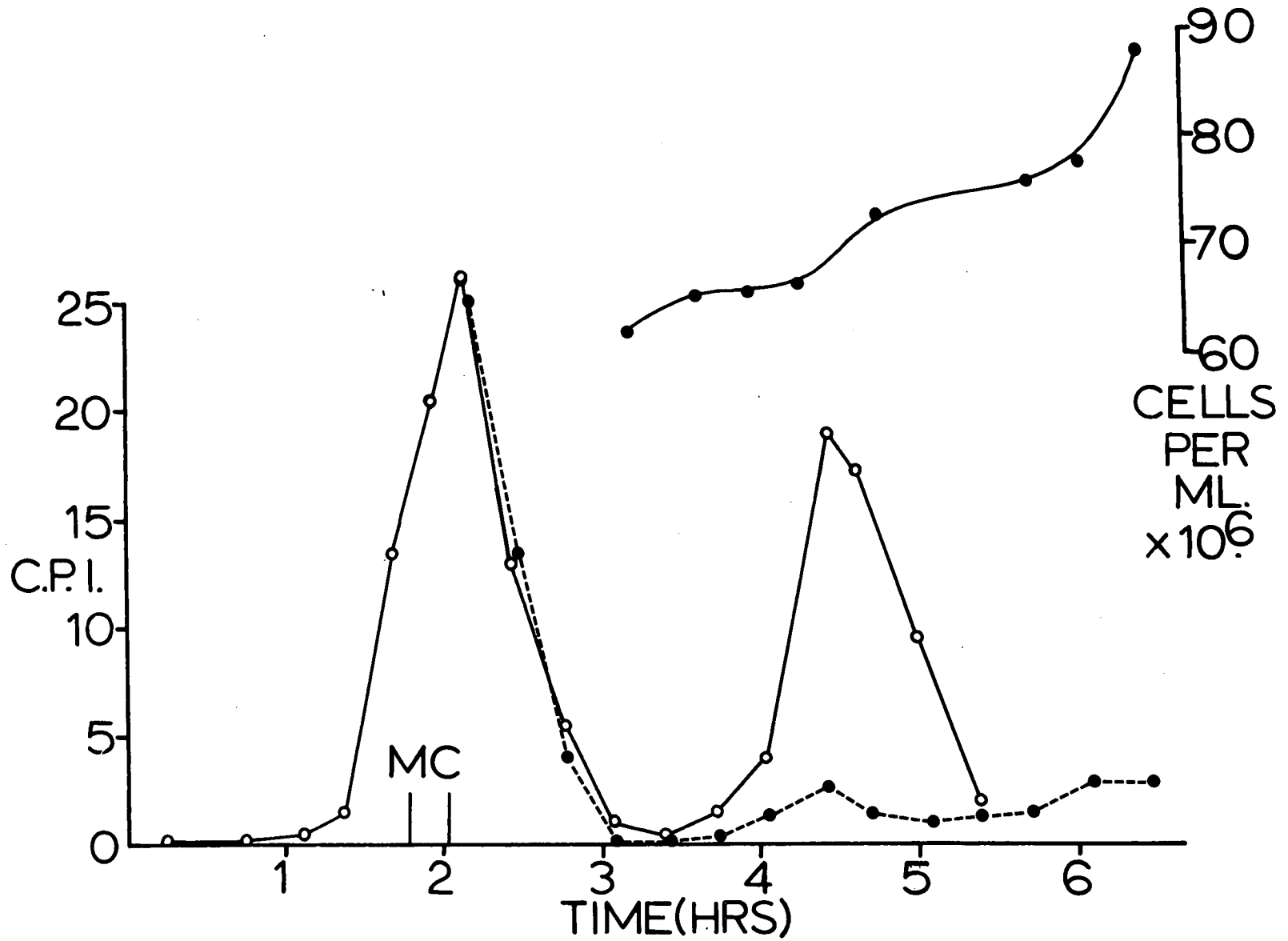
the cells at high cell concentrations during cell collection and found that there was indeed a drop in the amount of DNA per cell, hence this slight delay may well be an artifact.

Another obvious feature of the result shown in Figure 26. is that bulk culture DNA does not double but increases by about 70%. This is quite simply accounted for by the fact that the synchrony of the very large cultures (approximately 1.5×10^9 cells) needed for DNA assays is not as good as in the normal smaller cultures and at the time of the pulse about a quarter of the cells were still in their minor period and thus suffered a first division and a delay in DNA synthesis.

Figures 27. and 28. represent an attempt to answer a further question about DNA synthesis in cells treated in their major period, namely, do the cells become arrested in G_2 or do they go on and synthesize any 'extra' DNA in the course of their long delayed cycle by undergoing a further S period ? Figure 27. shows the result of measuring DNA synthesis for some $3\frac{1}{2}$ hours in the treated cells after the initial S period. The DNA shows approximately a further half doubling over this period but the cell plate data shows there is some residual division in the culture which is attributable to cells of the class mentioned above which were treated in their minor period and thus suffer only a short delay. The experiment shown in Figure 28. is an attempt to decide whether this low but sustained level of division can in fact account for the observed increment in DNA. As may be seen the residual division does in fact lead to a quite substantial increase in cell number and the result on treatment in the minor period predicts that these cells will be synthesizing DNA. Thus these two isolated results suggest that no 'extra' DNA is in fact synthesized

but that the cells become arrested in G_2 in a similar fashion to those cells treated in the minor period of sensitivity. Since it was found to be impossible to obtain a homogeneous population of cells in the major period without resort to a second inhibitor, this approach was not pursued any further.

The conclusion drawn from these results is thus that MC cannot be regarded as an inhibitor of DNA synthesis in these experiments but as an agent with the power to temporarily arrest the progression of the cells through what Mitchison ('69b) has termed the DNA division cycle in the G_2 phase of that cycle.

These results may now be fruitfully compared with those obtained by Williamson and Scopes ('62a) on the effects of continuous treatments of MC upon cells of S. cerevisiae made to divide synchronously by the method of Williamson and Scopes ('62b). The relevant events in the first cycle of such cultures are thus: firstly there is a round of DNA synthesis some 80 minutes after inoculation of the cells and this is followed within a few minutes by the cells becoming resistant to the inhibition of the first division by the continuous application of 400 μg/ml MC. During the 30 minutes after DNA synthesis the nucleus migrates to the neck of the daughter bud and after a further 30 minutes the nucleus can be seen to be clearly divided by staining. Williamson ('66) was able to produce evidence from electron micrographs of the dividing nucleus that the actual time of chromosome separation in the nucleus is between DNA synthesis and the migration of the nucleus to the neck of the bud; thus the transition point for MC inhibition and chromosome separation are closely associated in time in these cells. Williamson ('66) shows that in S. cerevisiae chromosome separation occurs about 30 to 40 minutes before the nucleus is scored as divided by Giemsa staining;

in S.pombe the first transition point precedes nuclear division scored by this criterion by exactly the same period. The evidence thus suggests that nuclear division is a MC sensitive process and that the first transition point is attributable to completion of some stage of nuclear division, possibly chromosome separation.

The argument above leads naturally to the question, is the chromosome itself sensitive to MC damage? There are compelling reasons for accepting that this might be the case. Firstly MC and UV irradiation are both known to cause damage to the structure of DNA. Secondly, nuclear division has been shown to be sensitive and the first transition point is well correlated with the time at which chromosome separation may be inferred to occur. Thirdly, the second transition point is also associated with another event of the chromosomal cycle namely DNA synthesis. The two events are particularly well associated in the case of the MC transition point, the estimates being separated by only 0.03 of a cycle; in the case of UV irradiation the correlation is not as good but it must be remembered that no estimate of the position of the S period was made at the time of $\underline{Gill's}$ work and that growth in $\underline{Gill's}$ experiments was on solid medium.

The working hypothesis which has emerged is thus that sensitivity fluctuates with the chromosomal cycle and that a pulse of MC is far more effective in the G_1 phase than in the G_2; sensitivity thus changes dramatically when the G_2 chromosomes separate prior to nuclear division and when the G_1 phase is ended by the completion of replication. One proviso must be made, however, and that is that this hypothesis may be thought to imply that MC damage is greater to the G_1 chromosome; this is unjustified. It is the effectiveness of MC in producing division delay which

fluctuates and this fluctuation appears to be correlated with the chromosomal cycle. Effectiveness is a function of both damage and its repair and both may vary qualitatively and quantitatively through the cycle to produce the observed result.

Returning to the data of Williamson and Scopes ('62a) my results on DNA synthesis agree well with their finding that DNA synthesis occurs in the presence of continuously applied MC since in both their experiments MC treatment was initiated just before DNA synthesis whilst the cells were in the G_1 phase.

The findings of this section may be summarised as follows :-

1.   The effect of a pulse of MC was to delay nuclear division and it is this delay which accounts for the effect of MC upon cell division.   A pulse produces a delay in the forthcoming nuclear division at all times in the cell cycle excepting a short period from the first TP at 0.57 to the separation of the daughter nuclei at 0.75; treatment during this interval causes a delay in the subsequent nuclear division.

2.   No effect of MC upon growth as measured by increase in cell size or the synthesis of protein of RNA were detectable.

3.   A pulse of MC was not found to have a considerable inhibitory effect upon DNA synthesis although it is possible that MC may have a very slight delaying effect upon the S period if applied in the major period.   However, a pulse of MC will delay the S period if applied in the minor period but this is thought to be due to the cells suffering a G_2 arrest and failing to reach the S period at the normal time.

4.   The results above when considered with the pattern of sensitivity to delay through the cell cycle and the position of the

transition point found by <u>Williamson and Scopes</u> in S.cerevisiae have led to the hypothesis that the major and minor periods of sensitivity correspond to two phases of the chromosomal replication cycle, the major period of sensitivity being that part of the cycle during which the chromosome is unreplicated and the minor period of sensitivity being that part of the cycle from the end of the S period to the time of chromosome separation.

CHAPTER 4.

The action of DNA synthesis inhibitors on periodic fluctuations in sensitivity to MC induced delay.

Whilst the work described in the preceding chapters was being performed Mitchison and Creanor ('71a) were investigating the effects of two of the inhibitors mentioned in the introduction, namely deoxyadenosine (AdR) and hydroxyurea (HU). Their findings proved to be of great use to this study for they provided a means of testing the hypothesis put forward in the last chapter. The method was just that proposed in the introduction, to distort the nuclear cycle and to examine the effect of this distortion upon the two markers of the MC sensitivity cycle, the transition points.

I have already employed the G1-S-G2 classification of the nuclear cycle introduced by Howard and Pelc. However, there is a problem when using this system with yeast for although the S period may be easily located the other marker which defines the cycle, mitosis, is absent in yeast as an easily scorable event. It is inherent in the hypothesis put forward in the last chapter that 'mitosis' should occur between the first transition point and visible nuclear fission, and before proceeding to consider evidence which concerns the second transition point and is thus unaffected by this difficulty, I should like to deal further with the evidence for this assumption.

Williamson's ('66) work upon the nuclear events in synchronously dividing cells has already been described and provides a basis for the hypothesis, but since that work was performed further evidence has emerged to support the idea that yeast nuclear division resembles normal mitosis. The subject has been recently

reviewed by Matile et al and they report that several authors have found using electron microscopy an intranuclear spindle consisting of a bundle of microtubules attached at both ends to a modified area of the nuclear membrane which has been called the centriolar plaque. They point out that this spindle has probably been observed previously by light microscopists who termed it the lateral granule or intranuclear fibre. This intranuclear spindle appears in the nucleus of many Ascomycetes including S.pombe (Robinow and Bakerspiel) and microtubules have been observed in the nucleus of S.pombe by Barker and Scmitter. The division of the nucleus in yeasts is thus preceded by events which strongly resemble mitosis in the cells of higher eucaryotes although it is not yet clear whether the intranuclear spindle has a role in effecting chromosome separation. It consequently seems quite reasonable to expect that the act of chromosome separation preceeds visible nuclear fission by a significant period of time.

No less than three inhibitors of interest became available to distort the cycle and all of them appeared to effect DNA synthesis; they are AdR, HU and an inhibitor which was studied by Bostock ('68 and '70b) 2- phenyl ethanol (PE). PE was used by Bostock at a concentration of 0.2% in the medium as a continuous treatment and this results in a long cycle of about 240 minutes with the S period occurring at about mid-cycle. However, it was found to be exceptionally difficult to obtain good synchronous cultures of cells growing in 0.2% PE. The work of Stebbing ('72) on PE treated cultures provides a probable explanation of this difficulty, he found that there was little synthesis of protein and hence growth during the G_1 part of the PE affected cycle. This means that when making a synchronous culture the small cells

selected by the gradient technique will have a much wider distri-
bution of cell ages than normal and the synchrony will fall
accordingly.

Concentration was thus focussed upon the action of AdR
and HU. Mitchison and Creanor found the action of these two com-
pounds was very similar. When added to a synchronous culture at
the appropriate concentrations it is found that the first division
occurs normally but that the subsequent division fails to occur.
There is little effect on the growth of the cells by either of .
these inhibitors at the concentrations used to affect division
and thus the result is cell elongation as is seen with MC treat-
ment. If the treatment is continued there is some recovery of
the ability to divide.

At higher concentrations some effect on growth is seen,
especially with HU; this manifests itself by a broadening and
heightening of the cell plate peak as the cells take longer to
traverse the cell plate phase. However, if protein, RNA and DNA
synthesis are measured it is clear that the major effect of the
two drugs is on DNA synthesis which shows a dose dependant reduct-
ion in rate and may be completely inhibited by 1.1×10^{-2} M HU.

The underlying basis of the effect is in both cases
most probably the inhibition of the enzyme ribonucleoside
diphosphate reductase. This enzyme has been made the subject of
intensive study by Brown and Reichard and has been found to be
sensitive to reversible repression by deoxyadenosine triphosphate
(dATP) which appears to bind the enzyme protein. Prior to this
finding it had been suggested by Overgaard-Hanson and Klenow that
dATP accumulates in AdR treated cells. The same enzyme has been
shown to be sensitive to HU by Krakoff, Brown and Reichard; in this

FIGURE 29. - The effects of a pulse of deoxyadenosine upon
the division of a synchronous culture, and the
effect of a pulse of MC upon a culture so
treated.

Counting graphs 1 - 5 downwards:

Graph 1 - Control.

Graph 2 - The effect of a pulse of 2×10^{-3}M AdR from
25 minutes to 2 hours 55 minutes.

Graphs 3,4 and 5 - 15 minute 500 μg/ml MC pulses were at times
2 hours 0 minutes, 2 hours 35 minutes and
3 hours 55 minutes respectively.
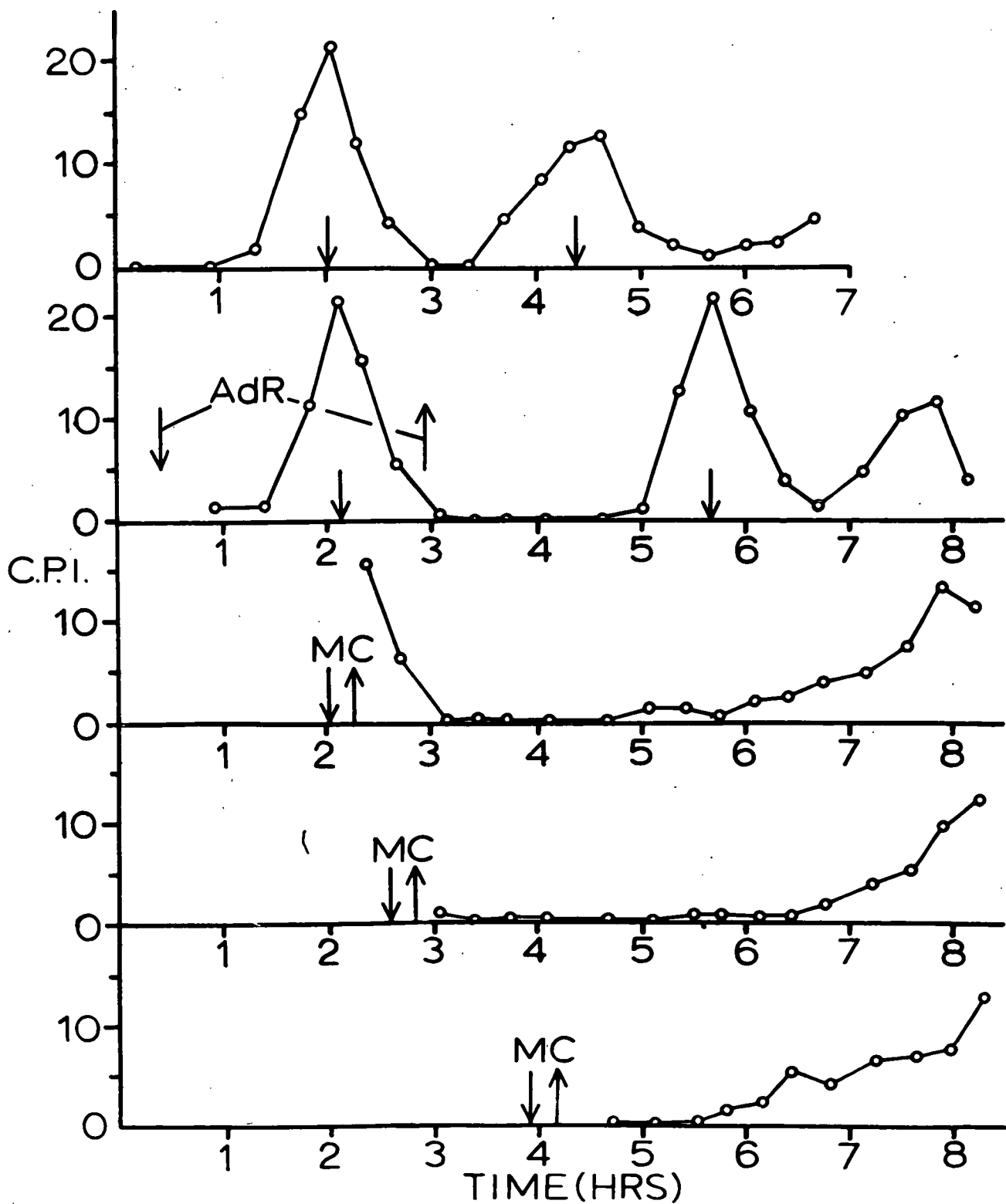
case the inhibition is irreversible as one of the protein sub-
units of the enzyme is inactivated. It suggested that it is
hydroxlamine formed from the breakdown of HU which causes this
effect as exactly the same result can be obtained with this sub-
stance. This conclusion was also reached by Young who studied
analogues of HU and the reversibility of HU inhibition with
deoxyribonucleotides. Direct evidence of the action of HU was
obtained by Neuhard who was able to measure and show the depletion
of the pool of deoxyribonucleotides in HU treated E.coli.

Another finding of possible relevance to the growth
effect in S.pombe was made by Rosenkranz who studied carbamoxy-
loxyurea, an oxidation product of HU and found that it had a
strong effect on protein and RNA synthesis in E.coli. It was in
light of this particular side effect of HU that most attention
was concentrated upon the effect of AdR.

Mitchison and Creanor ('71a) found that if AdR is added
to a synchronous culture about 20 minutes after inoculation at a
concentration of 2.0×10^{-3}M and is withdrawn $2\frac{3}{4}$ hours later
a highly elongated second cycle results lasting about 210 minutes.
Moreover DNA synthesis does not occur at the normal time but is
delayed by about 150 minutes. Hence the second cycle consists
mainly of a long G_1 phase which is absent in the normal cycle.
This provided a good situation to test the hypothesis put forward
in the last chapter since if the hypothesis is correct the period
of major sensitivity should, in the AdR treated cells, extend
well into the second cycle and the second transition point should
be delayed by the same amount as DNA synthesis.

Figure 29. shows the result of one such experiment in
which three MC pulses were placed in the AdR modified cycle; as

FIGURE 30. - An experiment to test whether the addition
of AdR to a synchronous culture modifies the
sensitivity of the cells to MC induced delay
during the first cycle.

Curve A - Control.

Curve B - AdR treated culture pulsed with 500 µg/ml MC
for 15 minutes at 0.28.

Curve C - AdR treated control.

Curve D - AdR treated culture pulsed as above at 0.55.

Curve E - AdR treated culture pulsed at 0.39.

N.B.   AdR was added at a concentration of 2×10^{-3}M to cultures
B, C, D and E at time 20 minutes. The MC used to pulse
cultures B, D and E was dissolved in medium plus AdR.
The delays in cultures B and E were 61 minutes and 76
minutes respectively, and the culture D shows that the
cells pass through their first transition point quite
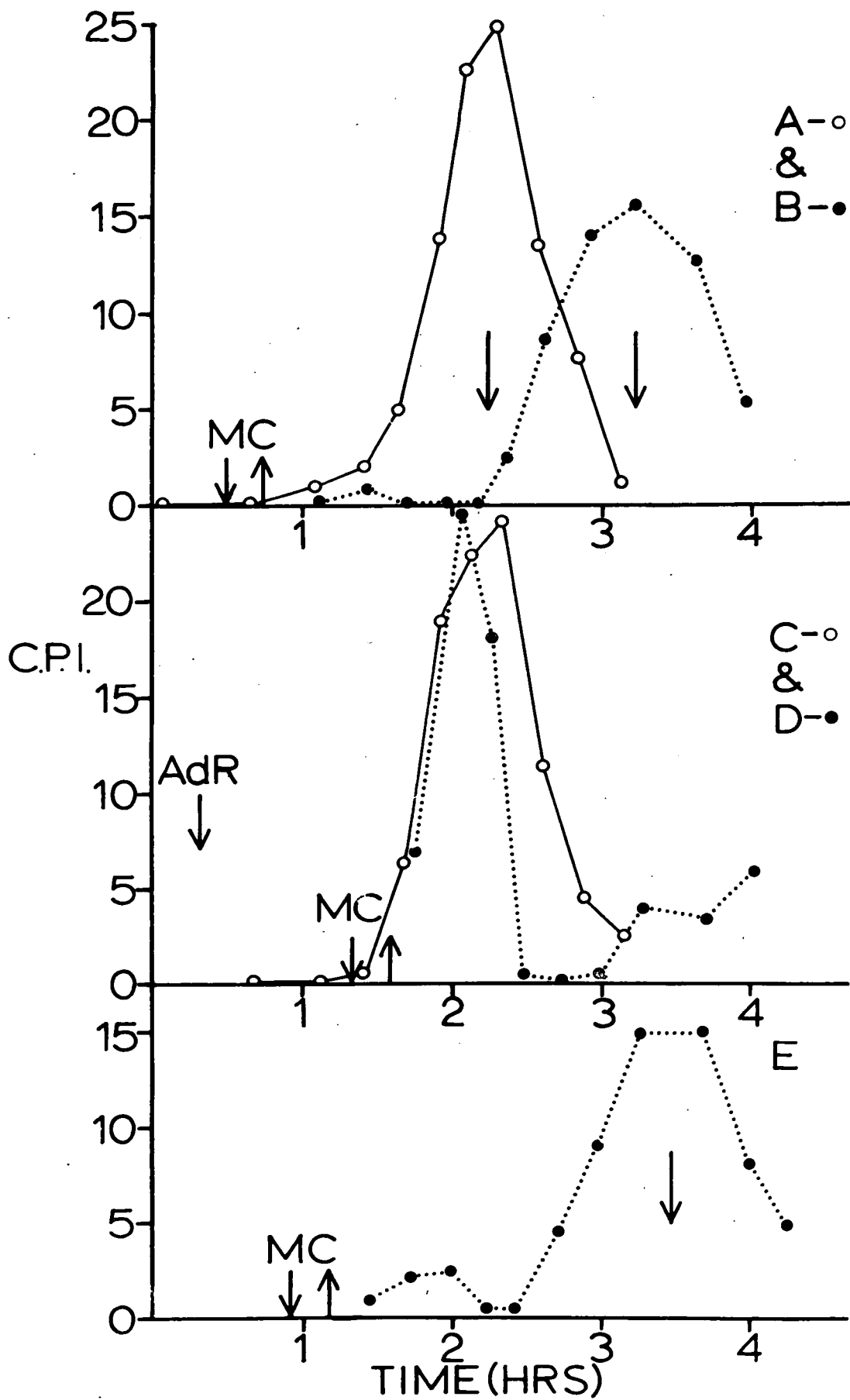normally in the presence of AdR.

FIGURE 31. - The relationship between the length of an AdR

pulse (2 x 10^{-3}M) starting at 20 minutes after

inoculation and the delay induced in the second

division.

N.B. See Figure 29 for an example of AdR induced delay in

the second division of a synchronous culture.

This experiment was performed with a single synchronous

culture.
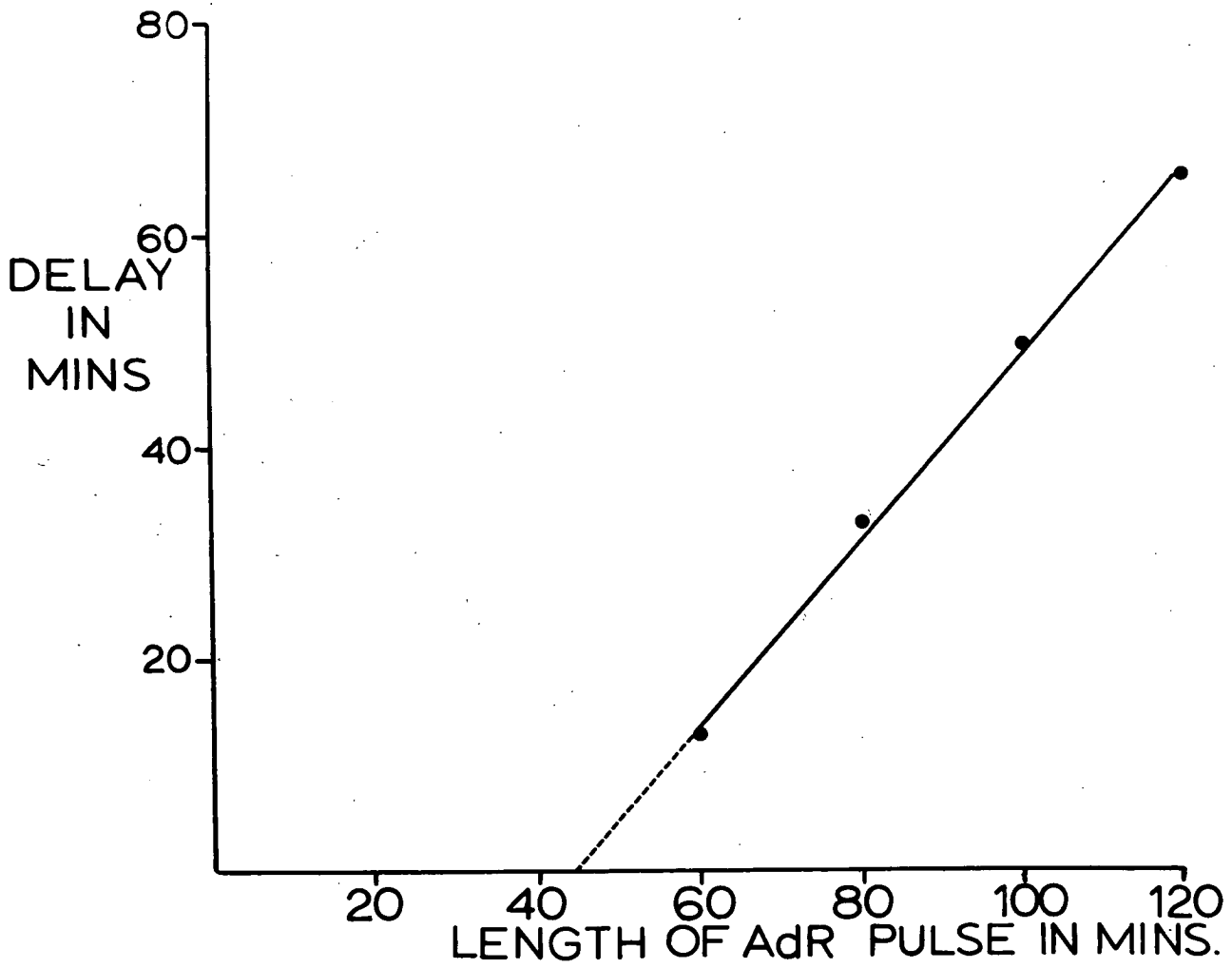
can be seen the cells display high sensitivity throughout most of
the second cycle and it is only in the latest pulse that there is
any sign of cells suffering only a short delay. Furthermore,
observation of the cells which contributed to the 10% dividing
fraction at around 8 hours showed that most of these cells were
undergoing their third division and that the majority of cells
were delayed before the second division.

This result is only significant, however, if AdR can be
shown to have no fundamental modifying effect upon the sensitivity
of the cells to MC induced delay. To test for such an effect AdR
was added to a synchronous culture after 20 minutes and three
pulses were applied in the continuous presence of AdR. The result
can be seen in Figure 30.; uniform delays of durations typical of
the pulse positions were obtained and the cells undergo their first
transition point in the normal way. Hence AdR does not appear to
affect MC sensitivity directly.

Whilst the results shown in Figure 29. are in agreement
with the idea of G_1 sensitivity this was not the ideal experiment
for testing the hypothesis since the G_2 phase is so reduced in
this situation that it is impossible to show that it retains its
characteristic sensitivity. It was thus decided to try and con-
struct a cycle with a G_1 phase, a mid-cycle S period and an observ-
able G_2 phase simply by manipulating the length of the AdR pulse.

Figure 31. shows the result of shortening the AdR pulse
on the delay produced in the second division of a synchronous
culture. The result suggests that a pulse of about 40 minutes
duration starting at the usual 20 minutes after inoculation would
not delay the second division at all, whilst the slope of the graph
suggests that increasing the pulse length in excess of 40 minutes

FIGURE 32. - DNA synthesis in a synchronous culture

pulsed with 2×10^{-3}M AdR for 53 minutes

from 20 minutes to 1 hour 13 minutes.

MD represents the time at which the bulk culture DNA had

increased by 50%. This time corresponds to a cycle position

of 0.42.
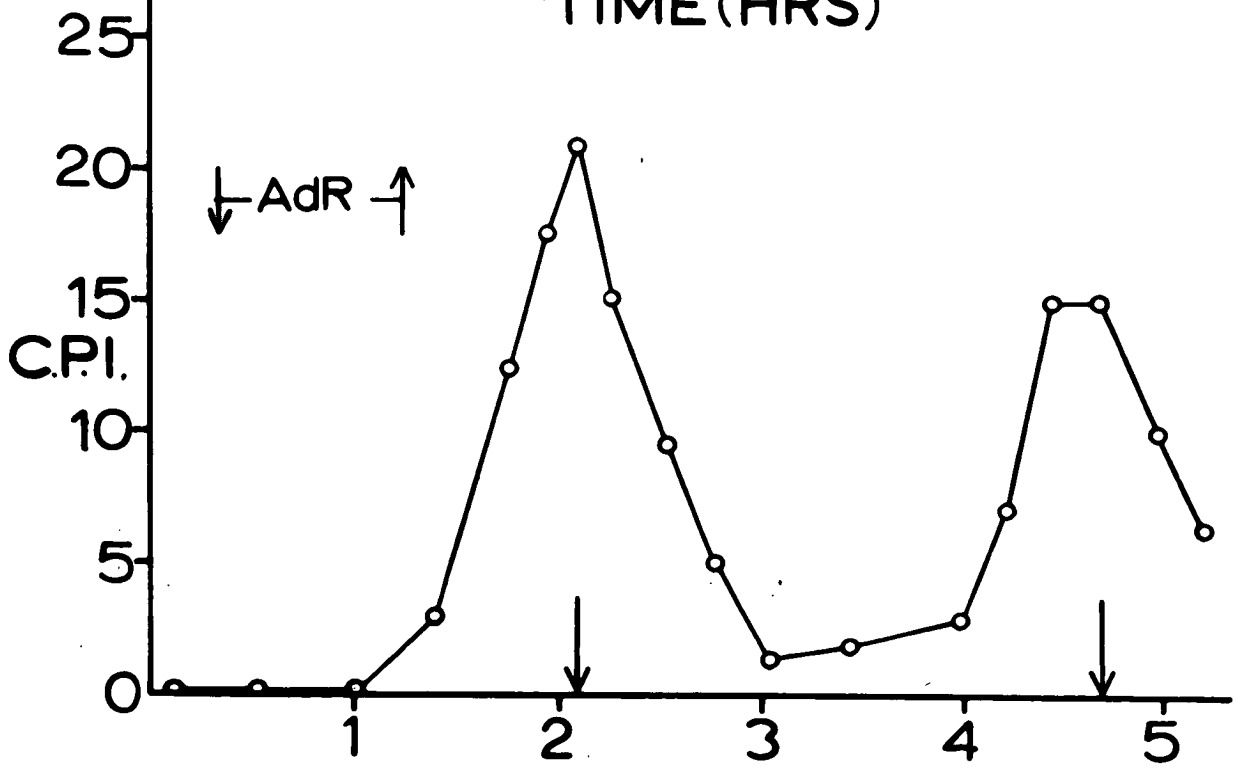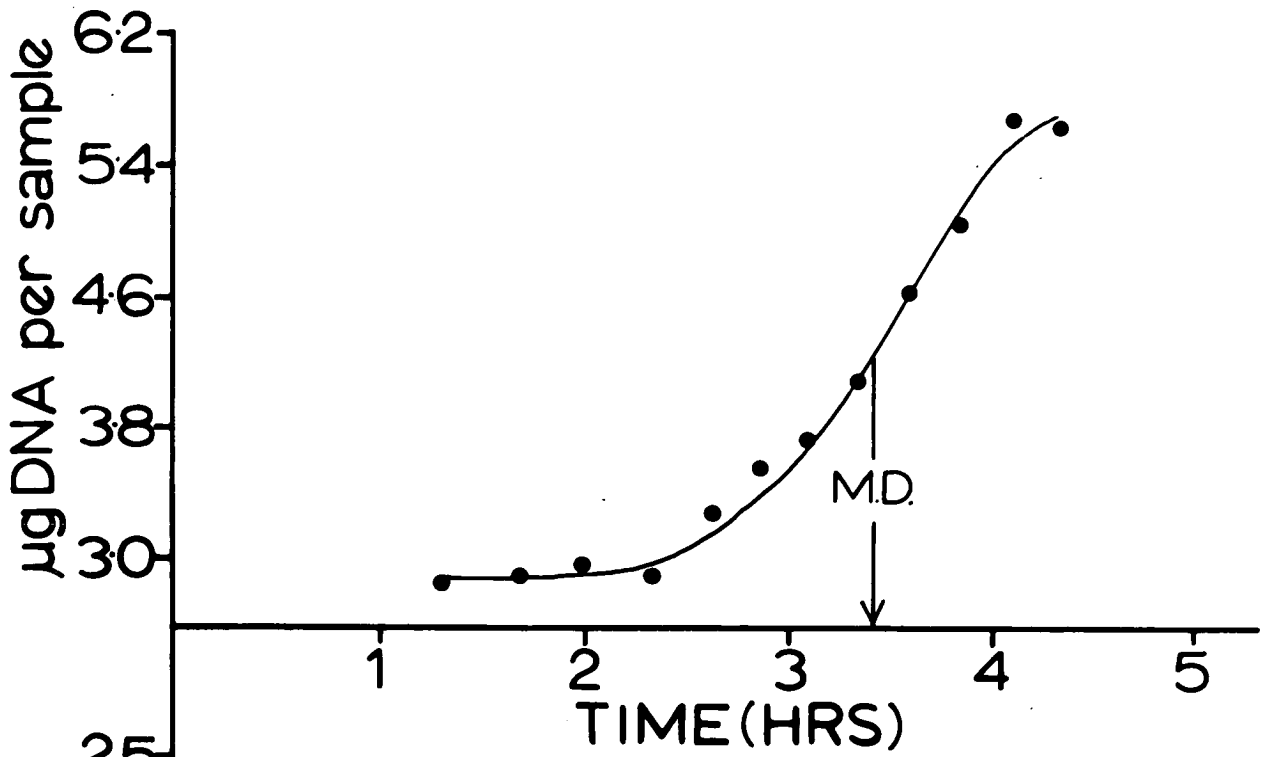
FIGURE 33.   -   The effect of a pulse of MC upon DNA synthesis
in a synchronous culture previously pulsed
with AdR.

AdR pulse   -   23 minutes to 1 hour 42 minutes. The concen-
tration was 2×10^{-3}M.

MC pulse   -   15 minutes 500 μg/ml pulse at 1 hour 45 minutes
(0.80).

The mid-point of the rise of DNA synthesis corresponds to a cycle
position of 0.42.
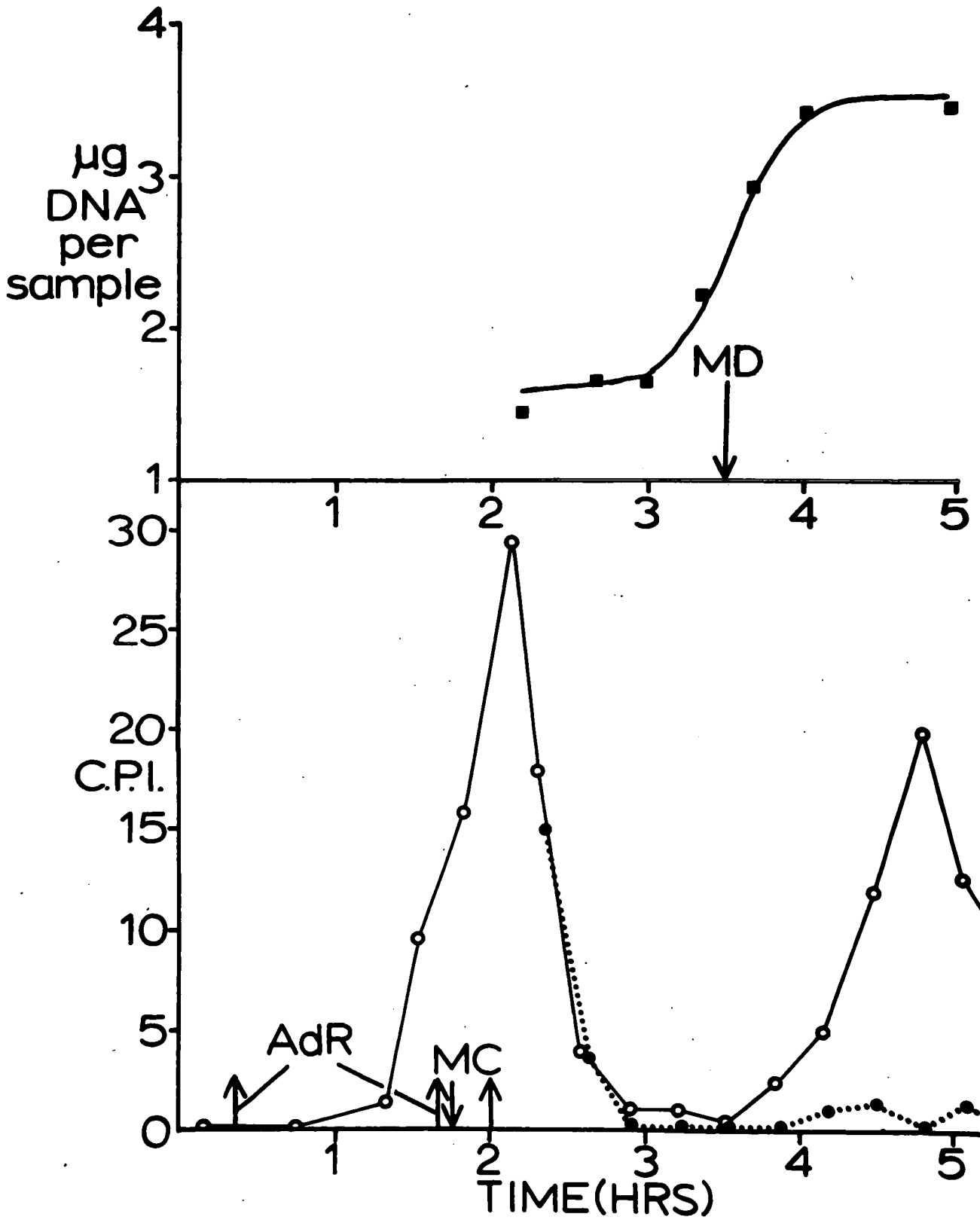
FIGURE 34. -   The effect of four pulses of MC upon a
                synchronous culture previously treated
                with a pulse of AdR.

Top graph   -   Control - AdR treated from 20 minutes to
                  1 hour 15 minutes (open circles).
                  Curve a: MC pulse (15 minutes 500 μg/ml)
                              at 0.94 (closed circles).

Middle graph -   Curve b: MC pulse at 0.03.

Bottom graph -   Curve c: MC pulse at 0.18 (open circles).
                  Curve d: MC pulse at 0.31 (closed circles).

                  The delay was 64 minutes.

The cycle time of control culture (treated AdR) was 2 hours
36 minutes.
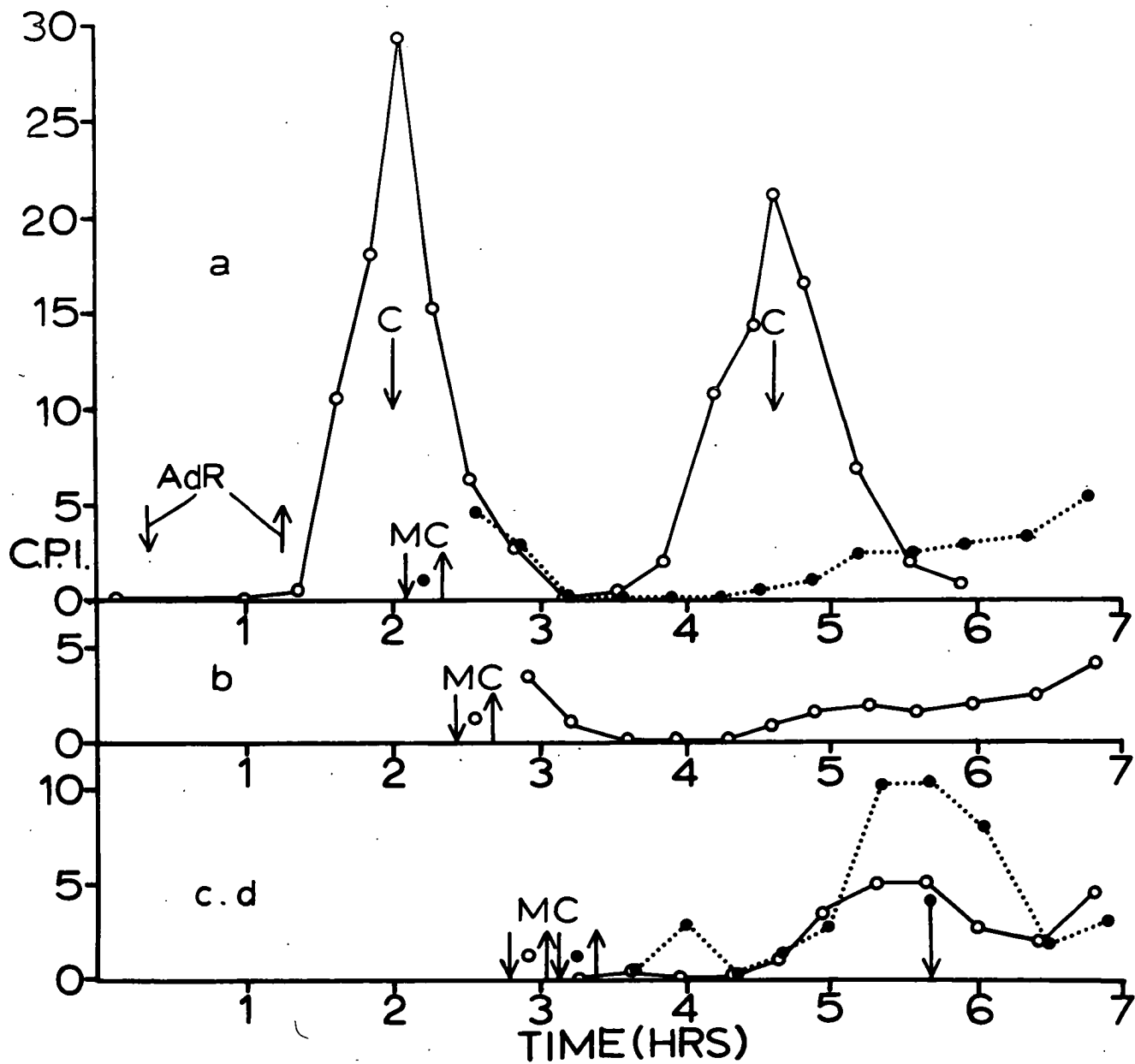
FIGURE 35. - Two MC pulses upon a synchronous culture

pulsed with AdR.

The first peak of the synchronous culture only is shown. It

was pulsed with AdR from 20 minutes to 1 hour 15 minutes

and the position of the MC pulses (500 μg/ml 15 minutes) was

0.91 and 0.11. The cultures were observed by photomicroscopy.

Open circles   -   0.91 pulse - increase in number of cells

in field due to 2nd synchronous division

only.

Open squares   -   total increase in number of cells in field.

Closed squares and circles - as above for 0.11 pulse.

The estimated delays assuming a typical cycle time for the

control were 203 minutes and 260 minutes for the 0.11 and 0.91

pulses respectively.
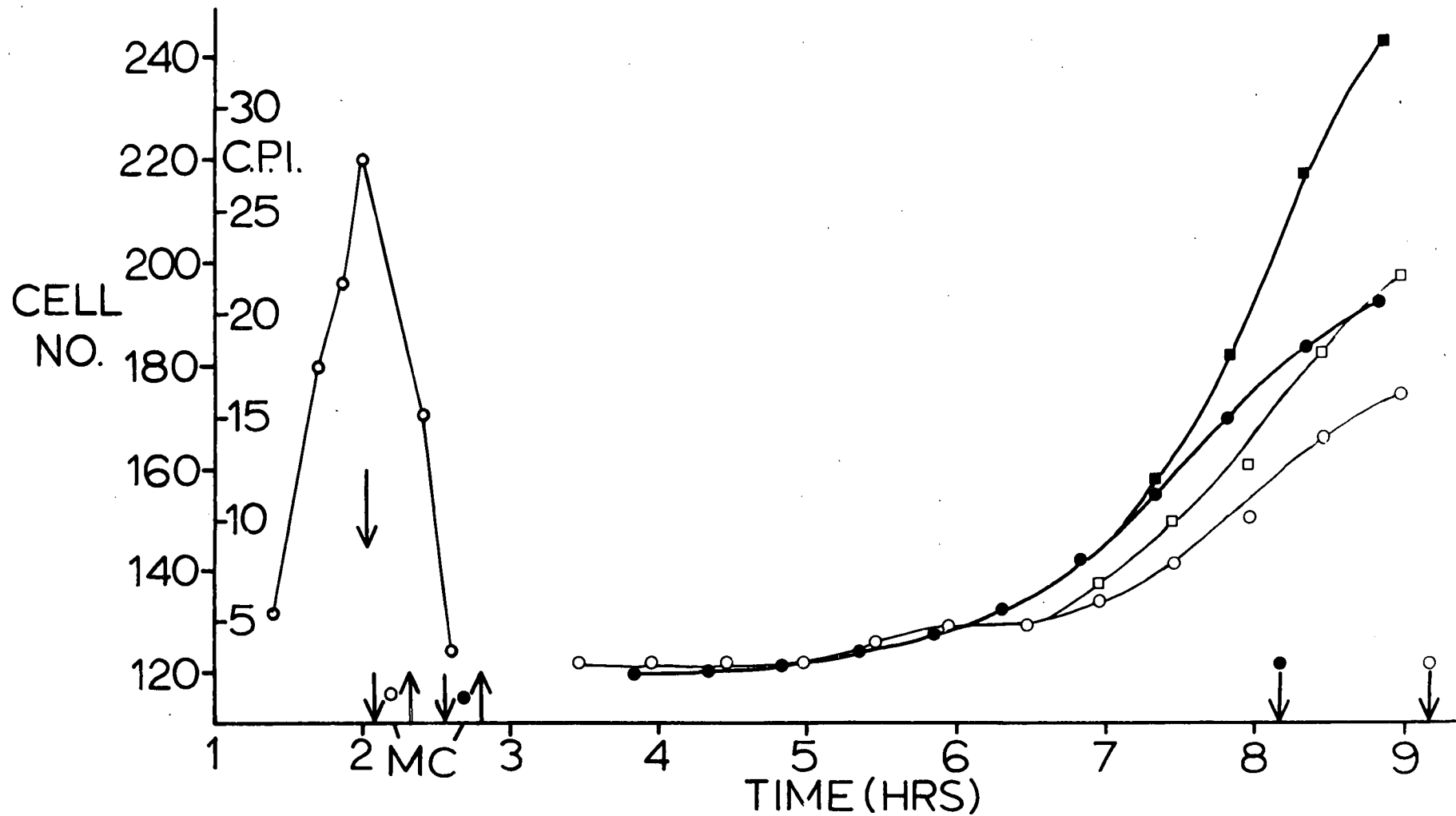
causes a one to one increase in the delay of the second division. The most likely explanation of this is that the S period can be moved in the second cycle without any effect on the second division but that as the pulse length is increased a point is reached where an effect is seen on the second division since there must be a minimum time from the end of the S phase to the end of the cell cycle to allow nuclear division and other end of cycle events to occur.

Assaying DNA synthesis in a synchronous culture treated with a short AdR pulse of 50 minutes duration confirms this idea. The result is shown in Figure 32. The mid-point of DNA synthesis is moved to 0.42 and the cycle is probably just slightly elongated. One further control was now performed to guard against the remote possibility that treatment with AdR might make DNA synthesis sensitive to inhibition by MC, and this was to assay DNA synthesis in an AdR pulsed culture. Figure 33. shows the result; clearly the S period is unaffected which confirms the results of Chapter 3. upon the effect of MC on DNA synthesis.

One final check was made on the delays induced in cells which had been treated with AdR and that was to ensure that the delays suffered in the AdR lengthened G_1 period were in fact comparable to those found for untreated cells pulsed in the major period. Figure 35. shows data, obtained by photomicroscopy, from two pulses upon a culture treated with a 55 minute pulse of AdR in the extended G_1 phase; the delays caused are both in excess of 200 minutes and are very similar to those reported in Chapter 1.

Having obtained these results it was then possible to test the idea that the major and minor periods corresponded to the G_1 and G_2 phases respectively. Figure 34. shows the result of an experiment in which four pulses of MC were placed in the second

cycle of synchronous culture treated with a 55 minute pulse of AdR.
The two earliest pulses show the cells to be highly sensitive
but the two later pulses show that a rising proportion of the cells
are only suffering a short delay typical of those elicited in the
minor period. The latest of the four pulses at 0.31 of a cycle
produces a measurable delay which is in excellent agreement with
the delay produced in an untreated culture (see Figure 8.) in the
minor period and a small "escape peak" of cells which have passed
their first transition point may also be seen. Since this experi-
ment was obviously important it was repeated; the second experiment
contained a pulse at 0.54 of a cycle which showed an even larger
"escape peak" confirming that the first transition point occurs
normally in these cultures. The other three pulses in this second
experiment were at 0.96, 0.17 and 0.34 and once more the results
clearly indicated that the effect of the AdR pulse was to move the
second transition point to a mid-cycle position. Once again the
pulse at 0.34 produced a measurable delay of 88 minutes which is
very similar to the control values.

That the delays produced by these pulses in the shortened
G_2 period are typical of the cell cycle position and not those
delays which normally follow immediately after the second transition
point is interesting and suggests that the rising pattern of delay
in the minor period may be due to the fact that a constant time is
needed to repair the damage done by a pulse.

Whilst these results lend good support to the hypothesis
put forward in Chapter 3., one finding of Mitchison and Creanor
(personal communication) upon the effect of AdR raised certain
doubts as to the cause of the extension of the minor period. This
finding was that the delay of DNA synthesis in synchronous cultures

FIGURE 36. - The effect of a pulse of hydroxyurea upon

division in a synchronous culture and the

effect of two MC pulses on a culture so

treated.

Top graph - Open circles - control.

closed squares, dotted line - culture treated

from 1 hour to 2 hours 10 minutes with

$1-1 \times 10^{-2}$HU.

Bottom graph - Open circles - MC pulse (500 μg/ml 15 minutes)

at 0.76 upon the HU treated culture.

Closed circles, dotted line - MC pulse at 0.05

on the treated culture.
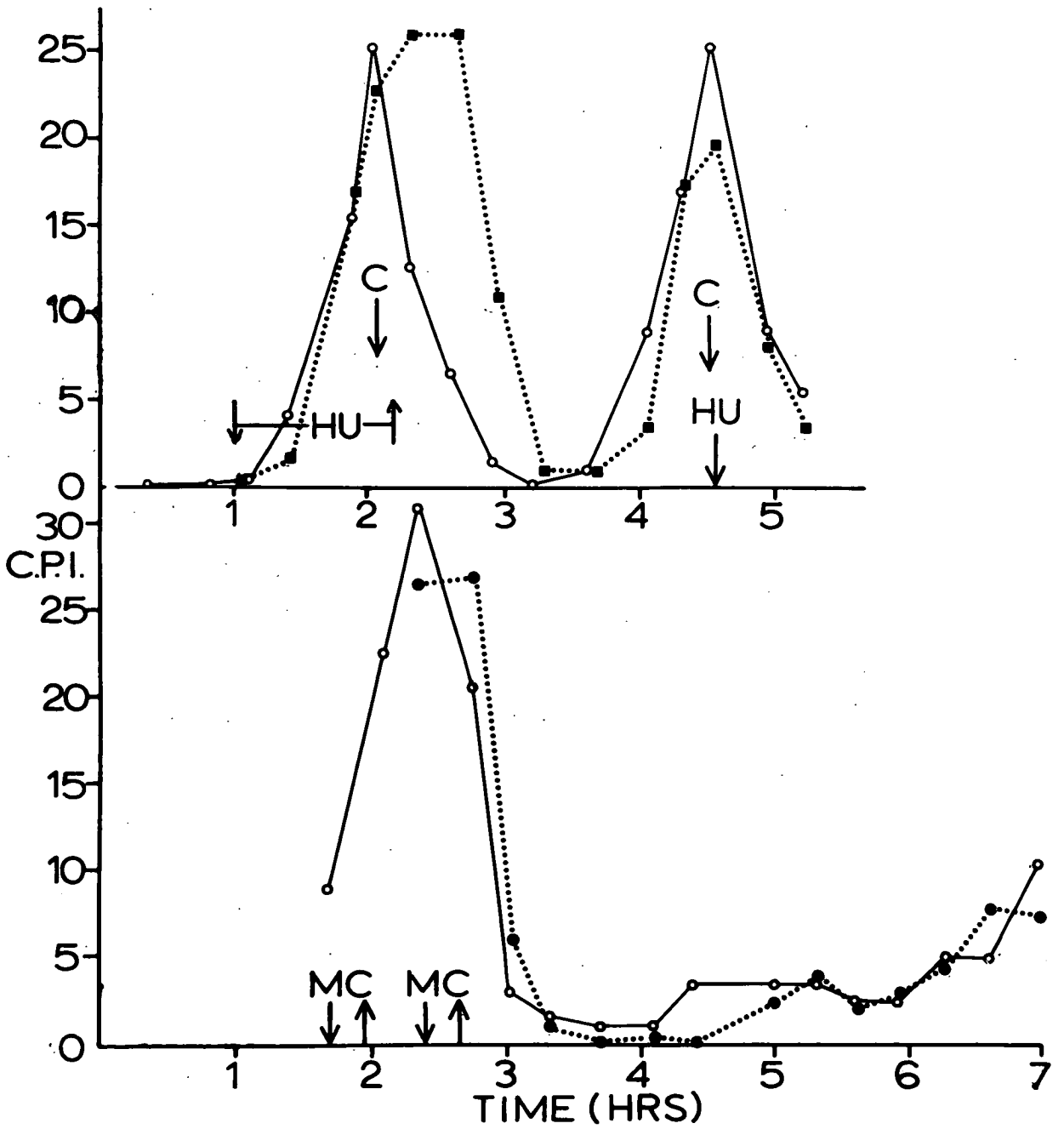
treated with AdR was possibly not complete for there was a notice-
able slow rise in bulk culture DNA during the period of inhibition.
This effect could have been caused by either a small proportion of
poorly synchronized cells in the culture, this is always a risk in
the large synchronous cultures used for DNA estimations, or all
the cells in the culture initiating DNA synthesis and undergoing
synthesis at a highly reduced rate. The danger was thus that the
AdR affected cycle did not have a protracted G_1 phase but a much
extended S period.

In an attempt to resolve this question it was decided
to investigate the relationship between DNA synthesis and sensi-
tivity to MC induced delay. This question was also relevant
since Gill suggested that his period of high sensitivity to UV
induced delay might correspond to the S period. This prediction
did not prove correct for logarithmically growing cells but his
results with cells synchronized by reinoculation support this view.

The first experiments were performed with HU: Mitchison
and Creanor found that this inhibitor used at a concentration of
1.1×10^{-2}M completely inhibited DNA synthesis when added to a
synchronous culture one hour after inoculation (personal communi-
cation). Figure 36. shows the result of a short pulse HU on a
synchronous culture and the effect of two MC pulses upon the HU
treated culture. One result of HU treatment is immediately
noticeable, the broadening effect upon the cell plate peak. This
is due to an inhibition of growth by HU causing the cells to spend
longer in the cell plate phase. There is, however, no effect with
this length of pulse treatment on the timing of the second division,
which indicates that this effect is not unduly severe. The two
MC pulses both give CPI profiles which are typical of cells in the

FIGURE 37. - The effect of a pulse of MC at around the
S period upon synchronous cells treated with
HU compared to its effect upon similar untreated
synchronous cells.

Open circles, solid line - Control CPI.

Closed circles, dotted line - HU treated culture CPI.

HU pulse was from 1 hour 2 minutes to 2 hours 17 minutes.

In both cases the MC pulse position was 0.88 and the

second cycle lasted 145 minutes.

Both these cultures were observed by time lapse photomicroscopy.

Control - thin line and open triangles represent increase in
numbers due to the second synchronous division
(i.e. the delayed division). The open squares
represent the total rise in all numbers in the
observed field. The closed triangles and thick
line represent the same as the above for the HU
treated culture.

The delays were 250 minutes and 234 minutes for control and
treated cultures respectively.
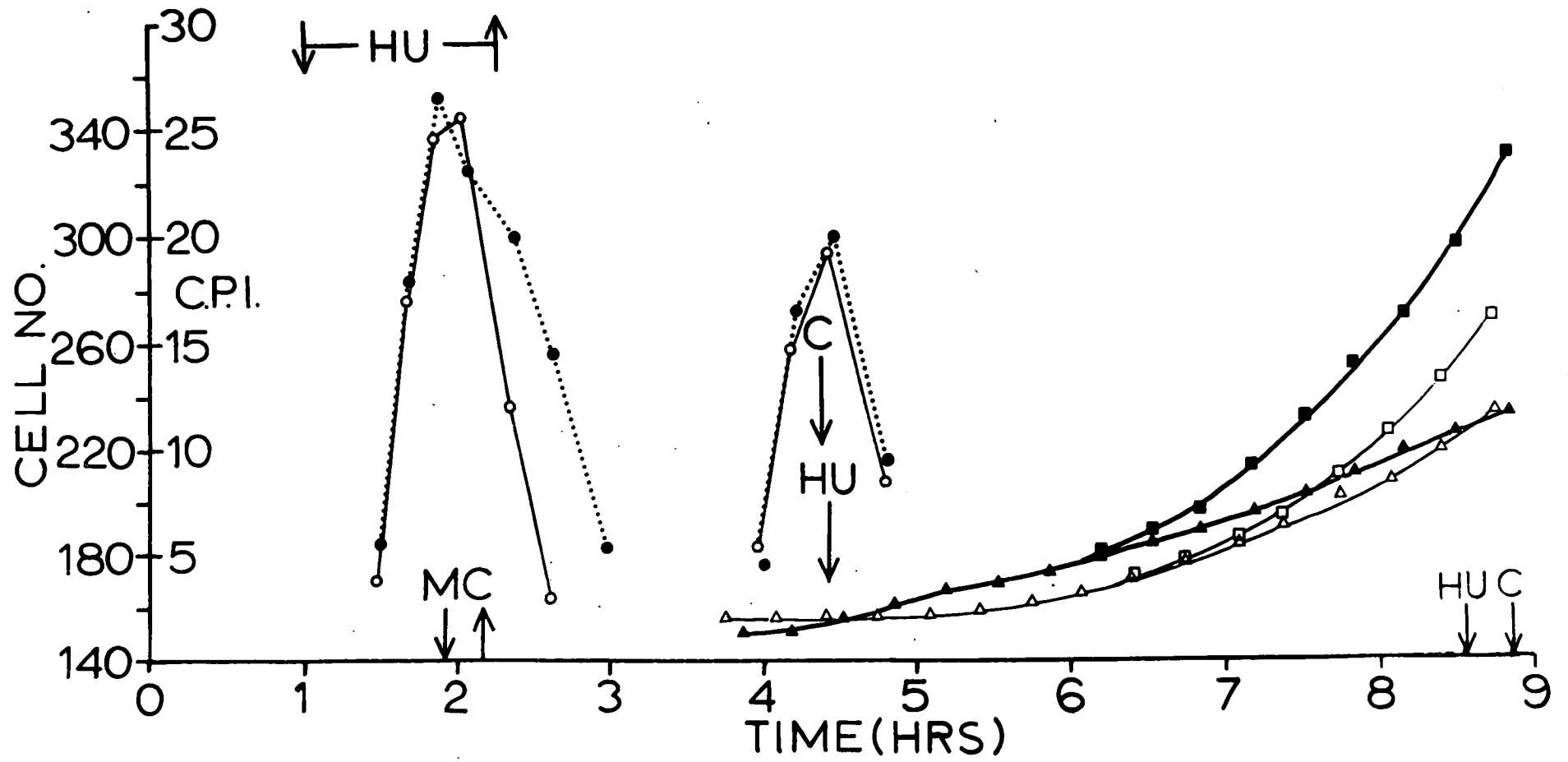
major period, showing that as with AdR the second transition point has been delayed.

Having established that a pulse of HU gave results which are comparable with those obtained with AdR the experiment shown in Figure 37. was performed. The aim of the experiment was to compare the delay caused by a MC pulse made at the time when the maximum number of cells would be in the S period with the delay caused by a pulse at the same time upon cells which had had their DNA synthesis inhibited by treatment with HU. The delays produced were measured by time lapse photomicroscopy and clearly both pulses give highly similar delays, the figures being 234 minutes for the treated culture and 250 minutes for the control culture.

Only a tentative conclusion is possible from this result which is that the inhibition of DNA synthesis does not unduly alter the sensitivity of the cells. Apart from the difficulty of interpreting photomicroscope data mentioned in Chapter 1. another weakness of this experiment is that as the duration of the S period in S.pombe is unknown there is also uncertainty as to the proportion of cells which are actually in the S period during the fifteen minute pulse; if the S period is very short this proportion may be very small. This uncertainty could have been reduced if the degree of synchrony of the culture had been greater; there is no means of doing this with the conventional density gradient method of producing synchronous cultures but S.pombe can be induced to undergo a particularly synchronous round of DNA synthesis by exploiting the reinoculation system developed by Bostock.

Bostock showed that if a four day old stationary phase culture grown in the low phosphate medium EMM I is reinoculated into fresh medium there is an exceptionally synchronous burst

FIGURE 38. - The effect of four MC pulses upon a culture
reinoculated from stationary phase.

The first pulse of MC was applied before inoculation of
the culture. The control growth curve is indicated by the open
circles and heavy line.

Thus the times of the pulses and resultant delays in the
times for a 50% increase in all numbers were :-

(a) pulsed from minus 15 minutes (15 minute 500 μg/ml - open
    circles) delay - 28 minutes.

(b) pulsed at 20 minutes (closed circles) delay - 54 minutes.

(c) pulsed at 52 minutes (open squares) delay - 58 minutes.

(d) pulsed at 1 hour 46 minutes (closed squares) delay - 54 minutes.

The left hand ordinate refers to the open triangles which
show the increase in DNA in the control culture during the first
three hours. (The zero time point was derived from the value
of 1-92 x 10^{-14} g of DNA per stationary phase cell, grown in EM 1.
This value may be found in Bostock '68).

N.B. The low phosphate medium EM 1 was used for this experiment.
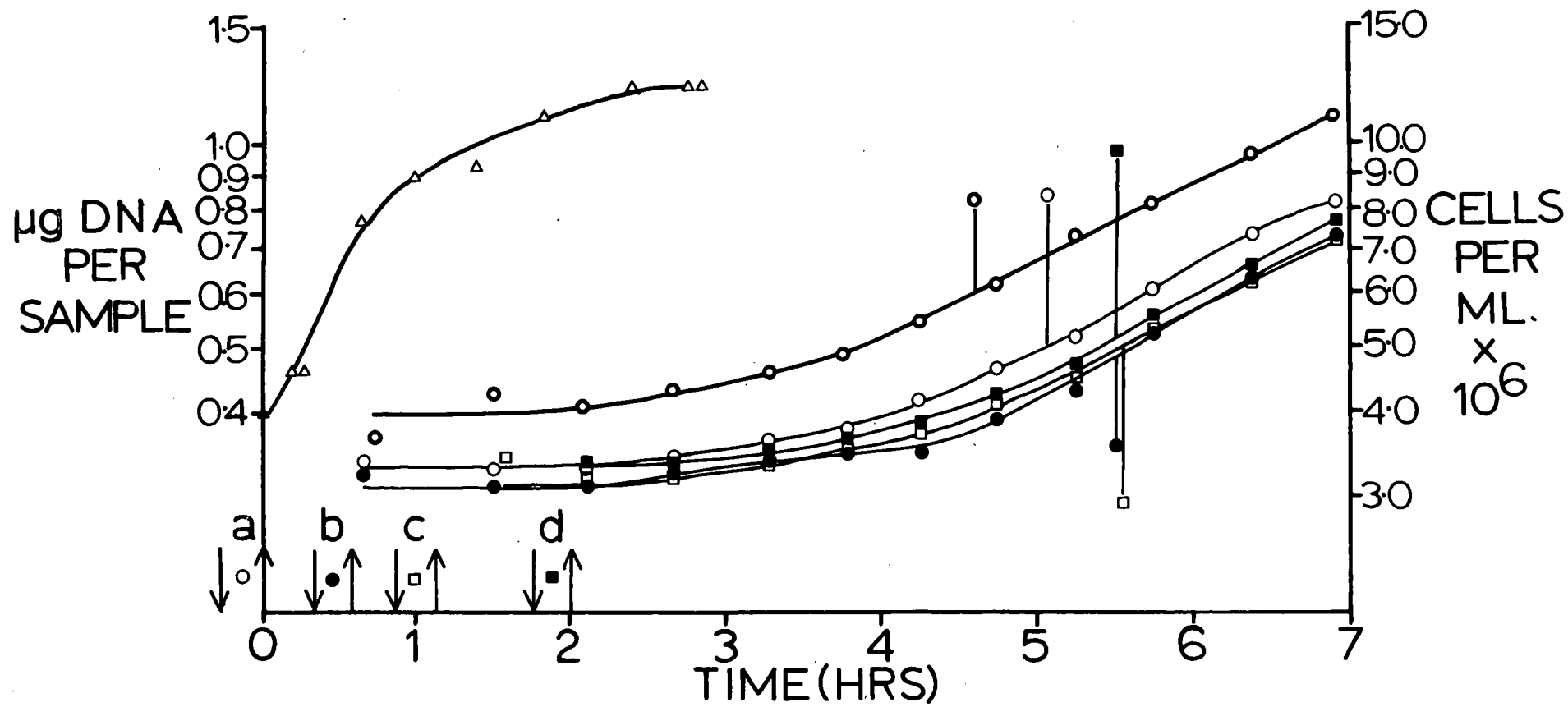
of DNA synthesis and that after a lag averaging four to five hours
the cells divide with a low degree of synchrony.

Figure 38. shows such a culture and the result of making
four pulse treatments with MC on it, the first pulse being adminis-
tered before the cells were inoculated. Also shown is the change
in bulk culture DNA with time for the control culture. The
vertical bars to the curves of cell numbers indicate the points
at which the individual cultures show a half doubling and are
used to calculate the delays. As can be seen the delays are
relatively modest relative to those induced in synchronous
logarithmic phase cultures and although the second pulse (labelled
b.) was made at the time at which DNA synthesis reached its maxi-
mum rate no high sensitivity is evident and the delay differs
little from that caused by subsequent pulses. To a certain extent
the same criticism applies to this result as to the time lapse
photography data in that the delays are calculated by scoring the
division of only half the cells; hence the calculated delays may
be a considerable underestimate of the mean delay. However, the
rise of cell number in the treated cultures parallels the increase
in the control up to the first doubling of cell number and this
does not suggest a hidden sensitive fraction in the pulsed cultures.

This result contrasts markedly with the findings of
Gill who showed that if cells were irradiated with low doses of
UV light at different times after reinoculation there was a marked
peak of sensitivity to delay associated with the first 30 minutes
of culture growth, the period of maximum DNA synthesis. The
result is similar, however, in that the absolute sensitivity of
the cells was less than that seen in normal logarithmic phase cells,
the maximum delay being in the order of 100 minutes whilst the

logarithmically growing cells suffered maximum delays of 250 minutes when irradiated in their major period with the same dose.

Once again this result has failed to show any effect of DNA synthesis upon the sensitivity of the cells, but interpretation of this result would, like the study of the dose delay relationship, greatly benefit from additional information on the uptake of the drug since it is quite possible that there are changes in permeability during the physiological transition from the resting state to logarithmic growth which change the effective dose received.

The major findings of this chapter may be summarised thus.

1. AdR may be used to inhibit the synthesis of DNA and thus to change the relative lengths of the G_1 and G_2 phases of the cycle without the introduction of any significant division delay.

2. MC pulses on such a distorted cycle show that the major period of sensitivity is extended in exactly the same way as the G_1 phase of the cycle and the results are in full agreement with the idea that the changes in sensitivity to delay are correlated with the events of the chromosome replication cycle.

3. Using HU treated cultures and cultures in which DNA synthesis is naturally synchronized it has not been possible to show that cells undergoing DNA synthesis possess any special sensitivity to MC induced delay.

CHAPTER 5.

The effects of heat shocks and short periods of protein synthesis inhibition on the division of S.pombe.

Whilst the last chapter dealt with factors which effect the overall pattern of sensitivity through the cycle this chapter is concerned only with the question of the rising pattern of delay within the minor period. This was of special interest because this pattern of delay is by no means unique to S.pombe; it has been found to be caused in Tetrahymena by a large variety of agents and has been the subject of a great deal of work.

The study of division in Tetrahymena was pioneered by Zeuthen and Thormar and the early work and basic hypothesis which stemmed from it is described by Zeuthen. More recently the whole body of work which grew out of those early experiments has been reviewed by Zeuthen and Rasmussen. The basic finding was that a temperature shock, either hot or cold, or a pulse treatment with inhibitors of protein synthesis or treatment for a short duration with various other agents could induce a division delay by a phenomenon which Zeuthen named 'set back'. This name was given to the process because the induced delays rose with cell age in a way which suggested that treated cells had to repeat their preparations for cell division; the cells were set back to the beginning of their cycle.

It was to account for the 'set back' phenomenon that Zeuthen propounded the 'division protein hypothesis' which, put very simply suggests that the increasing delay is caused by the accumulation through the cycle of a labile protein which has to reach a critical level for division to occur, treatment with any one of a number of agents causes the inactivation of this protein

and it is the time required for its resynthesis which explains the delay.

One of the most striking features of the 'set back' phenomenon is the wide variety of treatments which cause it; apart from the two main treatments mentioned above azide, fluoracetate, high hydrostatic pressure, mercaptoethanol and anaerobiosis can all cause 'set back' to some extent. The question thus arises, is it possible that MC could act in a similar manner? Bulk protein synthesis appears to be unaffected by MC, but it is not impossible that MC could interfere with the metabolism of a specific protein by an indirect mechanism such as the alkylation of a long-lived messenger RNA for example. Additionally the experiments necessary to answer the above question would also provide evidence as to the wider applicability of the division protein hypothesis which is an interesting question in its own right. Finally, the preliminary experiments were also necessary to study the effects of inhibition of protein synthesis on the repair of MC damage prior to the delayed division.

The two treatments which were studied were the two which have received by far the most attention from the Tetrahymena workers, heat shocks and a pulse treatment of a protein synthesis inhibitor.

Fortunately, when starting the work on the effect of a temperature shock some information was available from an earlier study of the effects of fluctuating temperature by Harnden who had attempted to synchronize S.pombe by this means. Harnden had tried various regimes and had enjoyed some success but had never managed to achieve a useful degree of synchrony by this method. However, he had identified the highest sub-lethal temperature at which S.pombe may be cultured which was 41.5°C.

FIGURE 39. - The division delay caused by various

35 minute heat shocks around mid-cycle.

The open and closed circles indicate two different synchronous

experiments (see Figure 40 for typical experiment).

Open circles - shock position (calculated from mid-point

of shock) - 0.55.

Closed circles - shock position 0.58.
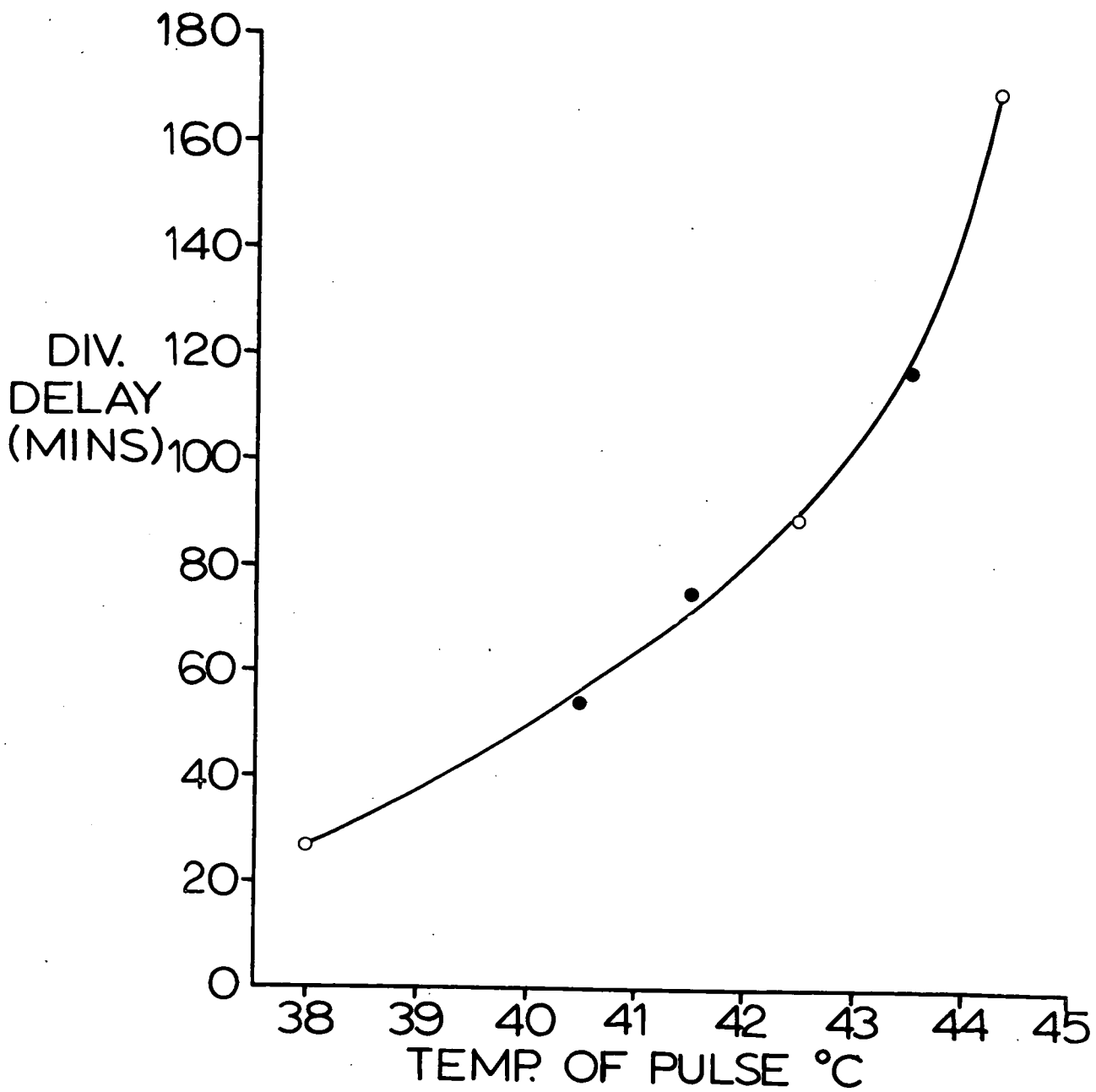
FIGURE 40. – Two experiments showing the effects seen
when a synchronous culture was exposed to
a heat shock of 40.5°C for 25 minutes at
varying points in the cycle.

Top graph – Open circles – control.
Closed circles and dotted line – culture shocked
at 0.22. This culture shows the peak sharpen-
ing effect of a heat shock in the beginning of
the cycle.

Bottom graph – Open circles – control.
Closed circles and dotted line – culture shocked
at 0.93. The failure of the CPI to drop in the
shocked culture was due to the effect of the
heat shock on fission, some 10% of the cells
were arrested in the cell plate phase.
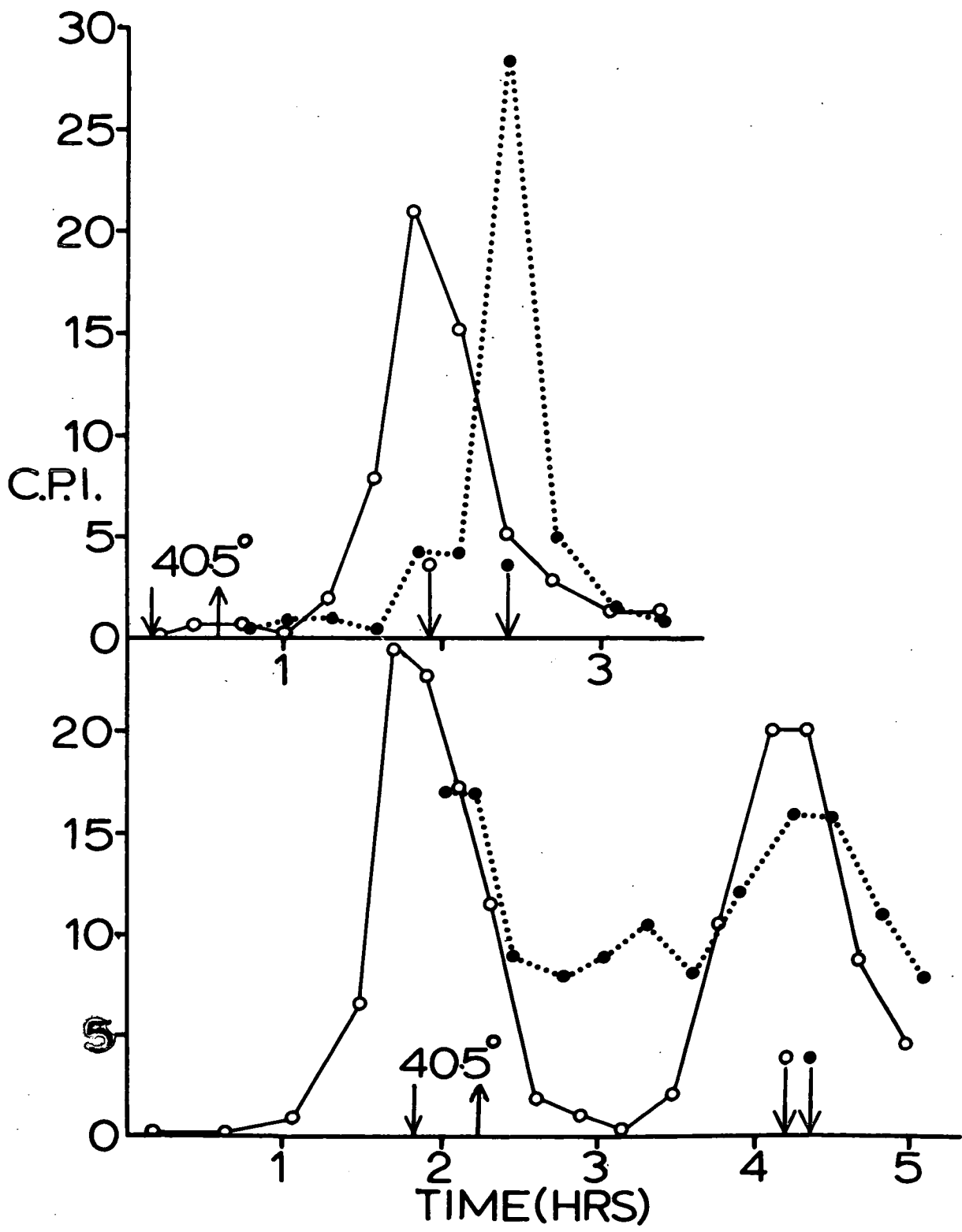
FIGURE 41. -   The relationship between the cycle position,

heat shock and induced division delay for a

25 minute 40.5°C shock.

The closed squares show first division delay (i.e. in the same

cycle as the shock), and the open circle indicates a second

division delay (in the cycle after the shock).

TP indicates the transition point from first to second division

delay.

Xs delay was excess delay which was total delay minus the shock

period.
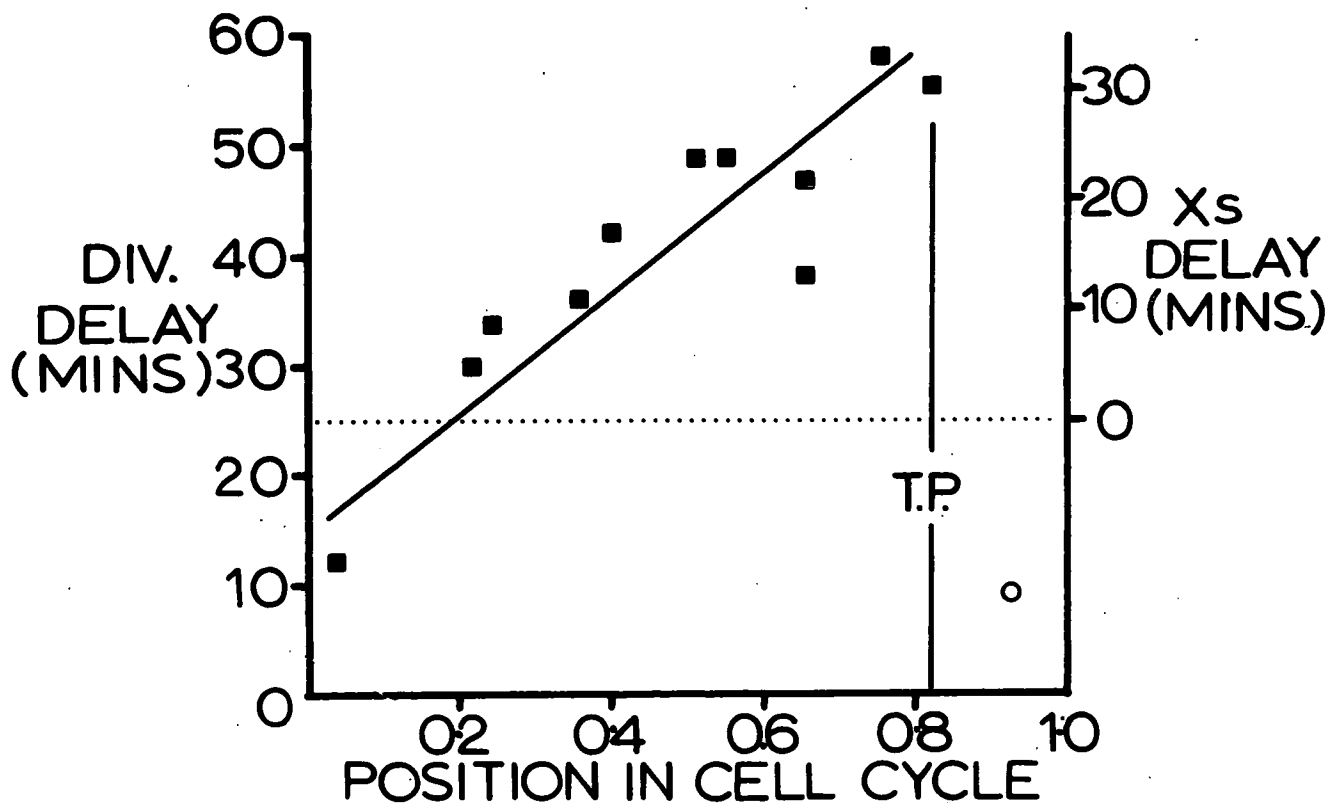
The cycle sensitivity to heat shock was investigated by heat shocks of 25 or 35 minutes duration with the warming up period, which was approximately 5 minutes, included in this period. Figure 39. shows the result of the first experiment which was performed to select a suitable temperature for the investigation of the whole cycle. 35 minute heat shocks were applied to a synchronous culture with the mid-point of the pulse (heat shocks positions are defined by the time at mid-pulse) just after mid-cycle. The delays produced were uniform and, as may be seen, increase sharply with increasing temperature. The data in fact would have been better plotted on a semi-logarithmic plot as it gives a straight line when treated in this way.

This one result alone is at variance with the Tetrahymena situation for the temperature induced delay which produces set back is not highly temperature dependent; I shall return to this point. One effect noticeable in these cultures is that significant lethality, as judged by the appearance of highly refractile cells, occurred at temperatures in excess of $40.5^{\circ}C$. It was interesting that these dead cells did not appear after the pulse but only after the first division. In view of this lethality a shock temperature of $40.5^{\circ}C$ was selected and to assist the spacing of the shocks a standard shock length of 25 minutes was adopted.

Two of the effects observed when the shock was applied at various positions through the cell cycle can be seen in Figure 40. whilst the collected results of three such experiments each measuring delay at four separate cycle positions may be seen in Figure 41. The early shock in Figure 40. shows the typical effect seen when the cells are shocked in the first half of the cycle, a considerable sharpening of the cell plate peak which suggests that

the cells have become more synchronous. The later shock at the end
of the cycle shows another effect, a failure of the cell plate
index to fall towards the baseline after division of the control
due to the fact that a sizable proportion of the cells fail to
undergo fission but remain fixed in the cell plate phase. This
effect was especially pronounced in cultures treated between 0.6
to 0.9 and the resultant distortion of the cell plate peaks made
the delays produced very difficult to estimate. This effect of
heat treatment was also noted by Harnden; the cells so afflicted
are able to undergo subsequent divisions but give rise to only
three 'cells' on division, the innermost daughters remaining
paired.

This effect upon the cell plate index must be borne in
mind when considering the collected results. The position of the
transition point is only tentative and would probably be a little
earlier if entry into the cell plate phase was considered the end
of the cycle. However, this uncertainty only really affects the
estimates at 0.75 and 0.82 and leaves the overall pattern of sen-
sitivity reasonably clear; once more it is an increasing pattern
through the cycle with a gentle slope and a maximum delay of about
50 to 60 minutes, and again there is a transition point at the end
of the cycle.

The rising pattern of delay and the sharpening effect of
an early pulse on the cell plate peak of a synchronous culture
are two results which are in excellent agreement with one another
since this pattern of delay will predictably have the effect of
differentially delaying the older cells and will tend to align the
time of division in cells of different ages. The effect is probably
too weak to allow an appreciable synchronization of a synchronous

culture with a single shock, but it may be that reinforcement
of synchrony can be achieved if the correct spacing of the shocks
can be found. If this is so synchronous cultures of S.pombe may
perhaps be produced by this method just as they are in Tetrahymena;
one complication would be the accumulation of cells blocked in the
cell plate phase but these cells may disappear by dilution or may-
be another strain could be found which lacks this response.

One very interesting possibility to be raised by the
discovery of this effect is that it may be possible to maintain
the synchrony of a synchronous culture with one heat shock per
cycle. If this is indeed possible it could lead the way to the
production of giant synchronous cultures especially if resort
can be made to continuous culture techniques. Whether the culture
could be made to lock on to a cycle of shocks is, however, doubtful.
Multiple shocks in Tetrahymena lead to adaptation and to division
at the shock temperature (Zeuthen) but in that case the shock
serves to completely delay division not merely to align it. The
real question is whether the sensitivity to heat shock cycle is
entrained to the cell division cycle, if it is all may be well,
if not the synchrony will be steadily lost. The preliminary ex-
periments to test which is the case are relatively easy and cer-
tainly the prospect of large quantities of synchronous cells is a
very tempting goal.

Viewed in terms of the division protein hypothesis the
two results above are sharply conflicting. One of the corner
stones of the division protein hypothesis is that it resembles
an all or nothing effect; (Zeuthen) varying the temperature of
the shock has little effect upon the delay produced since the
shock only serves to discharge the pool of division protein; the

delay is fixed being the time for resynthesis. The rate increasing
curve which relates delay to shock temperature in S.pombe is thus
the exact opposite of the relationship predicted by the division
protein hypothesis. However, the pattern of sensitivity is roughly
in line with the theory as is the effect on the degree of synchrony.
I shall return again to this point later.

The next question to be investigated was the effect of a
short period of protein synthesis inhibition. The choice of in-
hibitor to use was fortunately quite simple as the antibiotic
cycloheximide (CHX) had been shown by the work of both Stebbing
('69) and of Mitchison and Creanor ('69) to be a rapidly acting
and effective inhibitor of protein synthesis in S.pombe. The
properties of CHX as an inhibitor have been reviewed by Sisler and
Siegel and the following points emerge as important. CHX acts
in some but not all fungi (some being CHX resistant) as a rapidly
acting inhibitor of protein synthesis and is active both 'in vivo'
and 'in vitro'. Its action is reversible and protein synthesis
resumes after CHX has been washed out of the culture. The exact
mode of action of the drug is unknown, but it is thought to act by
blocking the transfer of amino-acid sRNA's into polypeptides.
It does not inhibit the synthesis of RNA but RNA metabolism is
affected and this has been studied in S.pombe by Taber and Vincent.
DNA synthesis is inhibited by CHX but this effect only manifests
itself 'in vivo' and may well be a secondary consequence of protein
synthesis inhibition.

The concentration of CHX chosen at the time that these
experiments were performed was 100 μg/ml CHX as this concentration
had been shown by Mitchison and Creanor (personal communication)
to have had the effect of reducing C^{14} leucine incorporation to

FIGURE 42. - The effect of various lengths of treatment

with 100 μg/ml cycloheximide upon division

in an asynchronous culture.

Graph A :- control.

Graph B :- Culture continuously treated with 100 μg/ml

cycloheximide from time 0 minutes.

Graph C :- Cycloheximide withdrawn after 20 minutes.

Graph D :-   "   "   "   30 minutes.

Graph E :-   "   "   "   40 minutes.
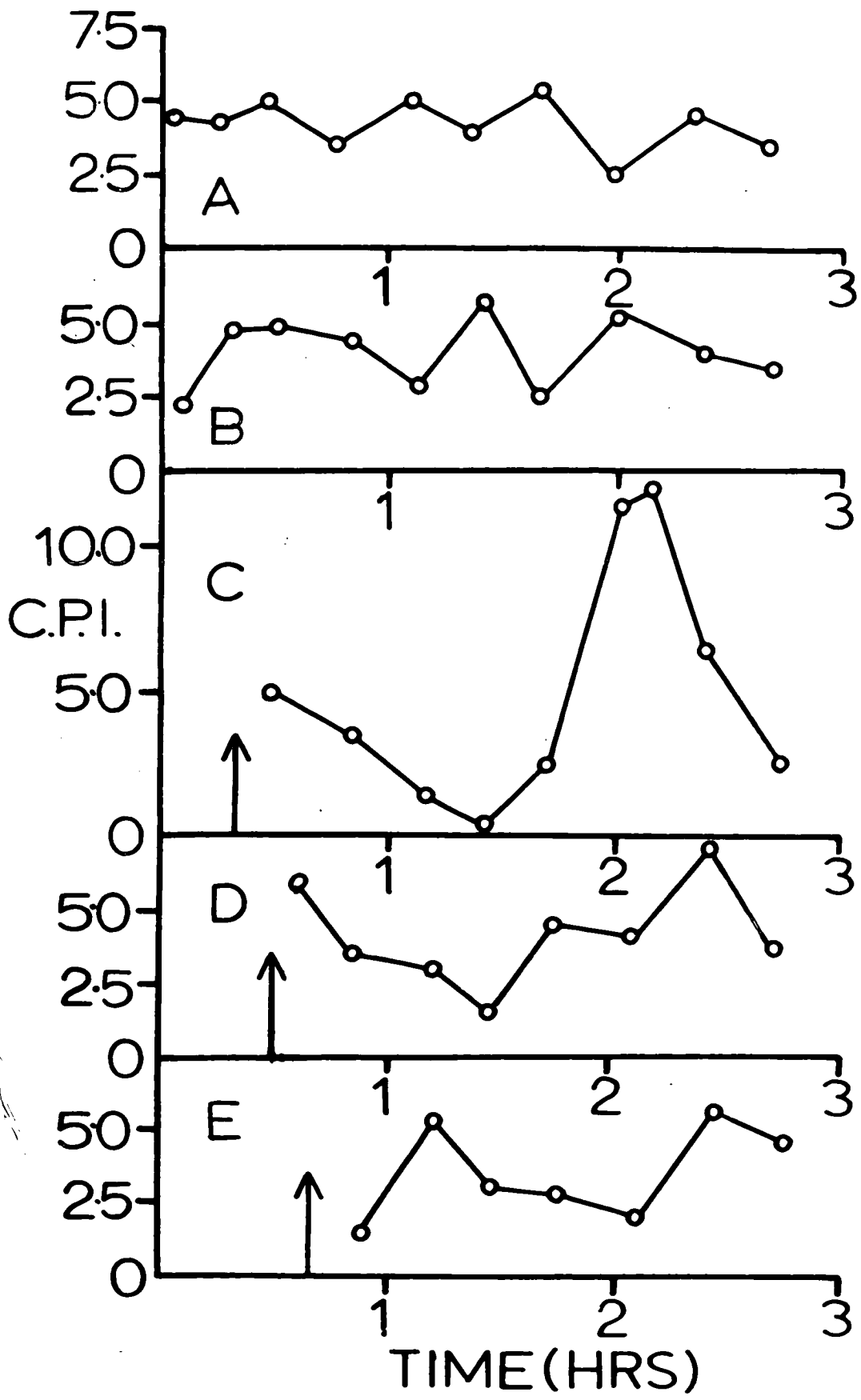
about 8% of the control value in about 10 minutes. Subsequent
work in this laboratory has shown that the same effect could have
been achieved by using much lower concentrations but that the
effects of 100 µg/ml are quite rapidly reversible (H.Polanshek,
personal communication).

The preliminary experiment shown in Figure 42. was then
performed to decide the optimum length of pulse to use and to see
if a single pulse of CHX could produce any synchronising effect,
which would suggest a rising pattern of delay. Clearly graph C
in Figure 42. shows that a 20 minute pulse of CHX does in fact
produce a fair peak in the cell plate index, hence this length of
pulse was adopted in the subsequent experiments with synchronous
cultures.

Figure 43. shows the effect of a CHX pulse of 100 µg/ml
for 20 minutes upon a synchronous culture; as in the case of the
heat shocks, the pulse position is calculated from the mid-point
of the pulse in view of the fact that the inhibitor takes about
10 minutes to exert its full effect. Three features of the data
are immediately evident. Firstly that a pulse just after mid-cycle
causes a delay of about an hour in the first division. Secondly
that once again the sharpening effect upon the synchrony of the
first division has occurred and finally that there is clear evi-
dence of a transition point between the two pulses shown at 0.59
and 0.69 which is not obscured by any effect upon cell fission.

Figure 44. shows the collected data from six separate
experiments of the type shown in the previous figure. The cell
cycle appears to be divided into three distinct regions; from the
beginning of the cycle to about 0.35 there is a period in which the
cells suffer little excess delay. Excess delay is a term used by
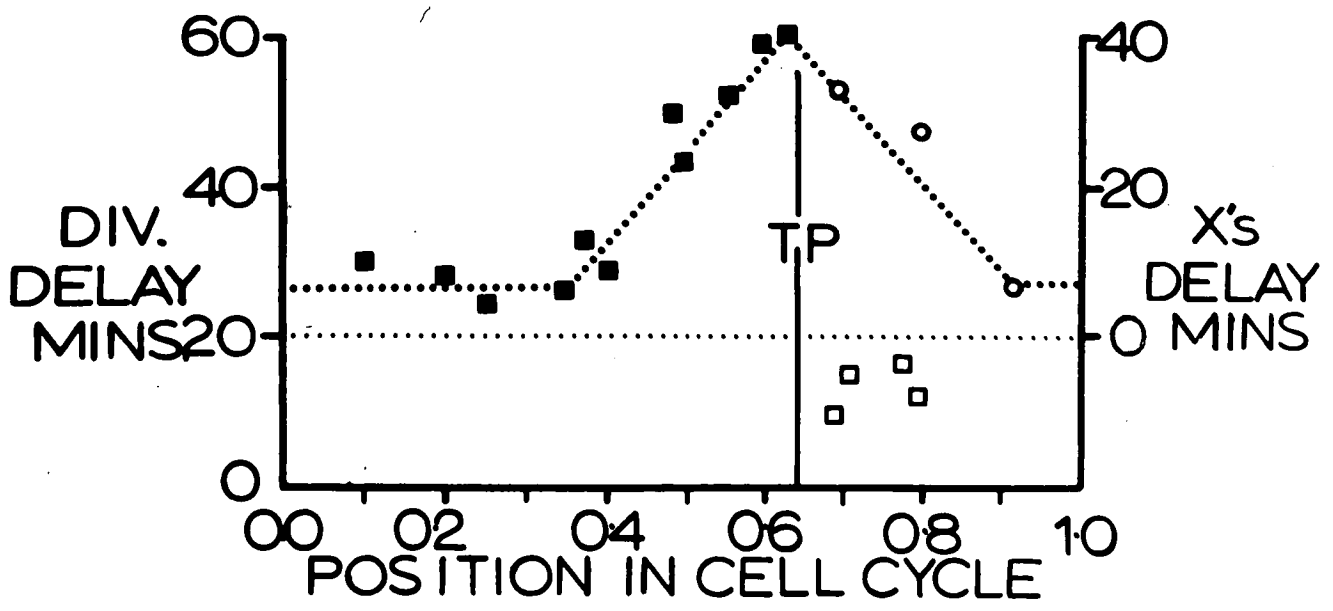
FIGURE 44. - The relationship between induced division delay and the position in the cell cycle of a 100 µg/ml 20 minute CHX pulse.

Closed squares - first division delay.

Open squares - very short first division delays induced after the transition to second division delay (TP) which were thought to be due to a lengthening of the cell plate phase.

Open circles - second division delays.
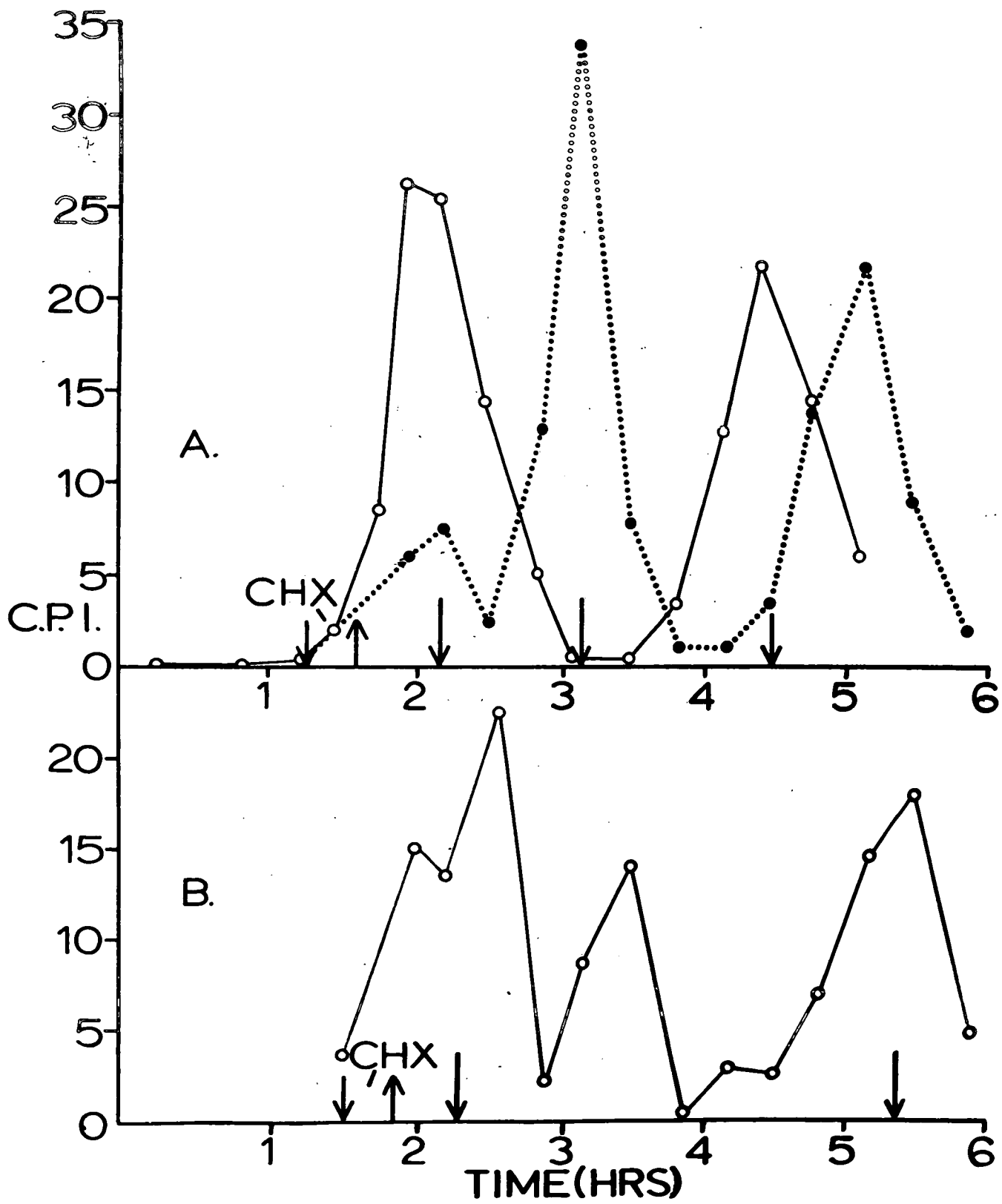
FIGURE 43. — The effect of two 100 Hg/ml pulses of CHX
upon division in a synchronous culture.

Graph A :— Open circles — control.

Closed circles and dotted line — culture pulsed
at 0.59. (There was a peak sharpening effect
similar to that seen with a heat shock).

Graph B :— CHX pulse at 0.69. There was a slight first
division delay (probably due to a slight
lengthening of the time spent in the cell plate
phase) and a small peak due to cells which had
not passed their transition point at the time
of the pulse. The second division was con-
siderably delayed for all cells.

the Tetrahymena workers to describe delay which is in excess of
the length of the pulse treatment; it is assumed that cells cannot
be expected to progress towards division during the actual pulse
and thus excess delay is a better measure of the true delay.
During the next phase of the cycle there is a rising pattern of
delay with cell age and doubtless it is this which explains the
synchronizing effect of a 20 minute pulse of CHX on a synchronous
culture. At approximately 0.64 of a cycle the cells undergo a
transition and become resistant to long excess delays. After
this point in the cycle the cells appear to suffer very short
delays in the region of 10-15 minutes; this is probably not a real
delay but is due to an elongation of the cell plate phase which
broadens the cell plate peak and makes the mid-position of the
peak slightly later. There does, however, appear to be an effect
on the second division when the cells are treated after the
transition point, but more data is needed before anything definite
can be said of this, especially as an effect during this part of
the cycle may be due to an interference with DNA synthesis. It
has been shown by <u>Cummins and Rusch</u> for example that in Physarum
protein synthesis is essential for a complete round of DNA syn-
thesis to occur.

One more observation was made upon CHX delayed cells and
that was to measure the size of the delayed cells at division. In
Tetrahymena general protein synthesis is not affected to any great
extent by the heat treatments which produce 'set back' and the
cells synchronized by multiple heat shocks are consequently quite
significantly enlarged. The size at division was measured on two
types of culture. A synchronous culture treated with a 20 minute
pulse was observed and a result very similar to that seen in

Figure 42. was obtained; the sample for length measurements was taken from the cell plate peak and a sample was taken from the control at the same time. The other comparison was made between synchronous cells pulsed at 0.59 (as shown in Figure 43.) and the control cells. In both cases the mean lengths of the treated cells in the cell plate phase was higher than the control by 5% in the asynchronous experiment and by 9% in the synchronous experiment. Both of these differences are significant at the 0.5% level when tested by Bailey's modification of student's 't' test. This observation suggests that the cells are not delayed because of a general effect upon growth which prevents the cells from attaining a critical size for division but that the effect which causes delay is upon a specific system involved directly or indirectly in the control of division.

The results above are reasonably consistent with the division protein hypothesis if one proviso is accepted and that is that the synthesis of the protein is restricted to only that part of the cycle from 0.35 to 0.65 during which the delay rises with cell age. These results with others thus raise the question of the wider applicability of the division protein hypothesis. This question has recently received attention from Mitchison ('71) who has produced several different models to account for the effects of heat shocks and pulses of division inhibitors upon various systems. I shall return to this topic in the forthcoming discussion.

Returning to the question of whether MC might act in the same way as heat and CHX by an interference with protein metabolism comparison of the results with all three inhibitors allows two conclusions. Firstly, that there is a broad similarity in the

findings with all three agents since all cause a variable age
dependent delay during the first part of the cell cycle, and
secondly that MC differs from heat and CHX in that it does not
cause a sharpening of synchrony.

The failure of MC to sharpen the cell plate peak is
probably due to the gradient of the delay against cell age curve
being steeper than is found with heat or CHX. Consideration of
the 'set back' model shows that at any given cell age the delay
resulting from a single treatment is equal to the time from zero
sensitivity to the transition point minus the interval between
the cell age and the transition point. There is only one variable
which is the interval between the cell age and the transition
point, and this is clearly related in a linear fashion to cell
age. Thus delay is also a linear function of cell age. The model
also predicts that slope of the graph of delay against cell age
will be 45° in its rising portion. If, for example, cells are
set back by say 100 minutes in a cycle of length 150 minutes with
a transition point at 120 minutes the cells will start to be delay
sensitive when aged 20 minutes and delay will increase in a linear
manner with cell age until cells aged 120 minutes will suffer a
100 minute delay; hence from age 20 minutes there is a one to one
increase in increment in age and increment in delay. Cells aged
from 20 to 120 minutes all divide at the same time, and thus if an
asynchronous culture of these hypothetical cells is treated some
measure of synchrony will result. However, this synchrony will
only occur if the age dependent delay rises in a one to one
manner with cell age; the nearer the approach to this relation-
ship the better will be the synchronizing effect of a single pulse.

Reference to Figures 8, 41 and 44 for MC, heat shock and

FIGURE 45. -   The effect of a pulse of cycloheximide upon

a culture delayed by an MC pulse in the minor

period.

Top graph :-   Untreated control.

1st down   -   Control treated with 660 μg/ml pulse of MC

at 0.39; delay 110 minutes.

2nd down   -   100 μg/ml CHX pulsed with mid-point at

1 hour 5 minutes upon MC treated culture;

delay - 108 minutes.

3rd down   -   As above, but CHX mid-point at 1 hour

50 minutes; delay - 112 minutes.

Bottom graph:-   As above but CHX mid-point at 2 hours

15 minutes; delay - 115 minutes.

C.P.I.

MC

CHX

CHX

CHX

TIME in hours
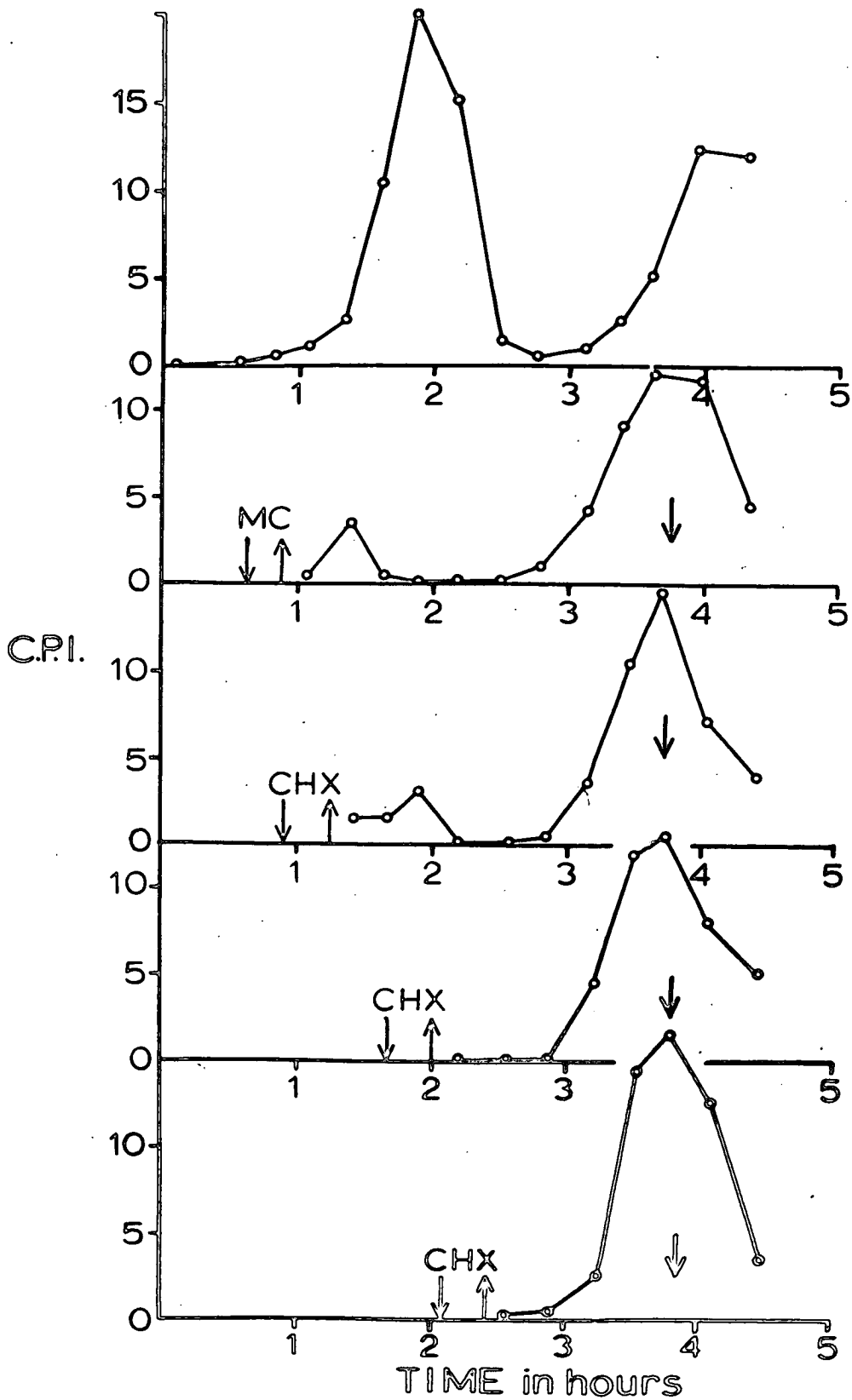
FIGURE 46. - The effect of a heat shock upon a culture
delayed by an MC pulse in the minor period
of sensitivity.

Top graph :- Open circles - control.
Closed circles and dotted line - MC treated.
Control culture (660 μg/ml pulse for 15 minutes
at 0.31). Delay - 74 minutes.

Upper middle
graph :- The effect of a 40.5°C 25 minute heat shock
mid-point 1 hour 8 minutes upon the MC treated
culture. Delay (relative to completely un-
treated control) - 105 minutes.

Lower middle
graph :- As above, shock mid-point is at 1 hour
53 minutes. Delay - 106 minutes.

Bottom graph:- As above, shock mid-point is at 2 hours
28 minutes. Delay - 108 minutes.
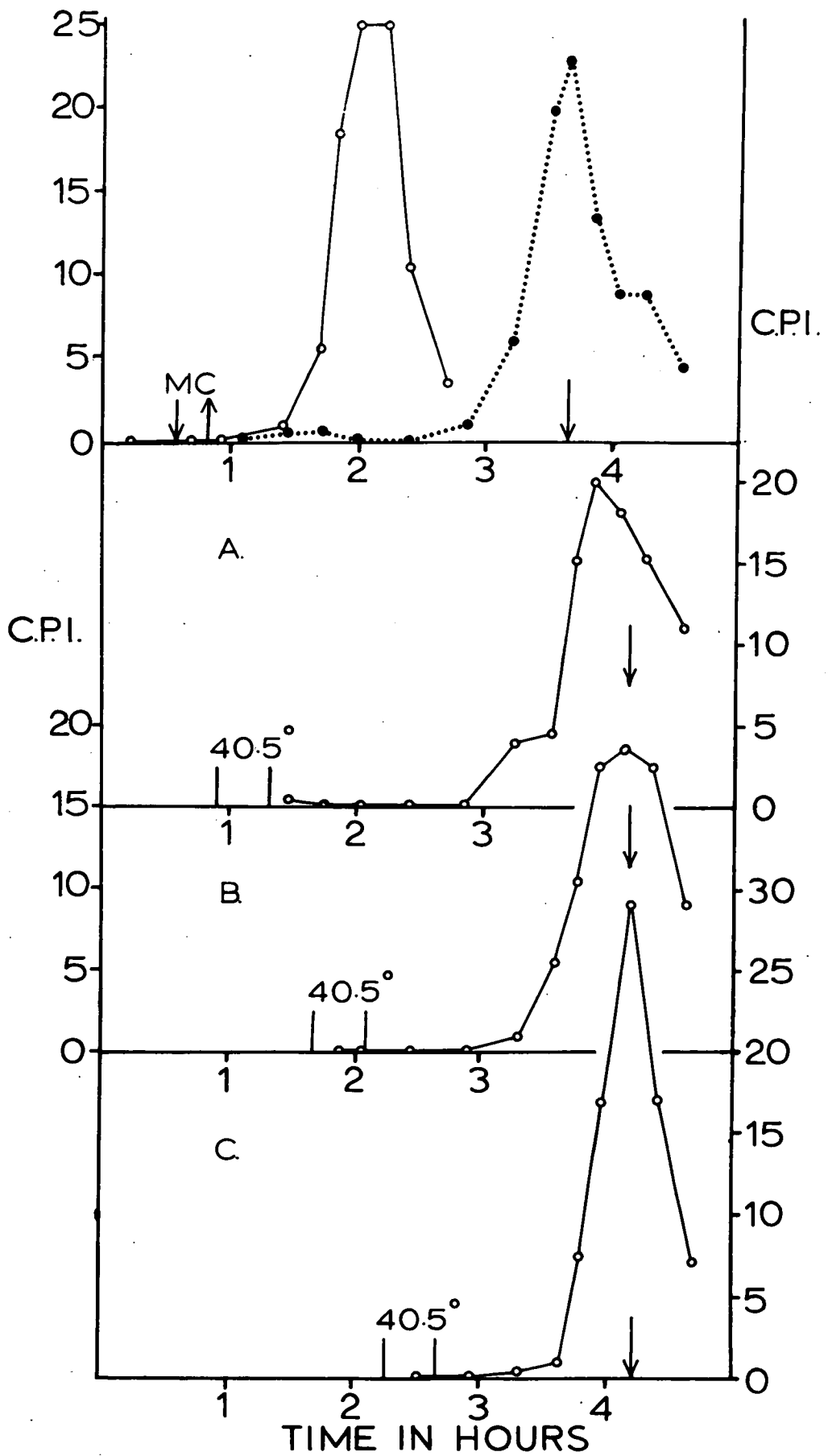
CHX data respectively shows that the gradient of the MC curve is greater than 45°, for heat shocks the gradient is less than 45° and for the ascending portion of the CHX curve the gradient is approximately 45°. However, the heat shock data is not particularly good at the two extremes of the cell cycle and does bear a strong resemblance to the CHX data in mid-cycle where most of the results were achieved. Over the period from 0.2 to 0.6 the excess delay does show a roughly one to one increase with cell age and it was pulses in this part of the cycle that showed the sharpening effect. The fact that the slope of the delay graph for MC is greater than 45° explains why no increased synchrony is seen with MC pulses on synchronous cultures and it also argues against the constant repair time theory to explain this pattern of delay since this theory also predicts a 45° slope (see Mitchison ('71). However, it may be that minor cycle dependent factors influence the delays and modify the sensitivity pattern and it is notable that the delay-cell age relationship in Tetrahymena for temperature shocks is not linear but is a shallow upward curve (see Zeuthen).

Having obtained some idea of the effect of heat shocks and CHX treatment on untreated cells it was possible to test whether there was any similarity in the effects caused by these agents and MC . Figures 45. and 46. show the results of the experiments which were simply performed by treating a MC delayed culture with pulses of CHX and heat shocks.

CHX appears to have no effect at all upon the division of cells inhibited by MC although the results above show that there is no time at all in the normal cycle when division cannot be delayed by CHX treatment. Thus to guard against the unlikely

possibility that MC might confer some type of resistance against CHX the experiment was repeated with exactly the same result and a comparison was made of the cell lengths at division between the MC treated culture and the cultures treated with both agents. The lengths of the CHX treated cells were 12-14% lower than the MC treated control and the differences were all significant at the 0.05% level. Tested against themselves, however, none of the three CHX treated cultures differed significantly at the 10.0% level. Evidently CHX was having its customary effect upon cell growth.

This result is not explainable in terms of the division protein hypothesis at all, in fact it suggests that MC does not affect the synthesis of division protein but that during the MC inhibited period enough division protein is made to not only provide for the coming division but also to counteract any effect of CHX. This idea of excess division protein is also consistent with the shortened second cycles found in Chapter 2. Perhaps the most straightforward conclusion to be made from this result is that the repair of MC damage is not dependent upon continuous protein synthesis.

Whilst CHX does not affect the inhibited cells Figure 46. shows that a heat shock does have an effect, although there is very little excess delay (6,7 and 9 minutes for the three heat shocks in order of lateness). However, the last pulse shows the effect of sharpening the cell plate peak and thus there is evidence that there is variable delay just before the inhibited division. In view of the lack of effect of CHX the simplest conclusion which may be drawn from this result is that the elevated temperature has an inhibitory effect upon the repair of MC damage.

These two results together suggest two further experiments

FIGURE 47. - The effect of a second pulse of MC upon

the division of a synchronous culture

previously delayed with MC.

Open circles and solid line - control.

Closed squares and dotted line - 15 minute 660 μg/ml MC

pulse at 0.22.

Open triangles and dashed line - effect of a second

identical MC pulse, 60 minutes after the first, upon the

treated culture.
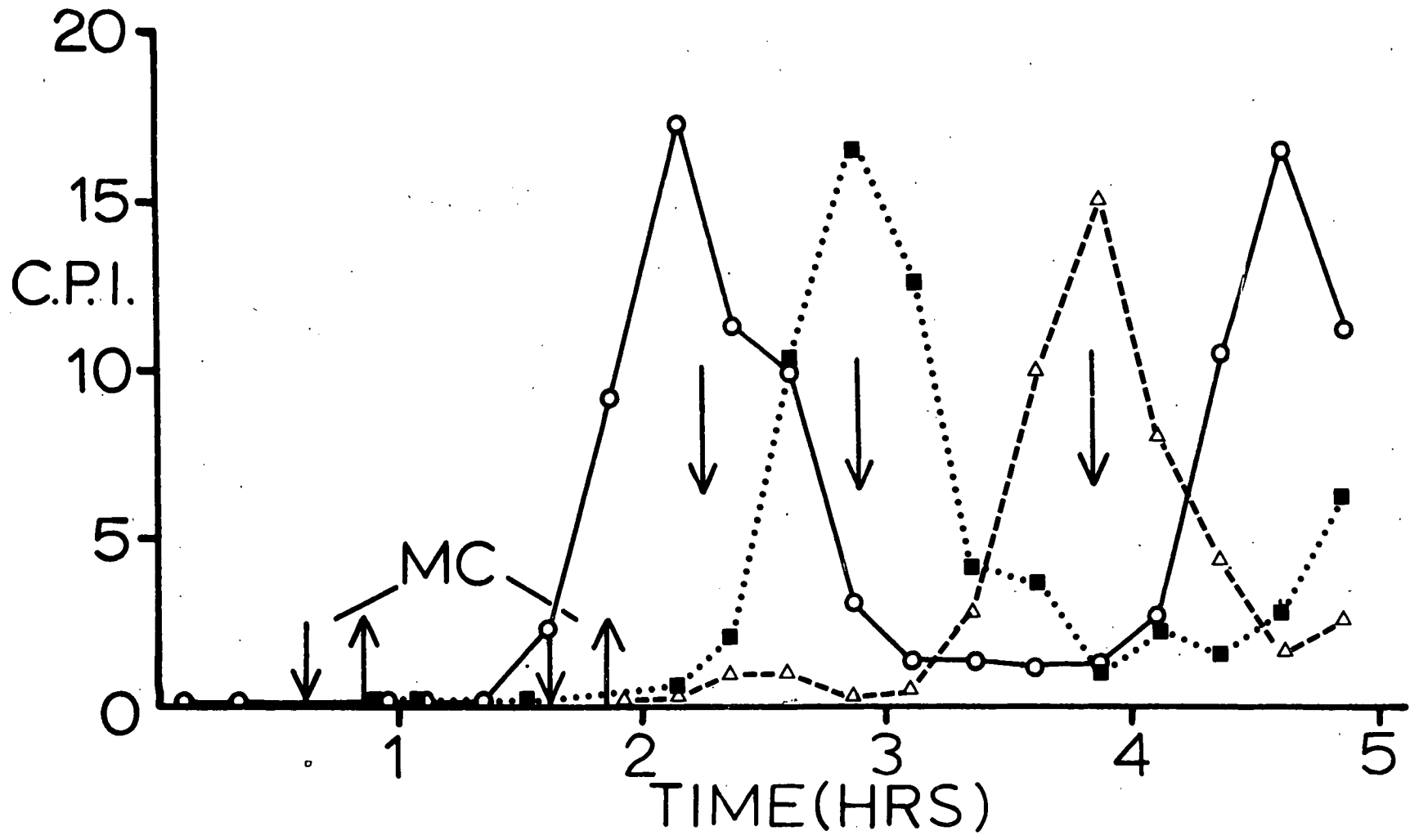
which might produce interesting results. The first is to determine how much further protein synthesis is required for cells delayed by MC treatment to initiate nuclear division and cytokinesis after the normal cell size for cell division has been reached. This could be done by the addition of CHX to the delayed cells at various times after the treatment with MC and following nuclear division and cell plate initiation. The second question is if division is limited in MC treated cells only by the repair of MC damage then the temperature optimum for this process may well be different from the normal growth temperature of $32^{\circ}C$. Thus simply shifting treated cells to a range of temperatures after the MC pulse could possibly result in a shortening of the delay.

The last result in this chapter is shown in Figure 47.; the experiment was performed to test whether cells treated once with MC and presumably in the process of repairing MC damage acquired any resistance to the effects of a second pulse. As can be seen a second pulse is quite effective in producing further delay and there is no reason to suppose that a second pulse is any less effective. This experiment again suggests a sequel which would yield interesting information which would be to plot the sensitivity of the MC inhibited cycle to further MC induced delay. Unfortunately, time did not permit this experiment to be performed.

The major results of this chapter may be summarised thus.
1. Both heat shocks and short pulses of the protein synthesis inhibitor cycloheximide will delay the division of synchronous cells. The cell cycle sensitivities are broadly similar to both agents delay rising with age during first part of the cycle with a transition point at around 0.6.
2. Heat shocks of $40.5^{\circ}C$ have an effect on cell fission and some

cells so treated become blocked in the cell plate phase.

3. The patterns of sensitivity loosely resemble that found in Tetrahymena for both agents and this raises the question of the general applicability of the 'division protein hypothesis' developed to explain the results of these inhibitory treatments on Tetrahymena.

4. Experiments in which both MC and either heat shock CHX were used did not indicate that either treatment shared the same mode of action as MC.

5. MC delayed cells acquire no resistance to further MC induced delay.

CHAPTER 6.

The effect of MC treatment upon enzyme synthesis.

The results described in this chapter illustrate the use of MC in the manner envisaged in the introduction, as a tool to distort the cell cycle. MC has been used in two ways to investigate enzyme synthesis and in both cases has yielded reasonably clear cut and interesting results.

The first use of MC was not by myself but by Robinson (personal communication) in a study of the factors affecting the synthesis of the enzyme alcohol dehydrogenase. In normal synchronous cultures of S.pombe growing in EMM II alcohol dehydrogenase shows a rise in activity, normally interpreted as being due to a synthesis of enzyme, which follows the pattern that Mitchison ('69a) has called 'stepping'. Thus it appears as if the enzyme is synthesized in a periodic manner with synthesis in this case being around mid-cycle.

The fact that enzyme activity rose at mid-cycle made the use of a pulse to distort the cycle inconvenient and thus a continuous treatment of MC was used at a concentration of 100 μg/ml. The drug was added to the culture just after inoculation from the gradient. This had the effect of producing a highly delayed first division which occurred some 5-6 hours after inoculation with a poor degree of synchrony; naturally it also led to a great elongation of the cells. My results suggested that the cells would be arrested in G_2 by this treatment and thus DNA synthesis would also be highly delayed. This was confirmed by DNA assay.

Assaying for the rise of alcohol dehydrogenase in cultures treated as above showed that this treatment had no detectable

effect upon the rise in activity for the first two synchronous cycles, the first two 'steps' in activity occurring as in the control. This result is quite consistent with those found by other workers as was mentioned in the introduction. I shall return to this point in the discussion.

The other way in which MC has been utilised was to test the gene doubling theory put forward by Mitchison and Creanor ('69). This theory arises from the finding that some enzymes which are synthesized in a linear fashion throughout the cell cycle show two types of periodic changes in their rates of synthesis. The first change is found when the basal level of enzyme is measured through the cell cycle; the rise in activity is found to be linear but with periodic doublings in the rate of synthesis. The second type of periodic change is found when synthesis of the enzyme is derepressed at intervals throughout the cell cycle and the resulting rate of synthesis plotted against the time in the cell cycle at which the rate of derepressed enzyme synthesis was measured. This shows that the ability of the cells to make enzyme when challenged (the so-called enzyme potential) increases in a step-wise manner. The gene doubling theory emerges because in bacteria both the rate changes and the steps in potential have been interpreted as gene dosage effects.

However, in S.pombe both these changes happen about 0.2 of a cycle after DNA synthesis (when allowance is made for the delay in the assembly of the enzyme protein from its sub-units,) (see Mitchison and Creanor ('69) and thus Mitchison and Creanor have postulated that they are due to gene dosage effects but that there is a delay between chemical replication (DNA synthesis) and the production of a functional gene which is able to express

itself (functional replication). In the prokaryotes the dosage
explanation of step-wise changes in potential is supported by
experiments in which increases of potential have been shown to be
dependent upon DNA replication (Donachie and Masters, Pato and
Glaser); it is just this experiment which can be performed with
MC. Potential changes rather than rate changes were measured in
these experiments since it is more difficult to estimate the
position of a rate change than a potential change.

Whilst DNA synthesis cannot be inhibited with MC it can
be easily displaced in time by pulsing the cells in their minor
period and producing arrest in the G_2 phase. The question was
thus, would such a treatment also displace the step in enzyme
potential ?

The enzyme which was assayed was sucrase; details of the
method used to perform this assay may be found in the Materials
and Methods section. Briefly, the design of the experiments to
measure sucrase potential was as follows; aliquots were taken from
a synchronous culture at intervals of 15-30 minutes and were de-
repressed by twentyfold dilution into medium without glucose.
From these sub-cultures samples were taken at intervals of 25,50,
and 75 minutes after inoculation and the sucrase activity of these
samples was assayed, and from this data which gives a linear plot,
the rate of rise of sucrase activity can be calculated for each
sub-culture. The sucrase potential curve can then be drawn by
plotting the rates of change in activity in the sub-culture 50
minutes after inoculation against elapsed experimental time. This
convention has been adopted by Mitchison and Creanor ('69) because
the cells continue to grow in the derepressed culture, and it is

further discussed by them.

A preliminary experiment was performed on an asynchronous culture in order to ensure that MC treatment did not interfere with the estimation of enzyme activity. A 15 minute 500 µg/ml pulse was used and the results showed that sucrase potential continued to rise for about an hour after the pulse and then levelled off. As this is the expected pattern of DNA synthesis in a pulsed asynchronous culture this initial result was encouraging and a series of experiments were made upon synchronous cultures.

The results may be seen in Figures 48. and 49. The first Figure shows the effect of a pulse of MC applied in the minor period; this would have the effect of delaying DNA synthesis and it appears to have had just that effect upon the step in sucrase potential which occurs about 40 minutes after the division of the treated cells thus retaining roughly its normal relationship with cell division. A word of explanation is necessary here about the shape of the potential curve; the rise which is seen from 1-2 hours in this curve is quite commonly seen and is possibly due to the fact that cells which have just been removed from the glucose gradients, which are used for these experiments, have absorbed exogenous glucose and thus do not derepress normally.

Figure 49. shows the effect of a MC pulse in the major period of sensitivity; in this experiment DNA synthesis should occur at around the normal time and as can be seen the step in potential also occurs normally. It is also noticeable that no further step in potential seems to occur.

Both of these experiments were repeated with very similar results; relative to the control cultures the two experiments shown gave cell cycle positions for the rise in potential of 0.61

and 0.37 respectively and the two duplicate experiments gave positions of 0.59 and 0.31 for minor and major pulses respectively. Mitchison and Creanor ('71b) have recently published an estimate of the position of the step in sucrase potential in the stock of strain 132 used for these experiments and they found that the mean of ten estimates was 0.34 of a cycle with the latest estimate of the ten being at 0.40. The chances of obtaining such results by chance variation of the position of the step thus seem highly remote.

This data thus represents the first experimental support for the hypothesis that the changes in potential for enzyme synthesis are a direct reflection of gene dosage to be obtained with a eukaryote. The experiments also illustrate another advantage of MC which is not shared by the inhibitors of DNA synthesis described in Chapter 3 and that is that by manipulating the position of the pulse DNA synthesis may be either delayed in time or may occur at the same time as the control. This allows the experimenter to investigate the linkage between DNA synthesis and other events and be sure that if they show the same behaviour as DNA that the inhibition is not due to the action of the drug itself which is always a danger with inhibitor experiments.

One final point which is suggested by the data shown in Figure 49. is that the conclusion reached in Chapter 3 that extra DNA is not synthesized after the first S period in cells treated in the major period is supported by the finding that in neither of the cultures pulsed in the major period did the sucrase potential show a second step.

The conclusions drawn from this chapter may be summarised thus.

1. That a pulse of MC in the minor period will delay the step in potential for the enzyme sucrase.

2. That a pulse in the major period will not delay the step in sucrase potential.

3. That the two results above uphold the interpretation of Mitchison and Creanor that the step in sucrase potential is due to a gene dosage effect. These two results also serve to illustrate the efficacy of MC pulses as an experimental tool.

GENERAL DISCUSSION.

Introduction.

The results presented in the previous pages have, up to this point, only been related to the findings obtained by a few other workers, and an attempt has been made to refer only to very relevant findings and to work which has had a direct influence on the design of the experiments or on the conclusions drawn from them. I shall now try to relate my results to those of a variety of other workers and to speculate upon their significance. I must at this point apologise to the reader for producing this long and rather ponderous discussion section. Since so little is known of the control of division much of what is to follow is of necessity speculative, and I have adopted this format in order to try and keep speculation clearly separate from the observations.

In the pages which follow I propose to consider four major areas. Firstly, I shall discuss what is at present known of the action of MC and compare the findings of other workers with this drug to those reported in the preceding chapters. Secondly, I wish to extend the discussion to include the effects caused by UV irradiation in view of the known similarity of effect of MC and UV. Thirdly, I wish to consider what might be the fundamental physiological basis of the division delay induced by MC. Finally, I shall consider the value of MC and other of the drugs used as tools in the study of the cell cycle of S.pombe.

The chemical and biological properties of MC have been reviewed twice recently by Waring in 1966 and by Szybalski and Iyer in 1967. The latter of the two reviews contains more details of the chemistry of the mitomycins and closely related forfiromycins

but essentially they both report the same work and reach much the same conclusions.

The major effects of the mitomycins and porfiromycins are thought to result from their capacity to act as polyfunctional alkylating agents after being chemically activated by reduction within the cell. 'In vitro' they are both inert unless activated by chemical reducing agents. This alkylating activity allows MC to react with DNA to form thermostable covalently bonded cross-links between the two complementary strands of the DNA helix (Iyer and Szybalski '64). However, binding experiments performed by Szybalski and Iyer ('64) suggest that only about one in five to ten of the MC molecules bound to DNA participates in a cross-link and that most of the molecules react with one strand only. It has also been shown by Lipsett and Weissbach that all the bases in DNA are not alkylated equally but that porfiromycin reacts pre-ferentially with guanine although there is some reaction with all the bases.

The binding of MC is not restricted purely to DNA; Weissbach and Lisio report binding of MC to RNA, ribosomes, pro-teins and glycogen, but the greatest reaction is with the nucleic acids.

Pricer and Weissbach have made 'in vitro' studies of the results of MC alkylation on the ability of DNA to serve as a template for polymerase activity. They showed that DNA extracted from treated cells of E.coli shows reduced template ability to-wards DNA polymerase but an unaltered ability towards RNA poly-merase. However, they also showed that DNA alkylated 'in vitro' suffers impairment in both respects. The former result is supported by the finding of Smith-Kielland that fast labelled

'mRNA' in E.coli treated with MC is apparently normal as judged by its ability to hybridise with DNA.

Moving from the molecular effects to the effects of the drug at the cellular level reveals a variety of results depending on the type of cell and dosage involved. Basically there appear to be two types of response, an inhibition of division which, coupled with continuing growth leads to giant cell or filament formation, or an inhibition of division coupled with a breakdown of DNA and in some cases RNA (Kersten et al, Suzuki and Kilgore) leading to cell death. DNA synthesis is found to be affected in varying degrees. Additionally, in some bacteria, the response is further complicated by the induction of lysogenic phage.

The effect on bacteria is quite well documented. Kersten et al report the degradation of sRNA as a result of sub-lethal treatments with MC but the most common report is the depolymerisation of DNA. This latter effect has now been largely explained by the work of Boyce and Howard-Flanders, Terewaki and Greenberg and Mahler. Taken together the work of these authors shows that the depolymerisation of DNA as a response to MC treatment is highly strain dependent and may be correlated with the ability of the strain in question to repair lesions produced by MC and other agents in its DNA. DNA breakdown is thus thought to be a secondary response to MC treatment resulting from the enzymic repair of alkylated sites in the DNA. Additionally, both Terawaki and Greenberg and Mahler were able to show that a repair process was in operation by demonstrating the disappearance of MC induced cross-links from the DNA of treated bacteria with time during post-treatment incubation. In both cases the authors exploited the fact that MC induced cross-links render the DNA fast renaturing.

One piece of work which has appeared since the publication of the two reviews does much to put the results of previous authors in perspective; this is the work of <u>Suzuki and Kilgore</u> on the effects of MC upon E.coli B. Their results showed that most of the effects reported previously could be induced in E.coli B simply by manipulating the dose of MC used. Whilst at low concentrations of drug (0.1 μg/ml) the effects on macromolecular synthesis were slight and the major effect was the inhibition of cell division and subsequent filament formation; at higher doses the characteristic sharp inhibition of DNA synthesis was seen and also an inhibition of β-galactosidase synthesis and RNA synthesis. In addition there was a depolymerisation of both DNA and RNA. All these effects, with the exception of the DNA synthesis inhibition which was immediate, appeared with a lag of about 30 minutes after the start of MC treatment. Measuring the change in viability with time after the start of incubation showed that whilst the effects of 0.1 μg/ml were reversible treatment with 5.0 μg/ml was lethal. The authors conclude that MC has two actions, being cytostatic at low doses and cytotoxic at higher doses. Whilst they stress the action of MC on RNA synthesis as a possible basis for the effect at high doses they offer no suggestion for the mode of action at cytostatic levels.

With mammalian cells the effects are broadly similar. <u>Shatkin et al</u> using mouse L929 fibroblast cells and S91 mouse melanoma cells report giant cell formation accompanied by nuclear fragmentation and partial depolymerisation of the nuclear DNA. They conclude from this that the action of MC on higher cells may resemble its action on the bacteria, but reference to their

data shows that nuclear fragmentation is only seen after 50-70 hours exposure to doses considerably higher than that needed to inhibit division permanently and thus this effect may only represent the eventual demise of the cells. Using lower doses they show that it is possible to only partially inhibit division and that cells will undergo their cycle in the presence of MC with a much extended generation time. This is just the result obtained by myself using asynchronous cultures and by Robinson (personal communication) using synchronous cultures of S.pombe.

Studinski and Cohen examined the effects of a continuous dose of 0.1 μg/ml upon Hela cells and found that both cell division and DNA synthesis were inhibited completely whilst RNA and protein synthesis continued almost unchanged for some 48 hours. They were also able to show that elevated levels of two separate deoxyribonucleases found in the inhibited cells were due to 'de novo' synthesis, thus showing that the protein synthesis machinery of the cell remains intact.

Most workers using MC have concentrated on the effects of continuous treatments and the results outlined above show that the universal observation from this type of experiment is an inhibition of division accompanied by various effects upon synthesis. No clear explanation has emerged to account for this inhibition although all authors have considered the effect of the drug upon DNA metabolism. Before considering this effect myself, however, I wish to examine in a more detailed fashion the results of three studies which have special relevance to this work since they entail the treatment of synchronous cells with short pulse treatments of drug.

Nowell used human leucocytes isolated from whole blood

and stimulated to divide with phytohaemagglutinin, a system which produces a workable measure of synchrony. His preliminary studies showed that 1 μg/ml for 24 hours completely suppressed mitosis and that a pulse of 2-4 hours produced marked inhibition except in G_2. He thus used pulses of 1 hour of 1 μg/ml at four different times during the cell cycle and in addition the G_2 sample was treated continuously for the last four hours of culture. Treatments were made in the G_0, G_1, early S, mid S and G_2 phases of the cycle and all cultures were terminated after 72 hours with the addition of colchicine for the last four hours of growth. He collected his results by measuring the mitotic indices after 72 hours and comparing them with that of the control.

Nowell found that the cells were resistant to delay in G_0 but became sensitive during G_1, sensitivity fell during the S period and there was only a small effect upon G_2 cells. He also scored chromosome aberrations and found that in this case both G_0 and G_1 were maximally sensitive with sensitivity once more dropping to a low level in G_2. Most of the aberrations found were simple chromatid and chromosome breaks but there were also a considerable number of chromatid rearrangements.

The study of Stein and Rothstein ('68a) may be compared with that of Nowell in that they also used a system in which the mitotic cycle is started from the 'G_0' resting condition, namely isolation and culture of tissue from the eye of the adult bullfrog. This system is also only semi-synchronous, a wave of division passing over the explants and reaching a maximum at 87 hours after isolation. The results obtained by Stein and Rothstein are difficult to interpret since they used different doses at varying times in

the cell cycle. However, they do show that a continuous exposure to 0.01 μg/ml or HC will completely abolish the normal division wave in their system. They also show that an 8 hour pulse of 0.3 μg/ml of HC during their so-called G_2 period will reduce RNA synthesis by 34%, protein synthesis by 50% and DNA synthesis by 65%! These inhibitions are inferred from the uptake of the relevant labelled precursors and from these measurements they infer that HC may act by reducing essential G_2 RNA and protein synthesis.

A word of explanation is necessary regarding Stein and Rothstein's definition of G_2. They define this period as following that time (typically 64 hours after explant) at which the addition of 5-fluorodeoxyuridine (FUdR) does not inhibit the normal wave of mitosis (i.e. the transition point for FUdR). Their uptake data shows, however, that from 64-70 hours after explant the tritiated thymidine uptake, by which they measure DNA synthesis, is approximately 80% of the maximum level reached. Hartmann and Heidelberger showed that the action of FUdR is attributable to an inhibition of the synthesis of thymidine monophosphate, a precursor of DNA. It thus seems reasonable to assume that the transition which Stein and Rothstein designate as the entry into G_2 in fact marks the completion of synthesis of thymidine monophosphate. They did not perform the decisive experiment of directly measuring DNA synthesis after the addition of FUdR.

Additionally, a later report of Stein and Rothstein's (68b) suggests a possible basis for their earlier results. They show that RNA and protein synthesis, (once more inferred from uptake data alone) are not continuous in the first cycle of their cells but show two peaks of 'synthesis' during this cycle. Thus

if the effect of MC is to prevent the cells traversing their cycle
the cells may not reach the synthetic period at the same time as
the controls and this would account for the large difference from
the control. In the same article they also report that short ex-
posures to UV light at 64 hours after explant have the same
effects on division and synthesis as a pulse of MC.

The final study which utilised short pulse treatments
of MC is that by Djordjevic and Kim and is the most interesting.
They used Hela cells made synchronous by the selection of mitotic
cells from a monolayer by controlled agitation. Their prelimin-
ary results contrast strikingly with those of Studinski and Cohen
in that they found that 0.1 μg/ml MC had little effect on DNA
synthesis for the first 4 hours of treatment but they too found
that RNA and protein synthesis were little affected as well.
Their synchronous experiments were performed with 2 hour pulses
of 0.1 μg/ml MC and the parameter measured was cell survival as
judged by colony forming ability.

Once more the cells were found to be most sensitive
during the G_1 phase with sensitivity decreasing during the S
period and survival being maximal at around the S/G_2 boundary.
The G_2 phase is short in these cells and around mitosis survival
falls to the G_1 level. An attempt was made to compare the sen-
sitivity of G_2 and S period cells by preparing enriched popu-
lations of these two cell types by selective techniques this
showed that the S phase cells were most resistant.

After having established this cycle of fluctuating sen-
sitivity Djorjevic and Kim investigated the effects of post-
treatment incubation of the cells with acetoxycycloheximide, a

protein synthesis inhibitor related to CHX; they found that this treatment increased survival with a constant dose modifying factor. They interpret this finding as effect upon the repair of MC damage other than interstrand cross-links in the DNA which they felt would be irreparable. They attribute the fluctuation of sensitivity to a corresponding fluctuation of permeability or of the number of sensitive sites in the cell or possibly of the ability of the treated cells to undergo post-treatment repair.

Since none of the three studies outlined above were designed to investigate division delay specifically only a limited comparison with my results is possible. Two points emerge as important, firstly that in two of the studies, those of Nowell and Djorjevic and Kim, there is a clear fluctuation through the cell cycle of sensitivity to the effects of MC treatment and this effect may be occurring in Stein and Rothstein's system although the results are unclear. Secondly, that in the two cases which clearly show this fluctuation the most sensitive part of the cycle is found to be G_1 a result which is in good agreement with my findings.

Whilst I am not inclined to place too much reliance upon the results of Stein and Rothstein ('68a) their conclusions raise the simple question, does MC have any significant effect on synthesis? Several investigations have been made which are relevant to this question and they fall into two groups, studies upon enzyme synthesis in MC treated cells and studies of the ability of MC treated cells to support virus replication.

Experiments of the latter class show much the same results if performed on mammalian cells or bacteria. Ben Porat et al have shown that in rabbit kidney cells treated with a level of MC

which reduced their rate of DNA synthesis to some 2-3% of the control value will produce psuedo rabies virus if infected but that the virus particles produced were not infective. Magee and Miller showed much the same result with HeLa cells and Vaccinia virus using porfiromycin but they add that if much higher doses of porfiromycin are used virus replication may also be inhibited. In E. coli Sekiguchi and Tekagi showed that in the presence of 10 μg/ml phages T_2, T_3, and T_5 can carry out a lytic cycle but once more the resulting progeny virus is not infective.

Enzyme synthesis has been studied in mammalian cells by Kit et al and they were able to show that in mouse fibroblasts there was normal synthesis at doses as high as 50 μg/ml but as the enzyme was virally induced thymidine kinase this work is perhaps more comparable to the virus studies. Several workers have studied the effect on enzymes in the bacteria and the most popular system has been the induction of β galactosidase in E. coli which has been investigated by Shiba et al, Cheer and Tchen, and Suzuki and Kilgore. The results are in broad agreement and show that at doses which have a marked effect upon division and DNA synthesis β galactosidase may still be induced at rates which are comparable with the control. However, as noted above Suzuki and Kilgore showed that at high doses which quickly lead to cell death this capacity may disappear after a short time. Similarly Coles and Gross showed that in Staphlococcus aureus treated with 1.3 μg/ml MC penicillinase inducibility only falls below the control level when the synthesis of RNA and protein begin to be affected some 45 minutes after the addition of MC. Since in this system the half-life of the penicillinase mRNA is calculated to be in the region of one minute or less they conclude that the prin-

cipal effect of MC cannot be upon protein or RNA synthesis.

The studies of virus replication and enzyme synthesis thus both give results which lead to the same general conclusion as that reached in Chapters 3 and 6, that MC does not appear to damage the synthetic machinery of the cell. Normal synthesis occurs after treatment even in cells which are treated with high doses which lead inevitably to cell death. The lack of infectivity found in viruses produced in the presence of MC is perhaps surprising; the authors mentioned above attribute this effect to interstrand cross-links in the DNA of the progeny virus, but if these molecules contain cross-links how were they replicated? This problem brings us naturally to the question of DNA synthesis inhibition.

The demonstration that MC produces links between the complementary strands of DNA led naturally to the conclusion that such damage must present at least a steric hindrance to the replication of DNA and will probably produce a complete block. There is good evidence that in the bacteria DNA cross-links are the most important form of damage since lethality is directly proportional to the degree of cross-linking and calculations of the number of cross-links needed to kill a cell show that only one per genome is required (Szybalski and Iyer '67). Indeed Szybalski and Iyer ('67) state in their review of the effects of MC that 'the mitomycins are considered as standard, rapidly acting and selective inhibitors of DNA synthesis' but perhaps it might have been more accurate to add a proviso to this that this is only true in the bacteria.

The results cited above illustrate that in the higher cells a complete range of response is seen from the total inhibition of DNA synthesis to the complete absence of effect found by

Williamson and Scopes and myself. It is also pertinent that my
results indicate that MC is able to prevent the cells from tra-
versing the cell cycle in the normal way and any agent which will
cause this effect will of necessity reduce the rate of DNA syn-
thesis in an asynchronous culture because cells will be prevented
from entering the S period. This may, for example, explain the
directly contradictory findings of Studinski and Cohen and
Djordjevic and Kim who both used 0.1 μg/ml MC upon HeLa cells.

The explanation of these results may well lie in the
fact that DNA replication in higher cells is managed in a funda-
mentally different way from the bacteria. The effect of cross-
linking on DNA synthesis is presumably proportional to the proba-
bility of a polymerase molecule meeting a cross-link and this
will be related directly to the size of the replicon. Thus with
replication organised in the bacterial manner with only one or two
replicating forks per genome, the polymerase molecule will stop at
the first cross-link met and replication of the whole genome will
rapidly cease. However, with replication proceeding in the
mammalian fashion, as discovered by Cairns and Huberman and Riggs
with each molecule of DNA consisting of a large number of repli-
cating units obviously much more DNA can be synthesized before
all the polymerase enzymes meet cross-links. A rough calculation
confirms this theory; Huberman and Riggs give the length of the
typical replicating unit as 30 microns and since the replication
of this unit is bidirectional from the centre, the length of DNA
covered by one polymerase molecule is about 15 microns. Using
their value of 2.6×10^6 daltons per micron we see that this
length represents some 4.0×10^7 daltons of DNA. The figures of

Szybalski and Iyer ('67) show that for B.subtilis treated to 5%
survival there is an average of one cross-link per 10^8 daltons of
DNA;thus at the same level of cross-linking many mammalian repli-
cons would not contain a cross-link at all.

This argument also supposes that all the DNA in the
mammalian genome is equally susceptible to MC cross-linking, but
there has been a most elegant demonstration by Szybalski ('64)
that this is not the case. Szybalski started from his observation
that it was not possible, even when using MC concentrations in the
region of 100 μg/ml, to cross-link all the DNA molecules of over
10^7 daltons molecular weight from rabbit kidney cells but that
only 30-40% of the molecules were affected whereas in bacteria
there was complete cross-linking. This finding suggested that
part of the genome of these cells is protected against cross-
linking by the chromosomal structure; Szybalski put this idea to
test by comparing the degree of cross-linking of DNA in the rabbit
kidney cell nucleus with that of naked psuedo-rabies virus DNA
located within the same nucleus. The results showed that when
differences in base composition had been discounted that the viral
DNA was far more susceptible to cross-linking than the chromosomal
host DNA and Szybalski concludes that a large proportion of the
nuclear DNA is protected presumably by close association with the
chromosomal proteins.

Clearly this protection of part of the genome and the
possibility that many replicating units may not contain a cross-
link suggests that in higher cells DNA synthesis would be expected
to continue after MC damage as is indeed observed in a number of
cases. The successful replication of the whole genome would
depend on the ability of the cells to remove cross-links. Removal

has been shown for two bacteria, E.coli and B.subtilis by Terauaki and Greenberg and Mahler. Both of these studies used the same approach to show the removal of MC cross-links, the change in the renaturation properties of the treated DNA with post-treatment incubation and in the case of Mahler, who worked with B.subtilis, advantage was taken of the fact that MC is able to render transforming DNA heat resistant. Both studies also compared the effects of MC upon normal cells with those on UV radiation sensitive mutants. The results showed that the fast renaturing property of MC cross-linked DNA was lost with time after treatment in strains able to repair UV radiation damage; this property was absent or much reduced in radiation sensitive strains. However, Terauaki and Greenberg suggested their finding that some radiation resistant strains were still sensitive in spite of the fact that they could remove cross-links may indicate that there is more than one mode of action for MC.

In the higher cells there is also evidence that the damage caused by alkylating agents can be repaired. Crathorn and Roberts showed using S^{35} labelled sulphur mustard that HeLa cells are able to remove bound label with time after treatment and that this repair was associated with DNA synthesis.

Taken together the results reported above suggest that the cross-linking theory can successfully account for most of the effects of MC treatment and that since cells were probably able to repair cross-links it is feasible that they can survive low doses of MC which only introduce a limited number of cross-links. The two results which require explanation are those of Williamson and Scopes ('62a) and myself that MC does not effect DNA synthesis in yeast.

Certainly the most important question to be answered regarding this synthesis is, does MC treatment introduce cross-links into yeast DNA? Since it is now possible to isolate high molecular weight DNA from S.pombe (Bostock '69) this problem is directly answerable by the application of Cs_2SO_4 density gradient centrifugation as applied to mammalian cells by Szybalski ('64).

If it can be shown that MC treatment does cross-link yeast DNA then the most likely explanations of this effect are either that most of the DNA is resistant, as shown by Szybalski for mammalian DNA, or that the number of cross-links introduced is small and only a small fraction of the DNA is affected. It is pertinent to this latter point that the way in which yeast DNA is replicated is not known but that if the figures for the speed of replication and replicating unit size given by Huberman and Riggs are applied to S.pombe the length of the S period is estimated to be about 10 minutes and this is just the figure arrived at by Bostock. Thus a small number of cross-links would affect only a very small proportion of the DNA. A third possibility is that cross-linking is extensive but that repair is very efficient and that the division delay in the case of a pulse is caused by the necessity to remove every last trace of damage or by another mechanism.

All of the suggestions above would result in the bulk of the DNA being replicated at the normal time and only a small proportion of the genome remaining unreplicated. Certainly the diphenylamine assay used by myself and Williamson and Scopes ('62a) would not be able to detect a small under-doubling of the bulk culture DNA. Indeed, it is a very difficult problem to detect a small unreplicated fraction of DNA in yeast without the advantages of the radioactive and density labelling techniques which are

normally used for the fine study of replication in mammalian and bacterial systems.

Thus, to summarise the conclusions up to now, several points have emerged. Firstly, the general effects of MC on other systems are comparable with the results obtained with S.pombe in that cell division is found to be sensitive and that protein and RNA synthesis do not appear to be the primary targets of MC damage. Secondly, the cross-linking theory accounts for most of the effects of MC upon cell viability and on DNA synthesis but that further investigation is needed in the case of the yeasts. Thirdly, no clear explanation has emerged for the effect of MC upon the division of S.pombe treated in the G_2 phase or for the rising pattern of delay seen during the minor period. I have suggested that this pattern might be due to the cells needing a constant time to repair MC damage although as pointed out in Chapter 5. the data is not exactly consistent with this idea. MC cross-linking of DNA has been shown to be a repairable form of damage, but it is not clear how this could effect division during G_2 when conventional DNA synthesis inhibitors have no effect. I shall return to this last question again.

Finally, in the two studies in which constant doses of MC have been applied to cells at different times in their cycles the results have been consistent with those found for S.pombe in that the G_1 period has been found to be most sensitive.

It is this last point which I wish to pursue in the pages to follow by considering the effects of ultra violet irradiation; this is useful and relevant for the following reasons. Firstly, the effects of MC and UV are the same in several areas, notably in their ability to induce lysogenic phage and colicins

(Otsuji et al) their ability to induce the filamentation response
in sensitive bacteria (Grula and Grula) and since some mutations
causing sensitivity to UV also cause sensitivity to MC (Boyce and
Howard-Flanders, Terawaki and Greenberg, and Mahler. Secondly,
the pattern of sensitivity to UV induced delay found by Gill
is very similar to that found in this study for MC induced delay.
Thirdly, both UV and MC are potent inducers of mitotic recom-
bination in both the smut fungus, Ustilago maydis, and S. cerevisiae
(Holliday, '64). The final point is that UV irradiation is always
applied as a finite dose which is much more comparable with a
pulse of MC and more attention has been given to the study of
division delay produced by UV.

However, at this point I must stress that I am not postu-
lating a complete synergism between the two agents; differences
in action are plentiful. To give just two examples the potencies
of MC and UV in inducing the petite 'mutation' in yeast are widely
disparate (Williamson, '70) and the most UV resistant organism
known, Micrococcus radiodurans, is relatively MC sensitive (Moseley).

Because of the very large amount of work which has been
performed with UV and since nobody has yet faced the daunting task
of reviewing this body of work I shall restrict myself to the
consideration of a few fairly modern studies performed specifically
to investigate UV effects in relationship to the cell cycle.

The system of choice for the investigation of radiation
induced mitotic delay has long been the newly fertilised echinoderm
egg and especially those of the sea urchin, Strongylocentrotus
purpuratus or the sand dollar, Echinarachnius parma. This material
has the advantage of being highly synchronous and its activity is
limited to division with very little growth which makes it a good

model system, its major disadvantage for the study of UV induced
delay being that the cytoplasm is strongly absorbent and thus
shields the nucleus.

The sensitivity of this system to UV induced delay has
been investigated by several workers but the most recent and far
reaching study has been by Rustad who has also recently reviewed
this field (Rustad '71). In the first cycle there are four dis-
tinct phases, an initial high sensitivity associated with ferti-
lisation which does not reoccur, a plateau of sensitivity to delay
during which first division delay is constant, a period when sen-
sitivity falls rapidly to zero and an insensitive phase in which
no first division delay is elicited but this last phase consti-
tutes the plateau phase for the second division. DNA synthesis
in this cycle occurs just before prophase about 40 minutes after
fertilisation and it is at this time that the falling phase of
sensitivity is seen. The second round of DNA synthesis occurs
immediately after the first nuclear division and the next mitosis
follows shortly after that; there is thus very little G_1 or G_2
in the subsequent cycles.

Perhaps the most interesting facet of the echinoderm
system is that there are two pieces of evidence which directly
link UV induced delay in this system with damage to the DNA.
These are firstly that UV induced division delay may be reduced by
treatment with photoreactivating light and secondly that 5-
bromodeoxyuridine (BUdR) will sensitize cells to the effects of
UV. Both of these effects were shown by Cook in his study of delay
in the eggs of the Sand Dollar. Cook found that he could not pro-
duce BUdR sensitization of the first post-fertilisation division
and that UV treatment failed to extend the UV sensitive part of the

cycle, a result which ties in with that of <u>Zeitz et al</u> who showed that delay induced in the urchin egg by the UV treatment of the sperm does not delay the first S period. <u>Cook</u> concluded that delay could only be produced by irradiation of unreplicated DNA. Thus here again we have an example of the sensitivity of the unreplicated chromosome, a result which agrees well with the findings with S.pombe.

Rustad ('59), however, has put forward the idea that the major cause of delay is damage to the centrioles and to centriolar DNA. He considers that the sensitive event happens during the transition phase (Rustad '60) and this is the time of centriolar duplication although it is also the time of DNA synthesis. He also supports his theory by pointing out that multipolar mitoses are a common result of UV treatment and this indicates centriolar damage although he acknowledges that there is an alternative explanation that multipolar mitoses are the result of an extra duplication of the centrioles due simply to the extra time provided by the division delay. One rather surprising piece of evidence, however, argues against a chromosomal basis for delay and that is that UV irradiation prior to fertilisation of an egg made enucleate by microsurgery will produce division delay and this delay is photoreactivatable. Some 80-95% of the DNA in the egg is cytoplasmic so this DNA may also play a part in division delay (Rustad '71). Additionally, against this last result must be set the fact that irradiation of the sperm which has virtually no cytoplasmic DNA is more effective in producing post-fertilisation division delay than irradiation of the egg by a factor of ten. Clearly this system is complex.

The other naturally occurring synchronous system, the

slime moulds, also suffer from the disadvantage that cytoplasmic absorption makes it difficult to irradiate all the plasmodial nuclei equally. However, UV induced delay has been studied in Physarum polycephalum by Devi et al; their results indicate that maximum sensitivity to delay occurs during mitosis and during the S phase which directly follows mitosis. During the G_2 phase which constitutes the majority of the cycle sensitivity declined gradually. When irradiated in mitosis the delay is in the subsequent division. Once more these results point to the unreplicated chromosome showing maximum sensitivity to delay. Another finding of this study which agrees well with the results obtained with S.pombe is that the cycle which follows the delayed cycle is quite significantly reduced in length.

Studies of the effects of UV on mammalian cells have tended to concentrate on the lethal effects which are easier to measure rather than division delay, but several papers contain a little data on delay. No complete studies of delay through the cycle have been made using synchronous cultures, but recently one comprehensive study has been published by Thompson and Humphrey in which both lethality and delay were investigated by the use of time lapse photography in much the same way as Gill for S. pombe. They used mouse L cells and found once again that the G_1 phase was the most sensitive to delay with sensitivity falling during the S phase to reach a minimum at around the S-G_2 boundary; at this point the cells passed through a transition to zero sensitivity to first division delay but just as with S.pombe these cells suffer a severe second division delay.

Broadly similar results were obtained by Domon and Rauth using irradiated asynchronous cultures of mouse L cells and analysing

the cell cycle by the labelling method of <u>Whitmore</u>. They were able
to show that the transition point to first division delay was in
fact dose dependent and that the cells could be arrested in G_2
if higher doses are used. Once more they found that the most
sensitive part of the cycle to division delay was G_1 with the
absolutely most sensitive point being at the G_1-S boundary. Delay
fell during the S period and at the dose they used to measure delay
the transition point was around the S-G_2 boundary. They also
showed that the progression of the cells from G_1 to S was unaffec-
ted by UV and that the delay occurred in traversing the S period
with a consequent reduction in the rate of DNA synthesis.

Both <u>Thompson and Humphrey</u> and <u>Domon and Rauth</u> found, as
did <u>Gill</u> for S.pombe, that sensitivity to UV induced division delay
and lethality were well correlated and thus it is relevant that the
same general pattern of changes through the cycle for lethality
has been recorded by several other groups of workers with the one
proviso that the most sensitive point in the cycle in these studies
is generally from early to mid-S phase and not actually in G_1.
Those groups are <u>Sinclair and Morton</u> , <u>Han and Sinclair</u>, <u>Djordjevic
and Tolmach</u> and <u>Bootsma and Humphrey</u>. There is also broad agree-
ment that UV does not delay the cells at the G_1-S boundary and
that the delay occurs by protraction of the S period.

One of the studies above, that of <u>Han and Sinclair</u>,
shows that whilst maximum sensitivity is reached in mid-S phase in
the Chinese Hampster cell that they used, this high sensitivity
does not appear to be dependent upon DNA synthesis. They found
that the addition of HU or excess thymidine to the cells inhibited
the onset of DNA synthesis in their synchronous cultures, but did
not effect the decline in sensitivity to its mid-S level, further-

more the cells remained sensitive until the inhibitor was removed.
This result parallels very closely my result with AdR treatment
of S.Pombe inasmuch as it shows that DNA synthesis is responsible
for reducing the sensitivity to damage and does not itself confer
sensitivity. Han and Sinclair also found that CHX could inhibit
both the onset of sensitivity in late G_1 early S and the onset of
resistance in late $S-G_2$; they conclude that the fluctuations in
sensitivity are governed by an unknown cycle which is dependent
upon protein synthesis but which is only loosely linked to DNA
synthesis.

There is little data available for UV induced division
delay in the fungi except for the complementary studies of Swann
and Gill on S.pombe, but some data is available on the effects
upon survival from three studies of the changes of induction by
UV of mitotic recombination through the cell cycle. Holliday
('65) presents data which shows that in the smut fungus, Ustilago
maydis, there is a highly resistant and a highly sensitive part of
the cell cycle and interprets his results as indicating that the
cells are highly sensitive during the S phase. He considers the
possibility that the sensitivity may be correlated with the amount
of DNA per nucleus and mentions that his data fits this idea,as
the onset of sensitivity coincides with completion of mitosis,
but concludes that it is the S phase which confers sensitivity
since this is indicated in a parallel series of experiments on
S.cerevisiae, the results of which have not been published.
However, this report that the survival of yeast growing in syn-
chronous cultures shows S phase sensitivity does not agree with
the small amount of other data available on this system which is
found in a report by Esposito,who also used the yeast synchronous

system to investigate UV induced mitotic recombination.

Esposito found that survival at 105 minutes after inoculation which is towards the end of the first period of DNA synthesis in his cultures was higher than that at 150 minutes at which time there is no DNA synthesis at all. Other differences between these two studies are also interesting; both authors found fluctuations in the numbers of recombinants induced at different times in the cell cycle and both were able to show that high frequencies of induced recombination were correlated with low survival values. However, whilst Holliday correlated his maximum induction time with DNA synthesis, in yeast Esposito found that the induction of recombinants in his cultures reached a maximum just before the S phase and declined rapidly as the S phase progressed. Moreover, Esposito found the same pattern of induction for both X rays and UV and was able to show using FUdR that delaying the S period also delays the fall in induction of recombinants by X rays found during the S period. Once more the idea that the unreplicated chromosome is sensitive, in this case to recombination inducing damage, seems to fit the data well.

It would be interesting to repeat the experiments discussed above with MC which, as has been previously mentioned, is a potent inducer of mitotic recombination. Holliday's results suggest that the experiments could then be performed with both Ustilago and S.cerevisiae with no significant lethality which should assist the comparison of these two systems. Additionally, if AdR acts in these two synchronous systems in the same way as it does on S.pombe, it should be relatively easy to decide the importance of the S period for both induced mitotic recombination and the survival of UV irradiation.

The third study is that of _Parry and Cox_ who also used

S.cerevisiae to study UV induced mitotic recombination, but instead

of producing their synchronous cultures by the alternate feeding

and starving method of _Williamson and Scopes_ they used the gradient

method of _Mitchison and Vincent._ Their survival data does not

show the dramatic changes seen in Ustilago and the synchrony of

their cultures appears to be somewhat less than was achieved by

the other two authors but the data, which shows fluctuations in

survival of 20-30% through the cell cycle, tends to support the

conclusion of _Holliday_ that the S phase is maximally sensitive.

The final study of the effects of UV upon synchronous

cells that I wish to consider in this section is an especially

interesting one for it compares the effects of heat shocks and UV

upon Tetrahymena and is thus comparable in aim with Chapter 5 of

this thesis. This is the work of _Nachtwey and Giese_ who found

that low doses of UV applied to heat synchronized cells produced

a constant delay up to a sharp transition point some 30 minutes

after EH. (EH is the time of the end of the last heat shock in the

synchronizing regime). At higher doses, however, there is evidence

that delay may be caused later in the cycle and thus the transi-

tion point is probably dose dependent. The dose-delay relation-

ship for these cells is complex and resembles that found for MC

induced delay in S.pombe; the initial response is high and this is

followed by a shallower linear increase as the dose is increased.

Nachtwey and Giese also investigated the effect of photoreactiva-

ting light upon delay and found that the delay at higher doses

could be reduced by photoreactivation but they were uncertain as

to the effect on the initial high response at low doses. Having

thus found that the pattern of UV induced delay did not resemble

that induced by heat shock, they performed an experiment in which
a culture was first UV irradiated and then exposed to heat shocks
at intervals thereafter. The result showed that the effects pro-
duced by heat and UV were roughly additive in that UV appears to
delay the transition point for heat shock, and it is interesting
that they also report that UV delays the onset of stomatogenesis.

It is impossible to interpret these results in terms of
the nuclear cycle since these experiments were performed with a
strain of Tetrahymena which lacks a micronucleus (Williams).
Furthermore these synchronized cells do not appear to require to
perform DNA synthesis to divide since they become 'overmature'
with respect to DNA synthesis during the synchronisation procedure
and inhibiting DNA synthesis by 80-90% with FudR will not effect
the first division (Zeuthen). Nachtwey and Giese explain the
involvement of DNA suggested by the finding that delay is reduced
by photoreactivation by extending Rustad's centriolar damage
hypothesis to include damage to the kinetosomes which are also
thought to contain DNA. Certainly macronuclear involvement in
this delay is hard to envisage since the activities of the macro-
nucleus do not appear to be fully synchronized in these cells in
the first cycle after EH (Zeuthen) and the sharp transition point
and retention of synchrony by the delayed cells strongly implies
that a synchronized process underlies this sensitivity. Perhaps
the simplest and safest conclusions are that UV arrests the pro-
gression of the cycle for a constant time if the cells are exposed
before 30 minutes after EH, and that UV is unlikely to act by the
same mechanism as heat shock, although the two types of damage may
interact. One more difficulty which affects the interpretation
of these results is that there is evidence from a much earlier

study by Iverson and Giese that the first cycle after EH may be abnormally resistant to UV induced delay. They found that in the subsequent synchronized cycles when the DNA per cell approached the normal value that much higher delays were elicited per unit dose and concluded that delay was inversely proportional to the amount of DNA per cell.

There are two major conclusions which can be drawn from the work described above. Firstly, the fact that in the two systems where it has been attempted photoreactivation has been shown to reduce delay clearly implicates damage to DNA as being involved in the cause of delay. This conclusion is strengthened by Cook's demonstration that BUdR sensitizes cells to delay. It is interesting that whilst photoreactivation reduces delay it does not abolish it and thus thymine dimers do not appear to be the sole cause of delay. Possibly the effect of photoreactivation can be explained by the fact that there may be competition between thymine dimers and other forms of damage for the attention of another repair system (discussed for yeast by Kilbey and Smith.) Secondly, comparing the cell cycle sensitivities of several different types of cell to UV induced delay shows that many of the results tend to support the hypothesis that the most sensitive part of the cycle is found when the chromosomes are unreplicated. This last finding is in good agreement with the results found for S.pombe with MC and with the results obtained with pulses of MC on mammalian cells suggests that one form, and perhaps the major form, of damage which causes delay is to the chromosomes.

This brings us inevitably to the basic question, what causes division delay? During the section that follows I intend to examine some ideas on this subject and to try and sketch out

some further ideas for an experimental attack on the problem.
What we are seeking is a form of damage which will arrest a cell's
progress towards division but which the cell can then make good
to allow it to proceed to division.

The common factor which links the agents which cause
division delay without an effect upon growth is that the great
majority of them either damage DNA in a number of ways, some of
which are relatively well understood, or inhibit its replication.
Furthermore, we have the evidence of involvement of DNA in UV
mediated delay quoted above. What then are the known functions
of DNA which can be shown to be or thought to be pertinent to
the control of division? There are three processes which might
qualify, transcription, replication and the ability of the DNA to
be packaged for segregation into the daughter cells.

The first of these three, transcription, is unlikely to
be important since, as was shown for MC, considerable delay can
occur in the absence of any noticeable effect on protein synthesis
and this would be expected to follow an effect on transcription.
However, protein synthesis inhibition has been implicated as a
factor in division delay caused by ionising radiation both by
Rustad and Burchill working with the Sea Urchin egg and by Doida
and Okada working with cultured mouse cells. The role of protein
damage and protein synthesis inhibition has also been dealt with
by Mitchison ('71) who concludes that there is not yet enough
evidence to decide whether or not proteins play an important role.
The second function, replication, deserves much closer attention
for it has been amply shown in many systems that the gross inhibi-
tion of DNA synthesis leads to an inhibition of division and there
is clearly some form of control mechanism which ensures that the

cell does not initiate division until replication has been perform-
ed. The results obtained with AdR which are presented in this
thesis provide a typical example of this phenomenon. Another point
is that if replication is the sensitive process then this provides
a good explanation of the general sensitivity of the G_1 condition.

I have already dealt with this point for the results
achieved with S.pombe and concluded that if replication is sensi-
tive then the lesion or lesions must be restricted to a small
fraction of the total DNA. However, other delayed systems show a
variety of responses to delaying treatments. In general, mammalian
cells appear to respond to most delaying treatments with a pro-
longation of the S phase.

Domon and Rauth found that the division delay induced by UV in
their mouse L cells could all be accounted for by an increase in
the length of the S period. Using the same cells, Walker and
Thatcher showed that low doses of sulphur mustard had little
effect on progression through G_2, mitosis or G_1 but that the cells
suffered delay by an elongation of the S period. A particularly
interesting observation made in this work was that although the
mustard treated cells have completed their normal premitotic amount
of synthesis after 24 hours, the S period, as judged by incorpora-
tion of labelled thymidine, goes on for a further 26 hours. The
authors suggest that this incorporation may represent repair of
damaged DNA and if this is so it agrees quite well with the idea
of only a small fraction of the DNA preventing the completion of
replication.

Another report which suggests that a discreet minor
fraction of DNA may be especially sensitive to the action of sulphur
mustard comes from Flamm et al ('69). This group studied the

replication of mouse satellite DNA in cultured mouse cells using
labelled sulphur mustard and the BUdR density labelling technique
to identify newly synthesized DNA. They found that although, as
far as they could tell using high lethal doses, all the DNA was
equally alkylated, that at lower doses mustard treatment specifi-
cally effected the replication of satellite DNA. It is notable
that by equal alkylation Flamm et al refer to the alkylation of
DNA of different buoyant densities. Since sulphur mustard is a
bifunctional alkylating agent which like MC is capable of pro-
ducing interstrand cross-links (Lawley and Brookes) it would be
interesting to know whether, as Szybalski ('64) showed for MC
alkylated mammalian DNA, there is a fraction which is resistant
to sulphur mustard cross-linking. The result of Flamm et al
suggests that if there is such a fraction it is made up of all
buoyant density classes.

Other eukaryotic cells, however, do not show an exten-
ded S phase and against the results with mammalian cells must be
set the results of Zeitz et al for the sea urchin that UV induced
delay does not delay the cells in the S phase, my results in
S.pombe that the MC delayed cells are blocked in G_2 and the result
of Nachtwey and Giese that cells which do not require to synthesize
DNA at all may be delayed with UV. The results with X-ray induced
delay of mammalian cells are also noteworthy for in this case
Sinclair concludes, in his review of X-ray effects, that there are
two components of delay, a prolongation of the S phase and a G_2
block. These results, with the exception of the Tetrahymena
situation, can be attributed to an effect on the replication of a
small fraction of DNA constituting the block. This theory cannot,
however, account for one obvious feature of the S.pombe results

with both MC and UV, the fact that a delay may be elicited by treatment in G_2.

Delays produced by treatment in G_2 are found in several other cell types too; Domon and Rauth found that mouse L cells could be delayed in G_2 using high doses of UV and G_2 delays are shown in the nuclear division of Physarum by Devi et al. If these delays are to be explained by an action on DNA replication then clearly the DNA affected must either be outside the nucleus or must be an aberrant nuclear fraction. There is in fact evidence that extra-nuclear DNA is involved in division delay from two systems. The first piece of evidence is that delay may be produced by UV irradiation of Sea Urchin eggs made enucleate by microsurgery and subsequently fertilised to initiate the division cycle (Rustad '71) and the second is a report by Jagger that delay may be produced by irradiating only the cytoplasm of a flattened amoeba with a UV microbeam. In both cases DNA is implicated as the sensitive molecule since the delay may be shortened by photo-reactivation but similarly in both cases the nature of the DNA target is uncertain.

One possible target suggested by both the above authors is mitochondrial DNA (mDNA) and certainly this is the most ubiquitous of all cytoplasmic minor species. Mitochondrial DNA shares with bacterial DNA the properties of being naked and circular and certainly the effects of MC treatment or UV irradiation of bacteria are a rapid cessation of DNA synthesis and a delay in its reinitiation (discussed above for MC and see Haas and Doudney and Smith and o'Leary for the effects of UV). The feasibility of mDNA replication inhibition being important in division delay thus depends on two questions: Whether this inhibition would in fact

be expected to have a delaying effect upon division and whether

mDNA is still unreplicated in the G_2 period so that this effect

can account for delays induced in G_2.

Williamson ('70) has postulated that the control of mDNA

replication is under nuclear control as part of a model to account

for the petite mutation in yeast and it is not unreasonable that

such a control mechanism should also feed-back on the division

controls, since to maintain the cellular mDNA at a constant level

it is necessary to ensure that mitochondrial replication has

occurred before division.

The position of mDNA replication during the cell cycle

has now been investigated in several cell types although most

attention has been directed towards yeast and the slime moulds.

Cottrell and Avers using cultures of S.cerevisiae synchronized by

the feeding and starving method of Williamson and Scopes ('62a)

found that there was a minor component which replicated outside

the normal S period when DNA synthesis was estimated by direct

colourimetric estimation. Since the time of this minor 'step' in

DNA coincided with the stepwise increase of two enzymes located

in the mitochondria they postulated that this step may represent

mDNA synthesis. The conclusion that mDNA synthesis is periodic

was also reached by Smith et al using synchronous cultures of

Saccharomyces lactis and the mercury ion and caesium sulphate

equilibrium density gradient technique of Nandi et al,to estimate

the proportion of mDNA in total cell DNA at different times in the

cycle. The latest study by Williamson and Moustacchi reaches the

opposite conclusion. The authors combined direct measurement of

the mDNA by caesium chloride density gradient centrifugation with

labelling studies to show that synthesis of mDNA in cultures of

S.cerevisiae made synchronous by the feeding and starving method is continuous.

Certainly this last result is in better agreement with the results from all other systems studied which all suggest that synthesis occurs throughout the cycle. Thus, using human liver cells Koch and Stokstad showed that mDNA is made continuously with the maximum rate of incorporation of label during the G_2 phase, and continuous synthesis has also been shown in Tetrahymena by both Parsons and Rustad and Charret and Andre.

The most consistent results come from four studies of DNA replication in the slime mould Physarum polycephalum by Evans, Guttes et al, Holt and Gurney and Braun and Evans. All of these authors agree that mDNA synthesis in this system is continuous and additionally the last three groups of the list above show that there is a minor nuclear component which shows incorporation during the G_2 phase which makes up the majority of the cycle. Braun and Evans identified this component as being a heavy satellite DNA which comprised about one per cent of the total cellular DNA.

It thus appears that mDNA does constitute a potential target for delay inducing treatments and that the inhibition of its replication may be a possible mechanism whereby cells are delayed.

One great attraction of the hypothesis that the division delay caused by alkylating agents and UV irradiation is due to an interference with replication is that in some systems this theory may now be testable, although there is no one cell type which lends itself readily to this type of experiment. Clearly since all the cell types studied so far show significant fluctuations in sensitivity through the cell cycle, what is required for such an investigation is a convenient synchronous system. The traditionally

favoured naturally synchronous systems, urchin eggs and the slime moulds,both suffer from the drawback that it is difficult to control the dose of irradiation received by the nucleus since both have strongly absorbent cytoplasm, the use of an alkylating drug like MC might overcome this difficulty. There is one study of the effects of pulse treatments of nitrogen mustard upon Physarum by McCormick and Nardone and the results are rather surprising. DNA synthesis was blocked by pulses in either the S period or G_2 but the first division was not delayed. The two subsequent cycles are delayed but two mitoses appear to occur in the absence of any DNA synthesis. However, there is some doubt as to whether these 'mitoses' are real or just represent a synchronous cycle of chromosome condensation since giant nuclei appear and no telophase nuclei were observed. In addition growth is affected and the plasmodia do not recover their normal growth rate until three months after treatment!

The major factor which will decide the usefulness of a system is whether the most sensitive techniques may be applied to it to study its DNA replication. Physarum is good in this respect as its genome has been well studied and it will readily incorporate both labelled thymidine and BUdR density label; the sea urchin egg will incorporate both of these labels but suffers from the disadvantage that a large proportion of the egg DNA is cytoplasmic and of unknown function (discussed Rustad, '71).

The yeasts do not lend themselves particularly readily to the detailed study of DNA replication due to the fact that thymidine is not incorporated specifically into DNA in yeasts and thus both simple labelling experiments and BUdR density experiments are not possible with yeast. There is one report by Jannsen

et al that thymidine monophosphate is a specific DNA label in yeasts but apparently useful levels of labelling cannot be obtained by this means (B.Cox, personal communication). The yeast genome, however, is fairly well characterised and one recent report is of special interest. This is that the small closed circular DNA of nuclear buoyant density discovered by Hollenberg et al is possibly a cytoplasmic species (Clarke-Walker). This DNA thus represents yet another potential target for the action of UV and alkylating agents.

The mammalian cell genome has been the subject of several detailed studies of late and there is general agreement that during the S period there is a transition from the replication of high G-C DNA, which is synthesized first, to lower G-C DNA which is replicated later (Tobia et al, Flamm et al and Bostock and Prescott). However, the disadvantage of mammalian cells for the study of division delay is that although homogeneous populations of cells may be obtained in various phases of the cell cycle by inductive or selective techniques, the retention of synchrony by such cultures is poor. One technique which has been used with mammalian cells by several workers mentioned above is the use of labelled alkylating agents which allows precise localisation and quantification of the bound drug. MC may be useful in this type of experiment since a fairly simple method has been developed by Weissbach and Lisio for the synthesis of labelled porfiromycin using MC as a starting material.

The third type of effect which might explain the delaying action of DNA damaging treatments is that the chromosomal cycle which ensures the safe segregation of the replicated DNA to the daughter cells at division may not be able to function properly.

Chromosomal damage as the cause for division delay is by no means
a new idea. Carlson found that X-rays could revert prophase nuclei
to the interphase condition in the neuroblasts of the grasshopper
Chortophaga and a similar observation was made by Devi et al using
high doses of UV on Physarum. Additionally, there is the visible
effect of both DNA damaging agents and DNA synthesis inhibitors
namely the production of chromosome breaks and chromosome aberra-
tions (reviewed Sheldon Wolff, and for alkylating agents, Loveless).

Whilst the present state of knowledge of chromosome
structure does not permit very much of an experimental approach to
this problem there is one type of UV induced damage which certain-
ly might be expected to have a profound effect upon chromosome
structure; this is DNA to protein cross-linking. This damage was
first postulated by Smith on the basis of the observation that UV
rendered the DNA significantly less extractable than in normal
cells and he subsequently performed 'in vitro' experiments which
confirmed that the cross-linking was to protein (reviewed Smith
and Hanawalt).

Recently two groups have drawn attention to the impor-
tance of this type of damage in mammalian cells; Habazin and Han
argue that thymine dimers may not be important in determining the
survival of mammalian cells since survival does not appear to be
dependent on their excision from DNA. They showed that a dose of
UV which yields 0.1% of the DNA as dimers will render 5% of the
DNA unextractable and presumably cross-linked to protein. Smets
and Cornelis implicate DNA-protein in the sensitisation to UV
brought about by the incorporation of BUdR into the DNA of human
kidney cells; they found that most of the photo-damage in the sub-
stituted DNA was readily repairable but that there was a very

significant increase in the DNA cross-linked to protein. This effect was so great that at a dose of 1000 ergs per mm^2 the extractable DNA had dropped to only 20% of that found in irradiated controls without BUdR. Both of the above groups looked for any reduction of this effect on the DNA with post treatment incubation but found no evidence that this type of damage is repaired. They both also mention that at the high doses needed to clearly show this phenomenon survival is very low and the situation may be different at more physiological doses.

The relevance of DNA-protein cross-linking to this study is that there is one isolated report which shows that this type of damage may be caused by alkylating agents as well as by UV irradiation. This is the work of <u>Steele</u> who studied the increased toxicity to Ehrlich's ascites cells of bifunctional over monofunctional alkylating agents. He showed that a range of bifunctional mustards were able to link DNA to protein but that, as would be expected, monofunctional agents lacked this ability. He also showed that at doses allowing 10% survival that some 10-15% of the DNA is cross-linked to protein and isolated the DNA-protein complex. He concludes that nitrogen mustard cross-links protein through the purines of the DNA and the carboxyl groups of aspartic and glutamic acids and that the bound protein is not a histone but an acidic protein.

As MC acts as a polyfunctional alkylating agent and only one out of five to ten of drug molecules bound to DNA is thought to participate in a cross-link, there seems no reason why MC should not also cause considerable DNA protein cross-linking. The investigation of this effect seems doubly worthwhile since not only may the results yield some clue as to the cause of division delay

but this might also be a method for determining which proteins are in the most intimate contact with DNA. Whether this form of DNA damage is repairable or not remains to be seen but if the cell can succeed in removing an interstrand cross-link there seems no a priori reason to doubt that repair of this damage is feasible.

Before leaving the subject of damage to the chromosome it is perhaps as well to mention that it is quite possible that many kinds of damage to the DNA may affect the interaction between DNA and proteins and thus disrupt the chromosomal cycle, but until there are further advances in our knowledge of the interphase chromosome and chromosome structure generally, it is impossible to frame a testable hypothesis in these terms.

The last idea which I wish to discuss is that division delay may not be caused by damage to cell components which directly prevents those components undergoing their normal cycle leading to cell division, but that delay is an indirect effect which represents an adaptive response on the part of the cell to the presence of damage in its genetic material. The one immediate objection to this idea is that there are agents which are known to damage DNA which do not appear to have much effect upon division. For example McCormick and Nardone found that monofunctional nitrogen mustard had no effect on division of DNA, RNA and protein synthesis and in a preliminary study for this thesis I found that ethyl methane-sulphonate had little effect on the division of S.pombe.

Nevertheless the ability to repair lesions in the DNA appears to be a fundamental property of cells and is found at all levels of organisation from mammalian cells to mycoplasma(D.W.Smith and P.C. Hanawalt.) That the process of repair also appears to be

very closely related to the process of recombination is also an
observation which has been made both for the bacteria (discussed
K.C.Smith and P.C.Hanawalt)and in fungi (see Holliday '64 and
Esposito). Thus division delay may represent a physiological
response by the cells by which normal progression through the cell
cycle is halted possibly by the same mechanism which operates to
control the progress of the normal meiotic cycle. This view is,
in essence, an adaptation of the hypothesis discussed by Holliday
('64) that UV irradiation creates an intracellular environment
resembling that found in the meiotic cell. If this view is correct
then fluctuations of sensitivity through the cell cycle to both
induced delay and lethal damage may reflect underlying change in
the ability of the cells to perform repair and this view has been
expressed by several authors. Thus Sinclair suggests that there
may be a connection between survival and division delay since the
results with X irradiated mammalian cells tended to indicate an
inverse relationship between them. He argues that this may indi-
cate a greater degree of repair is possible in the cells which
suffer the longest delays. However, as mentioned above, this
relationship does not seem to hold for UV induced division delay
and lethality. Lange also concludes that his results with X-ray
dose fractionation experiments on HeLa cells indicate that repair
capabilities fluctuate during the cell cycle if the assumption is
made that the amount of damage per unit dose remains constant
through the cell cycle.

One corollary of this theory is that it predicts that
there may be a class of sensitive mutants which have lost this
ability to arrest the cell cycle to gain time for repair to be
completed properly. This is just the response found when the UV

sensitive mutant of S.pombe was challenged with MC (Chapter 1) and this suggests that a simple test of this idea in yeast would be to survey the considerable number of radiation sensitive mutants now available for UV induced division delay. Certainly this would be an interesting and simple task with the S.pombe sensitive mutant uvsl-1,as Gill's work would serve as a basis for comparison with the wild type,and the use of synchronous cultures combined with time lapse photography would considerably facilitate the analysis of cell cycle. It would also be very interesting to know whether yeast which is known to have a photoreactivating system and an efficient dark repair system (Patrick et al) suffers a reduced delay after UV treatment followed by photoreactivation.

In summary of my conclusions from this section of the discussion the following points emerge as important. Firstly, the inhibition of DNA synthesis cannot be overlooked as a possible cause for division delay as there appears to be some DNA replication throughout the cell cycle in most cell types. However, techniques now exist which may be able to detect small effects on replication,notably the combined use of labelling and the BUdR density labelling which have been widely applied in the study of repair. Perhaps the best material for the study of such an effect would be Physarum, using UV as the delaying agent.

Secondly, delay may be caused by damage to the chromosome which prevents it from undergoing its normal cycle. One form of damage which may be important in this respect is DNA-protein cross-linking caused by alkylating agents and this seems to merit further investigation.

Finally, delay may be a physiological response on the part of the cell to gain time for repair; if this is so then a

study of radiation sensitive mutants may reveal mutants with different patterns of division delay response from the wild type.

In the final section of this discussion I wish to examine the narrower question of what advantages the phenomenon of MC induced delay offers for the study of the cell cycle of S.pombe.

I have already dealt in the last chapter with what I consider to be the greatest use for MC, which is to determine whether or not any particular periodic event in the cycle is linked to the DNA synthesis cycle; in this capacity MC will complement AdR. Clearly exactly the same type of experiment can also be used to investigate linkage with the cytokinetic cycle again with the advantage that the experiments can be designed to control for the effects of the inhibitor. Linkage with nuclear division can also be studied using MC but in this case it is not possible to control for the effects of the inhibitor, and as AdR will permit such a control it is the agent of choice in this respect. The initial results obtained by Robinson and myself using MC to study enzyme synthesis are very encouraging. Certainly Robinson's results are quite unequivocal and support his result obtained with HU that steps in enzyme activity occur in the absence of DNA synthesis and division. This finding has a clear corollary for the sequential transcription theory. This is that the process which controls the ordered transcription of the chromosome must clearly be capable of reinitiating the cycle in the absence of DNA synthesis and nuclear division. This result thus agrees with the findings of both Eckstein et al and Culotti and Hartwell which were described in the introduction. However, Culotti and Hartwell's results suggest that reinitiation of the cycle may depend on the point in the cycle at which the cell is arrested. It would thus be interesting to

repeat Robinson's experiments with continuous treatments of MC but to delay the addition of the drug until after the first transition point; this will lead to the cells being treated in the major period and my results suggest that the first round of DNA synthesis will occur. It is possible that if the cell cycle is arrested at a different position from that achieved when treatment is started in the minor period then a different result may be seen when the activity of alcohol dehydrogenase is assayed.

Another interesting experiment which may be performed with MC concerns the location of the rate change points found by Mitchison and Creanor ('69). When the basal level of sucrase and several other enzymes is measured in synchronous cultures of S.pombe a pattern of synthesis is seen which is best explained by treating it as series of linear curves which have periodic increases in rate, but the level of synchrony of the cultures is not good enough to enable this conclusion to be reached without recourse to a complicated computer aided analysis of the data. This means that the rate change points are very tedious to locate since the collection of a great deal of data is required. Mitchison and Creanor ('69) postulate that the rate changes are due to dosage changes in the relevant genes which are not expressed at the time of DNA replication but with a slight delay for 'functional gene replication'. Thus rate changes might be expected to respond in the same way as the change in sucrase potential to a pulse of MC. However, it would also be useful to completely block division and DNA synthesis using a continuous treatment of MC. If the system responds as expected the pattern of basal synthesis of enzyme in a continuously treated culture should be a simple linear increase. Furthermore the location of the rate

change point should be easy to calculate simply by comparison of
the treated culture result with the control result.

In fact one such an experiment was performed by Creanor
and myself but the continuous dose of MC administered in this ex-
periment (50 μg/ml) was found to be too low to completely abolish
division and thus DNA synthesis. The basal sucrase level was
found to follow a shallow upward curve with time in this experi-
ment. Probably a result would have been obtained if a concen-
tration of 100 μg/ml had been used.

The two transition points found for the effects of MC
provide two useful cycle markers although it would be highly
desirable to have some information regarding the changes in position
of these transition points when pulses of lower doses are used.
The fact that to a very near approximation the major and minor
periods of sensitivity are the same as the G_1 and G_2 phases of the
cycle for standard pulse employed throughout this thesis suggests
that MC might also be useful, if in some future study it is wished
to determine which phase of the cycle cells are in at any given
time. Naturally this type of experiment must be interpreted with
caution but it might be useful where a DNA assay is out of the
question due to lack of material, and it has the advantage that
determining the sensitivity of the cells to MC induced delay is
much quicker than a DNA assay.

As a system for the study of the biochemistry of division
delay the problem, as mentioned above, is that it is not possible
to apply the most sensitive techniques to the study of DNA repli-
cation in yeasts. The situation is not aided by the fact that it
is difficult to isolate DNA from S.pombe although a method has

been developed by Bostock ('69) and the combined isolation and
density gradient analysis method described by Williamson,
Moustacchi and Fennell could also be successfully applied. The
usefulness of the system thus depends upon the development of very
sensitive techniques for the study of DNA replication; one tech-
nique which might be adapted to this end is polyacrylamide elec-
trophoresis of nucleic acid (see Loening). This technique, which
is currently used to study RNA metabolism in S.pombe, might well
be useful since it can effect a complete separation between DNA
and RNA and thus it allows the use of a non-specific nucleic acid
label to study DNA. Another approach to this problem would be to
use much larger synchronous cultures produced by the inductive
method using AdR or HU as described by Mitchison and Creanor (71a),
although such experiments would be rather costly.

The results presented in Chapter 5 of experiments with
One nucleic acid experiment which is feasible and which
would provide a very useful complement to this study is, as
mentioned above, to determine whether or not MC does cross-link
S.pombe DNA. The method for showing this effect is quite straight-
forward and depends on the fact that MC cross-linked DNA renatures
instantly after denaturation (see Sybalski and Iyer ('67) and
thus does not show a change in buoyant density after denaturation
and rapid cooling.

The results presented in Chapter 5 of experiments with
CHX and heat shocks,whilst they do little to clarify the effects
of MC,do raise some interesting questions regarding the control
of division in S.pombe. These questions have already been dis-
cussed quite thoroughly by Mitchison ('71) and are being actively
investigated by Mitchison and Polenschek;thus I shall only consider

these results briefly and have only one ancillary point to add of my own.

Mitchison has produced several models to account for the patterns of delay observed with delay inducing treatments and these may be classified into two types: pool filling models in which the cell is required to complete the synthesis of a certain amount of division protein before division, and sequential models in which the inhibition depends upon interference with a sequence of synthetic events. In the pool filling models Mitchison has relied on a feature of the Tetrahymena theory to explain the occurrence of excess delays, namely the breakdown of the pool of unstabilised division protein. There is, however, another mechanism whereby excess delays may be caused if the ability of the cell to resume protein synthesis after the period of inhibition varies through the cell cycle.

Consider the case of a cell which synthesizes its division protein over most or just part of its cycle. Should synthesis be inhibited in this cell and the pool of pre-existing division protein does not breakdown, the resulting delay to that cell will only be constant through the cycle if, when synthesis is resumed, the time taken to regain the control rate of synthesis is constant throughout the cycle and thus cells of all ages will take the same time to make the required quantity of division protein. Some cells, as Mitchison ('71) points out, appear to suffer a constant delay when treated with protein synthesis inhibitors and thus presumably do resume synthesis after inhibition in a uniform manner (see Walters and Petersen).

If, however, there is any effect of the age of a cell

upon its ability to resume the synthesis of division protein after inhibition, age variable delay would result and it might be expected that if this effect is operating the pattern of synthesis of the division protein through the cycle may have a profound effect upon the resulting delays.

If the pattern of synthesis is linear with time it is probable that this effect would only cause small variations in delay induced at different cell ages, but if the pattern is exponential it is possible for this effect to cause quite large variations in induced delay. As an example, consider the situation of a cell which makes its division protein exponentially and which after inhibition can only reinitiate synthesis at the same rate as it would normally at the beginning of the cell cycle. It becomes clear that a pulse of inhibitor early in the cycle will cause a much shorter delay than a later pulse since the time to re-establish the control rate of synthesis will be correspondingly shorter.

Since the synthesis of bulk protein during the cell cycle of S.pombe follows an exponential pattern (Mitchison and Wilbur) it seems likely that the further investigation of the effects of CHX pulses on synchronous cultures will yield some information on the way that reinitiation of protein synthesis is related to the cycle position at the time of the pulse. This, however, only provides a partial test of the theory, and my observation that CHX delayed cells are larger at division than control cells suggests that in S.pombe division protein does not respond in the same way as total protein. The conclusion that bulk protein patterns do not reflect those patterns expected from other evidence in S.pombe is also reached by Wain as a result of a

detailed analysis of soluble protein synthesis through the cell cycle.

It thus appears that there are various interesting experiments which can be performed with MC and with the other inhibitors used in this work in several areas of investigation. I would like to conclude this discussion by referring the reader to my acknowledgements which can be found overpage and by expressing the hope that some of the work presented in the preceding pages will prove useful to those who study S.pombe in the future.

ACKNOWLEDGEMENTS

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APPENDIX 1.

The composition of Edinburgh Minimal Medium 2.

Glucose	10 g
Ammonium Chloride	5 g
Sodium Acetate	1 g
Potassium Chloride	1 g
Magnesium Chloride	0.5 g
Sodium dihydrogen phosphate	300mg
Sodium Sulphate	10mg
Calcium Chloride	10mg
Inositol	10mg
Nicotinic acid	10mg
Calcium Pantothenate	1mg
Biotin	10Ug
Boric acid	500Ug
Manganese Sulphate ($MnSO_4.H_2O.$)	400Ug
Zinc Sulphate ($ZnSO_4.7H_2O.$)	400Ug
Ferric Chloride ($FeCl_3.6H_2O.$)	200Ug
Molybdic acid ($H_2MoO_4.H_2O.$)	160Ug
Potassium Iodide	100Ug
Copper Sulphate ($CuSO_4.5H_2O.$)	40Ug
Citric acid	1mg

The above constituents are dissolved and made up to 1 litre and the resulting solution sterilised by autoclaving for 15 minutes at 10 lbs. per square inch.

Edinburgh Minimal Medium 1 is identical to the above with the exception that the Sodium dihydrogen phosphate concentration is only 10mg per litre.

The method of estimating the rise in cell number in a synchronous
culture by numerical integration of the cell plate indices.

The method used was devised by N.Stebbing, and it must
be stressed at the outset that it is not a rigorous calculation
but a rough estimation of the rise in cell number. However, the
results achieved by this method compare favourably with those
obtained by direct cell counting. (J.M.Mitchison, personal
communication.)

The rationale behind the method is simply to derive the
cell count at any given time by increasing the original cell count
(if it is known) by a factor proportional to the area under the
curve of the cell plate index plotted against elapsed time. The
cell plate index curve cannot be rigorously treated in this way
because of the dilution effect of fission on the CPI as a measure
of the rate of division in the culture (discussed Chapter 3).

The first step of the calculation is to estimate the
CPI at intervals of 10-15 minutes throughout the cell plate peak;
the increase in cell number is then calculated by the formula

$$N_t = N_o \times \frac{\frac{E_t^{cp}}{2} \times N_o}{100}$$

Where N_o is the original cell count, N_t is the cell count at time
t. and E_t^{cp} is a summation of the cell plate indices according to
the following formula

$$E_t^{cp} = E_{t-1}^{cp} + CP_t + CP_{t-1}$$

where CP_t and CP_{t-1} are cell plate indices estimated from the
graph of CPI against time at a predetermined interval. E_t^{cp} is
thus a summation of the cell plate indices in which all but CP_t

occur twice.

Stebbing's original method was to make the interval t-1 to t equal to the cell plate time, i.e. 15 mins. However, in the one experiment in which this method was used, the interval t-1 to t was found empirically which gave a calculated 100% rise in cell number due to the first CPP. This same interval was then used to calculate the rise in cell number in the treated cultures.

APPENDIX 3.

Derivation of the method employed for the calculation of the
'corrected cell plate index'.

If, in a synchronous culture undergoing its first divis-
ion, the cell count is observed to increase by a certain percentage
x it follows that the percentage of divided cells in the original
population is similarly x. The proportion of newly divided cells
in a population of synchronous cells of which x% have divided is
thus

$$\text{proportion newly divided cells} = \frac{2x}{100+x}$$

which expressed as a percentage becomes

$$\text{percentage newly divided cells} = \frac{200x}{100+x}$$

Now the cell plate index is the percentage of the whole
population of cells which display cell plates but the corrected
cell plate index is (as explained in Chapter 3) the percentage of
the original population of cells displaying cell plates. Thus in
calculating the corrected cell plate index from the cell plate
index a correction must be made for the newly divided cells counted
during the estimation.

If 100 cells are counted and y cells have cell plates
the CPI is clearly

$$CPI = \frac{y \times 100}{100}$$

but of those 100 cells a certain proportion will have passed through
the cell plate stage and will be newly divided cells and this pro-
portion represent only half their number in terms of the original
population. The number of cells of the original population repre-
sented by those 100 cells is thus

$$\text{number of original cells} = 100 - \frac{\frac{200x}{100+x}}{2}$$

The corrected CPI (CCPI) is thus equal to

$$CCPI = \frac{y \times 100}{100 - \frac{\frac{200x}{100+x}}{2}}$$

$$= \frac{100y}{100 - \frac{100x}{100+x}}$$

$$= \frac{100y}{100} \left(\frac{1}{1 - \frac{x}{100+x}} \right)$$

$$= CPI \left(\frac{100+x}{100} \right)$$

The CCPI may thus be easily calculated from the CPI provided that the percentage increase in the cell number at the time of estimation of the CPI is known.

APPENDIX 4.

Sources of drugs used.

Deoxyadenosine, Hydroxyurea and Cycloheximide were all obtained from Sigma.

Mitomycin C was obtained from Kyowa Hakko Kogyo Co.Ltd., Ohtemachi Building, Ohtemachi, Chiyoda-ku, Tokyo, Japan. The drug is supplied in 2 mg ampoules which also contain 48 mg of sodium chloride. The removal of this salt is essential and it may be achieved by dissolving the mitomycin in absolute ethanol and recovering the drug by vacuum drying, the losses due to this procedure were estimated spectrophotometrically to be only 2%. If larger quantities of the drug are required it may be obtained salt free from the above source by application to the Pharmaceutical Foreign Trade Dept.