THE EFFECT OF CYCLOHEXIMIDE ON CELL DIVISION IN PARTIALLY SYNCHRONIZED PLANT CELLS

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Summary

Cycloheximide blocked cell division at interphase, prophase, prometaphase, metaphase, and telophase in Jerusalem artichoke tuber tissue. Blocking at anaphase did not occur or was very infrequent. In any cell, the location of the block depended upon the stage of the cell cycle reached at the time of cycloheximide addition. In general, cycloheximide caused similar blocks in interphase and mitosis in cells of synchronised broad bean root tips. A reduction of mitotic frequency occurred after about 8 hr in cycloheximide, indicating a reversion of chromosome coiling.

The cytological effects of cycloheximide included: "supercontraction" of chromosomes and prevention of breakdown of the nucleolus and nuclear envelope in prophase, prevention of orientation of prometaphase chromosomes, prevention of separation of the chromatids in metaphase, and prevention of nuclear membrane and nucleolus reformation in telophase. As cycloheximide is an inhibitor of protein synthesis, each of the above effects may be due to the absence of specific proteins whose synthesis extends well into mitosis and resumes in telophase.

I. Introduction

The effect of the antibiotic cycloheximide on mitosis in plant cells was first reported by Wilson (1950). This and subsequent studies in onion (Hawthorne and Wilson 1952) and pea roots (Bowen and Wilson 1954; Hadder and Wilson 1958) indicated that the two most conspicuous effects were mitotic inhibition and the formation of deviant prophases. The typical cycloheximide-induced prophase had an intact nuclear envelope and excessively condensed chromosomes and failed to advance to metaphase. It was thought that these effects were due to the same kind of primary action, possibly involving the nuclear membrane (Wilson 1963).

Cycloheximide is now known to be an inhibitor of protein synthesis in higher plant cells (Key 1966, 1969; Waters and Dure 1966) as well as other eukaryotic cells (Ennis and Lubin 1964; Siegel and Sisler 1965). It appears to act on the ribosome, inhibiting the transfer of amino acids from soluble RNA to polypeptide (Ennis and Lubin 1964; Siegel and Sisler 1965). The action on protein synthesis does not preclude the possibility of other effects within plant cells (MacDonald and Ellis 1969).

In the slime mould, *Physarum polycephalum*, cycloheximide added between telophase and nucleolus dissolution in prophase completely blocked the ensuing mitosis (Cummins, Brewer, and Rusch 1965). Cycloheximide does not appear to accumulate any mitotic stages in some mammalian cells, cells blocking in G2 (Tobey, Anderson, and Petersen 1966; Verbin and Farber 1967). However, in one case (L-strain cells) mitotic stages are affected (Neskovic 1968).

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I have extended the original observations of Wilson by using two types of partially synchronized plant cells. Besides finding some additional cytological effects of cycloheximide, my results indicate that cycloheximide can block cells at different stages of mitosis, depending on when, in the cell cycle, it is added. There may be protein synthesis requirements in prophase and telophase for the completion of mitosis. Cycloheximide was also found to affect the chromosome coiling cycle.

II. Methods

(*a*) *Artichoke Culture and Division Synchrony*

Artichoke tubers (*Helianthus tuberosus*) used in these experiments had been stored for some months in vermiculite at 3°C and the tissue was prepared as described by Adamson (1962). The tissue was incubated in the dark at 23±1°C with manipulations done in the light. Such tissue, treated with the hormones 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, is induced to divide in a partially synchronous fashion. Inclusion of 2% sucrose in the incubation medium increases the partial synchrony (Adamson, Low, and Adamson 1968), and a similar peak mitotic frequency to that using a more complex medium is obtained (Mitchell 1967). Another advantage of this tissue is that all cells initially are 2C (Adamson 1962).

(*b*) *Broad Bean Root Culture and Division Synchrony*

Broad bean (*Vicia faba*) seeds were soaked in distilled water for several hours, and germinated in vermiculite until the lateral roots were 3–4 cm long. The seedlings were then suspended in the various aerated aqueous solutions. The incubation was carried out in the dark at 23±1°C. The lateral root tips used were synchronized by suspending the seedling roots in 700 p.p.m. 5-aminouracil (5-AU) for 24 hr, rinsing in distilled water and transferring to the appropriate aqueous solution free of 5-AU (Smith, Fussell, and Kugelman 1963).

(*c*) *Cytological Preparation and Mitotic Frequency Counts*

Both tissues were fixed in 3 : 1 (v/v) ethanol–acetic acid, washed in 70% ethanol, and stored in 70% ethanol under refrigeration until required.

Free-hand sections were cut from individual artichoke slices and stained directly in aceto-orcein. The mitotic frequency (percentage of nuclei in mitosis) was obtained by scoring about 200 nuclei in each of eight slices.

Root tips were hydrolysed in 1N HCl at 60°C for 8 min and stained in aceto-orcein. The coverslips were removed using dry ice and permanent mounts made in Euparal. The mitotic frequency was obtained by scoring about 500 cells, in areas of active division, in each of four slides.

(*d*) *Cycloheximide*

Cycloheximide (Calbiochem) was used at 10^{-4}M. Preliminary experiments had shown that lower concentrations (10^{-5}M) caused similar effects except that reversion of blocked mitotic figures to interphase was much more rapid.

III. Results

The effect of cycloheximide on hormone-induced divisions in artichoke tuber slices is shown in Figure 1. Cycloheximide added 38 hr after excision completely blocked mitosis. Cycloheximide added at 42 and 46 hr after excision reduced the mitotic frequency, but blocked many cells in mitosis. After about 8 hr in cycloheximide the mitotic frequency declined, and eventually fell to zero. Cytological examination suggested that the blocked mitotic figures reverted to interphase, consistent with the observations of Bowen and Wilson (1954). A mitotic figure thought

to be a reverting prophase is shown in Figure 2, and should be compared with the cycloheximide-blocked and normal prophases in Figures 3–6.

In photographs depicting the cycloheximide effects in artichokes, incubation had been carried out in cycloheximide for a sufficient length of time (at least 4 hr), to suggest that the mitotic figures were actually blocked in mitosis, rather than that mitotic cells had recently entered mitosis and were still proceeding through it.

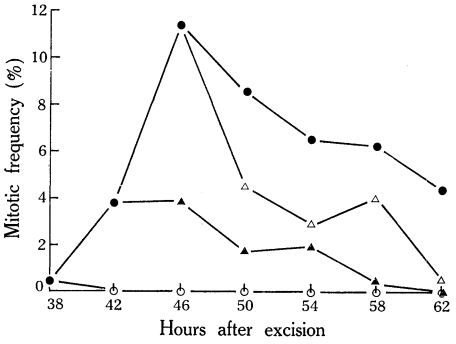


Fig. 1.—Percentage mitotic frequency in artichoke tissue slices incubated, after excision, in 2,4-D (1 mg/ml)+kinetin (1 mg/ml) (●) and when cycloheximide (10^{-4}M) was added at 38 (○), 42 (▲), and 46 (△) hr after excision.

The mitotic figures blocked by cycloheximide in artichoke slices were in all mitotic stages except anaphase. The main stages accumulated were prophase and metaphase, with a small number of telophases, as shown in the following tabulation:

	Mitotic Phase as Percentage of Mitotic Figures Present		
	Prophase+ Metaphase	Anaphase	Telophase
Normal tissue	48	5	47
Cycloheximide added	90	0	10

The cycloheximide prophases (Figs. 3 and 4) can be compared with the normal prophases (Figs. 5 and 6). Most prophases blocked by cycloheximide had a nucleolus, an intact nuclear envelope, and excessively condensed chromosomes (Fig. 3). Wilson (1950) first observed the effect of cycloheximide on chromosome coiling and termed it "supercontraction". There were a small number of blocked prophases with a nuclear envelope and supercontracted chromosomes, but without a nucleolus (Fig. 4). Of the blocked mitotic figures 90% were in prophase and metaphase, made up of 36% prophases and 54% prometaphases plus metaphases.

Prometaphases were not separated from metaphase when scoring mitotic figures in artichoke slices. The prometaphases arrested by cycloheximide had chromosomes as condensed as those in metaphase and this, together with the large number of small artichoke chromosomes, made many of them difficult to distinguish from metaphase. However, it was apparent that mitosis could be blocked at prometaphase when the chromosomes were not aligned at the equatorial plate (Fig. 7). A normal prometaphase is shown in Figure 8. Metaphases blocked in the presence of cycloheximide have supercontracted chromosomes (Fig. 9) in contrast to the normal chromosomes (Fig. 10), and do not appear to have a sharply delineated spindle (Fig. 11) such as seen in normal metaphases (Fig. 12).

In artichokes no anaphases were observed in cycloheximide treated tissue. A normal anaphase is shown in Figure 13.

Artichoke slices treated with cycloheximide showed 10% of the blocked cells in telophase. Most telophases showed no reformation of the nucleolus or nuclear envelope, and the chromosomes remained tightly coiled and closely associated within each daughter group (Figs. 14 and 15). The telophase in Figure 14 still shows the remnants of the spindle suggesting that it had blocked soon after the completion of anaphase. A normal early telophase is shown in Figure 16, while a later stage showing nucleoli and the nuclear envelope is shown in Figure 17. In the presence of cycloheximide only a few telophases showed reformation of the nuclear envelope and nucleoli as shown in Figure 18. In this particular cell the phragmoplast has failed to complete its development in the presence of cycloheximide.

Additional experiments with artichoke tuber slices were carried out using 2% sucrose in the medium to increase the synchrony, with similar results.

With broad bean root tips, the dividing cells were synchronized using a 24-hr treatment in 5-AU. Smith, Fussell, and Kugelman (1963) showed that division commences about 6 hr after the end of 5-AU treatment with some division between 6 and 12 hr and a peak at 14 hr. The results in Figure 19 are consistent with this pattern. Cycloheximide was added 4 hr prior to 5-AU removal, at the time of 5-AU removal, and subsequently at 2-hourly intervals until 12 hr after 5-AU removal. Figure 19 shows results for cycloheximide additions at 4, 6, 8, and 12 hr after 5-AU removal. Cycloheximide added before 6 hr after 5-AU removal almost completely prevented mitosis. Cycloheximide added at 6 hr after 5-AU removal blocked many more cells in mitosis, while cycloheximide given from 8 to 12 hr after 5-AU removal caused very large numbers of cells to be blocked in mitosis.

In root tips, cells were only scored as metaphase if the chromosomes were clearly aligned on the metaphase plate. All earlier stages were scored as prophase. No time of cycloheximide addition caused any significant accumulation of anaphases or telophases as shown in the following tabulation:

	Mitotic Phase as Percentage of Mitotic Figures Present		
	Prophase + Metaphase	Anaphase	Telophase
Normal tissue	80	10	10
Cycloheximide added	98	1	1

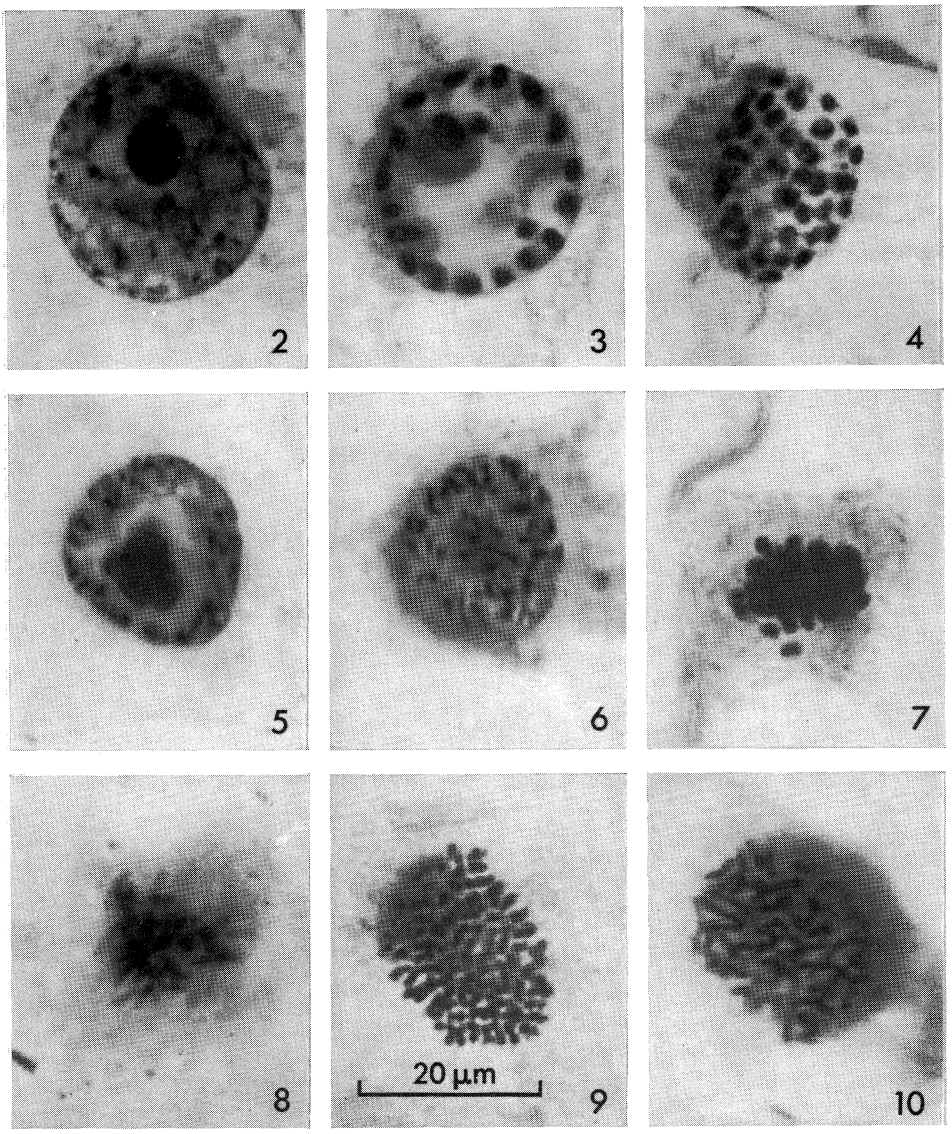
Fig. 6.—As for Figure 5, but focus on chromosomes.

Fig. 7.—Prometaphase from artichoke slice treated 4 hr in cycloheximide. No nuclear envelope present.

Fig. 8.—Normal prometaphase from artichoke slice.

Fig. 9.—Polar view of metaphase from artichoke slice treated 4 hr in cycloheximide. All chromosomes in one focal plane.

Fig. 10.—Polar view of normal metaphase from artichoke slice. All chromosomes in one focal plane. Some are partially obscured by background cytoplasmic basophilia.



Figs. 2–18.—Free-hand sections from artichoke tuber slices stained directly in aceto-orcein. Direct staining in aceto-orcein, besides staining the chromosomes, also stains the nucleolus. All figures ×1200.

Fig. 2.—Prophase blocked by cycloheximide reverting to interphase. Artichoke slice had been treated 12 hr in cycloheximide, therefore it is unlikely to be a normal early prophase.

Fig. 3.—Prophase from artichoke slice treated 12 hr in cycloheximide, showing nucleolus, intact nuclear envelope, and supercontracted chromosomes.

Fig. 4.—Prophase from artichoke slice treated 4 hr in cycloheximide, showing intact nuclear envelope and supercontracted chromosomes. No nucleolus seen at any plane of focus.

Fig. 5.—Normal prophase from artichoke slice, with focus on nucleolus. Intact nuclear envelope and chromosomes visible.

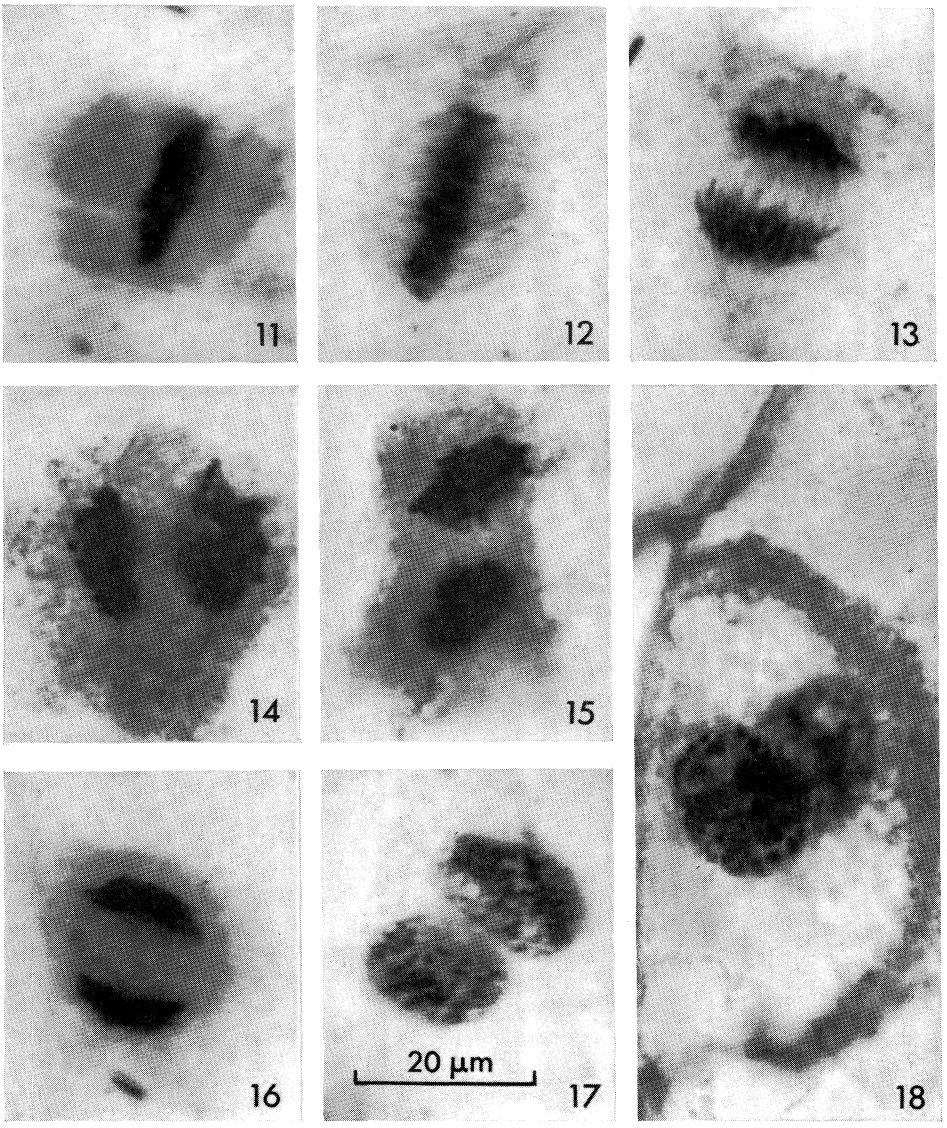


Fig. 11.—Side view of metaphase from artichoke slice treated 4 hr in cycloheximide.

Fig. 12.—Side view of normal metaphase from artichoke slice. A spindle is visible.

Fig. 13.—Normal anaphase from artichoke slice.

Fig. 14.—Telophase from artichoke slice treated 4 hr in cycloheximide. Remnants of spindle visible.

Fig. 15.—Telophase from artichoke slice treated 4 hr in cycloheximide. Blocked at slightly later stage than Figure 14.

Fig. 16.—Normal early telophase from artichoke slice.

Fig. 17.—Normal late telophase from artichoke slice. Focussed to show nucleoli, nuclear envelope, and chromosomes which are still uncoiling.

In this tabulation the data for the cycloheximide additions at 8 and 12 hr after 5-AU removal have been averaged. If taken separately, cycloheximide given 8 hr after 5-AU accumulated 96% of cells in prophase and 4% in metaphase, while cycloheximide given 12 hr after 5-AU accumulated 79% of cells in prophase and 19% of cells in metaphase. The root tip results suggest that cycloheximide blocks cells in interphase, prophase, and metaphase as the inhibitor is added at progressively later stages of the cell cycle.

In Figure 19 all cycloheximide treatments show a decline in mitotic frequency with time. However, the very small numbers of cells in phases after metaphase do not suggest that the cells move slowly through mitosis, but rather that they revert to interphase. Cytological examination also suggests that the chromosomes eventually uncoil in the presence of cycloheximide after they have been blocked.

The cytological effects of cycloheximide on the partially synchronized root tips were similar to those for the artichoke tuber slices, except for the absence of telophase.

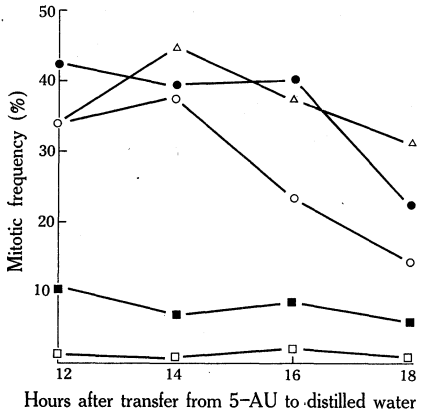


Fig. 19.—Percentage mitotic frequency in broad bean root tips synchronized by a 24-hr treatment in 700 p.p.m. 5-aminouracil and removed to distilled water (○) and when cycloheximide (10^{-4}M) added 4 (□), 6(■), 8 (●), and 12 (△) hr and mitotic frequencies determined 12, 14, 16, and 18 hr, respectively, after removal to distilled water. Root tips not synchronized with 5-aminouracil and grown in distilled water all the time had a mean mitotic frequency of 15% over the period shown in the figure.

IV. Discussion

By adding cycloheximide at different times of the cell cycle in plant cells, it was possible to block cells in interphase or in a greater number of different stages of mitosis than previously reported. These stages in artichoke tissue have been listed in the sequence they are thought to occur in the following tabulation:

Characteristics of Blocked Mitoses	Mitotic Stage Classification
Nucleolus+nuclear envelope+supercontracted chromosomes (scattered)	Prophase
Nuclear envelope+supercontracted chromosomes (scattered)	Prophase
Supercontracted chromosomes (clumped)	Prometaphase
Supercontracted chromosomes (on metaphase plate)	Metaphase
Supercontracted chromosomes (clumped)±spindle remnants visible	Telophase
Nucleolus+nuclear envelope+supercontracted chromosomes	Telophase
Nucleolus+nuclear envelope+supercontracted chromosomes+phragmoplast	Telophase

Fig. 18.—Late telophase from artichoke slice treated 4 hr in cycloheximide. Nuclear envelope present, chromosomes still tightly coiled, and a nucleolus appears to be present. Phragmoplast has apparently been blocked in this position by cycloheximide.

In both artichoke slices and bean root tips, cells were blocked in interphase, prophase, prometaphase, and metaphase. Blocks in anaphase were absent in artichokes and rare in root tips. In artichoke slices 10% of the blocked mitotic cells were in telophase, compared with only 1% in root tips. Possible explanations for the discrepancy in telophase percentages are the smaller proportion of time spent in telophase in root tips (see tabulations, Section III), absence of nucleolar staining in the acid-hydrolysed root tip squashes, or the need for cycloheximide additions for a longer period after 5-AU removal. Wilson (1950), Hawthorne and Wilson (1952), Bowen and Wilson (1954), and Hadder and Wilson (1958) in their studies with bean root tips also did not report telophase effects of the type noted in artichoke slices. However, I have also found telophases in excised dividing wheat coleoptiles treated with cycloheximide.

In order to prevent mitosis, cycloheximide had to be added prior to the appearance of any mitotic figures. In the artichoke tuber slices cycloheximide added at 38 hr (Fig. 1) completely blocked the initiation of mitosis. The controls were commencing division at this stage (0·4% mitotic frequency), and even though the G2 period is probably less than 1 hr (Mitchell 1967), at 38 hr many cells would have been in G2. The results for the broad bean root tips (Fig. 2) also suggest that cycloheximide given in early G2 can prevent the initiation of mitosis. The G2 phase is 4–5 hr in *Vicia faba* (Brewen 1964; Evans and Scott 1964), and no cells incorporate label into DNA of lateral root tips 7 hr after removal from a 24-hr 5-AU treatment (Brewen 1964). There would be some delay before cycloheximide had an inhibitory effect, and there is some variation in the time at which different root tips reach their peak of synchrony. With these considerations in mind, many bean cells would have moved from S to G2 about 6 hr after 5-AU removal. Cycloheximide added 6 hr after 5-AU greatly reduced the mitotic peak but at 8 hr 40% of the cells were blocked mainly in prophase. Additions of cycloheximide after 8 hr and at 10 and 12 hr accumulated more metaphases. The root tip results suggest that it is in the G2 and prophase periods that the requirements for the completion of prophase, prometaphase, and metaphase occur.

Cycloheximide is not just acting as a general metabolic poison, but shows selectivity in blocking the mitotic figures. This can be seen from the tabulations in Section III in which the distribution of mitotic stages of cycloheximide-treated tissue is markedly different from that of controls. Cycloheximide accumulates virtually no anaphases, suggesting that once the spindle units are formed and assembled cells move from metaphase to telophase. Cycloheximide does not apparently interfere with the mechanism of anaphase separation of the chromatids once separation is initiated.

Although it is not certain at what time of the cell cycle synthetic events necessary for the completion of telophase occur, the telophase blocks may reflect a need for synthetic requirements in early telophase in order to complete telophase and cytokinesis, in particular for the reformation of the nuclear envelope and nucleoli. Cell plate initiation and also its development is inhibited by cycloheximide in artichoke tuber slices. In *Trillium erectum* protein inhibitors added at the end of the second meiotic division inhibit wall formation and cytokinesis (Hotta and Stern 1963).

However a 3-hr treatment with puromycin, which did not prevent cell plate formation in maize root tips, did have an effect on the golgi apparatus associated with the process (Whaley, Dauwalder, and Kephart 1966).

The cycloheximide effects on mitosis may reflect the need for specific proteins in order for a cell to proceed through the various mitotic stages. Based on the various addition times, the synthesis of these proteins may extend well into mitosis and may resume very early in telophase. This possibility needs direct confirmation. It is well established that there is little protein or RNA synthesis in metaphase and anaphase (Prescott and Bender 1962; Davidson 1964; Das, Siegel, and Alfert 1965; Hodge, Robbins, and Scharff 1969), but this does not preclude the synthesis of important proteins at prophase (Cummins, Brewer, and Rusch 1965; Cummins, Blomquist, and Rusch 1966; Tobey, Petersen, Anderson, and Puck 1966; Sisken and Wilkes 1967) or telophase.

Parchman and Stern (1969) have shown that cycloheximide inhibits protein synthesis in meiotic prophase of lily microsporocytes. Cycloheximide added at successively later stages of the first meiotic prophase subsequently caused cells to block at successively later stages of meiosis. Cycloheximide also caused stickiness and supercontraction of the chromosomes.

Cycloheximide did not accumulate or prolong mitotic stages in some mammalian cells (Tobey, Anderson, and Petersen 1966; Verbin and Farber 1967). However, Neskovic (1968) found with L-strain cells that cycloheximide retarded the division of cells that were in prophase, metaphase, or telophase at the time it was introduced. Cycloheximide also prevented the disappearance of the nuclear envelope.

After about 8 hr in cycloheximide there is a reduction of mitotic frequency in artichoke and bean cells that cannot be related to progression of cells through mitosis, indicating a reversion of the supercontracted chromosomes to an interphase condition, presumably by uncoiling. The properties of the reverted interphase nuclei are not known. The observation of supercontraction in chromosomes caused by cycloheximide may provide a useful technique for studying the mechanism of chromosome coiling.

More direct evidence is needed to interpret the action of cycloheximide on cell division in plant cells. Although interference with protein synthesis is a plausible explanation of the mitotic effects of cycloheximide, a recent report (MacDonald and Ellis 1969) has implicated cycloheximide in the interference with energy transfer in beet root disks in a way reminiscent of 2,4-dinitrophenol. Webster and Van't Hof (1969) have shown that 2,4-dinitrophenol prevents entry of G1 cells into S and G2 cells into mitosis in pea root tips but they did not report any blocks in mitotic stages, so that the effects of cycloheximide on mitosis are unlikely to depend only upon an interference with energy supply. Regardless of its mode of action, cycloheximide is a useful tool to study the sequence of events in the whole cell cycle and especially in the mitotic stages.

V. Acknowledgments

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