STUDIES OF MITOTIC CYCLE TIME IN *PISUM SATIVUM*

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ABSTRACT

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The purpose of this study was the evaluation of a simple method, termed continuous treatment method, for measuring mitotic cycle times. This method involved continuous treatment with a low concentration of colchicine (1.88×10^{-6}M), and then determining the time when the polyploid cells appeared in the subsequent division. This time was taken as the minimum mitotic cycle time. At the same time, the continuous method was compared with the polyploid tag method of Van't Hof *et al*. (1960) and the tritiated thymidine technique. The tag method produced a polyploid population through short treatment (30 minutes) with a high concentration of colchicine (4.38×10^{-6}M). In the subsequent division, the rise and fall in polyploid frequency was determined, and the time of peak polyploidy was taken as the mean mitotic cycle time.

The control temperature was defined as 22.5°C. Mitotic cycle times were measured at 17°, 20°, 22.5°, 25°, 27°, and 30°C.

The pea root meristem of *Pisum sativum* var. Alaska was the experimental material. Samples were taken at the appropriate hours, prepared for microscopic examination and analyzed for degree of effect.

The continuous treatment method has been shown to be equivalent to the tag method for estimating mitotic cycle times. Both methods appear to be more reliable than the tritiated thymidine technique, since this was found to increase the cycle time about 1.4 times (Van't Hof, 1965). With the continuous treatment method the mitotic cycle time at 22.5°C was 10.5 ± 0.5 hours. An almost linear decrease in cycle times was observed in the temperature range of 17°C to 27°C. Above and below this range mitotic activity was erratic. At low temperatures, e.g. 17°C and 20°C, the tag method was characterized by double polyploid peaks.

STUDIES OF MITOTIC CYCLE TIME
IN *PISUM SATIVUM*

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

The mitotic cycle has been defined as "the series of events which occurs from the inception of one mitosis to the inception of the ensuing one" (Wilson and Morrison, 1959). This series of events includes all of the biological, biochemical, and physiological events which make the mitotic cycle an ordered process necessary for cellular growth and reproduction.

Major advances in the fields of biochemistry and physiology have made it possible to study the biochemical events operating during the mitotic cycle. The techniques available to measure this process are limited. In the early 1950's the advent of radioactive tracers provided a particularly useful tool. However, extensive experimentation using radioactive compounds has also shown that there are side effects causing some genetic and physiological damage. A less complicated method proposed by Van't Hof, Wilson and Colon (1960) is the production of a fairly synchronus dividing population through short treatment with colchicine in the appropriate concentration, and scoring for the rise and fall in the polyploid frequency with time. This method, too, has some drawbacks which will be discussed later.

A simple method for measuring mitotic cycle times would be ideal if it has few disadvantages and still provided an effective measure of the duration of the mitotic cycle. The purpose of this investigation was the evaluation of such a simple technique, termed the continuous treatment method.

LITERATURE REVIEW

In the literature review, the following topics have been considered: non-radiographic techniques (direct and indirect); radiographic techniques; and the effects of temperature on the mitotic cycle.

The experimentalist has available to him a variety of methods, both direct and indirect, for measuring the duration of mitosis and the mitotic cycle. Direct measurements require single cells or small groups of cells and have been limited to observations of cell cultures, microorganisms, and eggs of certain animals, utilizing phase contrast microscopy and/or photography. Fell and Hughes (1949), for example, measured the duration of mitosis and intermitosis interphase in mouse spleen cultures by phase contrast microscopy, and found the mitotic cycle varied from 8.8 to 19.5 hours. Mazia (1961) has listed mitotic cycle times for various other cell cultures and microorganisms, using both direct and indirect methods.

Indirect methods are used to measure such processes as cell division, each phase of mitosis, and cell extension in whole tissues or organisms. Brown and Rickless (1949), using Cucurbita pepo, devised a phase-timing technique. This method involved macerating excised root tips in a known volume of fluid, so that the tissue was separated either into single cells or groups of cells. The density of the cell suspension was then determined by placing a drop of the suspension on a haemacytometer slide and counting the cells. The number of cells in the suspension on the slide was calculated. From this the average number of cells in the root was determined. The average number of non-vacuolated cells per root was determined in the same manner. The length of each root was also measured. From the average root length, the average total number of non-vacuolated cells per root, and from the average total number of cells per root, the rate of cell division and the degree of cell extension were calculated. The overall rate of division in 2% sucrose medium at 25°, 20°, 15°, and 5°C are given in Table 1.

The alkaloid, colchicine, has become a very useful tool for the experimental cytologist who is working with mitosis. Evans, Neary and Tokinson (1957), using colchicine, determined the mitotic cycle time in Vicia faba root meristem. The technique involved short treatment (1-6 hours) with a high concentration of colchicine (0.5% or 0.1%), so as to block all cells at metaphase causing them to accumulate and increase lineraly with time. The plot of accumulated metaphases against durations of colchicine treatment gave the number of cells entering metaphase per hour, and from these data the mitotic cycle was calculated. The total mitotic cycle time under these conditions was found to be 24.6 ± 1.5 hours at $19^{o}C$.

Wilson, Morrison and Knobloch (1959) measured the minimum mitotic cycle time in Pisum sativum. Their method involved continuous treatment with a low concentration of colchicine. The time when the first polyploid cells appeared in the subsequent division was used to provide a rough estimate of the minimum mitotic cycle time. The first polyploids appeared about eight hours after treatment.

Van't Hof, Wilson and Colon (1960), also using colchicine, developed a method for measuring mitotic cycle times. The tag method, as it was named, involved exposing pea root meristems to a fairly high concentration of colchicine ($3.76x10^{-6}M$ or 0.015%) for thirty minutes. The time between colchicine treatment and appearance of a maximum number of tetraploid cells was taken as the mean mitotic cycle time. Under these conditions and at 22.5°C, the mitotic cycle time was 12 hours.

The advent of radiographic techniques opened whole new areas of research. Howard and Pelc (1951) were among the first to use phosphorus^{32} (P^{32}) to study cellular proliferation and biochemistry. Using P^{32} they were able to label the population of cells that was synthesizing DNA at the time of treatment. These cells could then be followed through the subsequent division. They found a mitotic cycle time of 30 hours for *Vicia faba* at 19°C.

Taylor, Woods and Hughes (1956) prepared tritium-labeled thymidine (H^3-T), and used it to study chromosome duplication. The synthesis of this compound has opened areas of research which, until this time, could not be investigated because of the lack of adequate techniques.

Quastler and Sherman (1959) used H^3-T to label intestinal epithelial cells in mouse during the S stage (time of DNA synthesis) of interphase. The subsequent appearance and disappearance of these labeled cells was used to estimate the mitotic cycle time.

Wimber (1960) using H^3-T estimated the mitotic cycle time of Tradescantia paludosa as 20 hours at 22°C.

Combining both H^3-T and colchicine, Van't Hof and Ying (1964) were able to label simultaneously two different segments of the mitotic cycle in peas. The tag method indicated the mitotic cycle time at 20°C was about 14 hours, and H^3-T indicated that the S period was located about mid-interphase.

Although the use of radioactive substances can be useful, research has indicated that results should be interpreted with caution. The reason is that the endogenous radiation delivered to the cells may result in chromosome breakage, changes in cellular physiology, mitotic inhibition, reduced clonal growth and cell death (Painter et al., 1958; Drew and Painter, 1959; Wimber, 1959; Sanders et al., 1961).

In a study more pertinent to the present research, Van't Hof (1965) has compared the tag method to the H^3-T technique. When measuring mitotic cycle times with H^3-T, colchicine, or both simultaneously, the first cycle following H^3-T labeling is 1.4 times longer than that measured with colchicine. For peas, at 20°C, the mitotic time was 14 hours when measured by the tag method, and 18 hours measured by H^3-T. The increase in cycle time was attributed to G_1 (pre-DNA synthesis). It was hypothesized that the increase was due either to chronic beta irradiation from tritium present in the cell, or the fact that H^3-T and colchicine mark two distinctly different cell populations in the pea root tip.

Considering the techniques available, little work has been done on the effect of temperature on the mitotic cycle *per se*. Brown (1951) concluded that in peas all stages of mitosis are accelerated by an increase in temperature from 15°C to 25°C. He supposed that above 30°C, conditions other than metabolic changes were limiting the mitotic cycle. His method for determining the lengths of the stages of the mitotic cycle was based on the assumption that divisions throughout the pea root meristem were random. Savage and Evans (1959) and Evans and Savage (1959) have concluded that as the temperature decreases from 25°C to 3°C, the cycle time increases. They used the c-metaphase accumulation method to measure the cycle times. Their results also indicated that temperature does not affect all stages of the mitotic cycle in the same manner.

A summary of the above observations on techniques, organisms, temperatures, and mitotic cycle times is given in Table 1.

TABLE 1.--Summary of literature review

REFERENCES	ORGANISM	TECHNIQUE	TEMPERATURE	CYCLE (hr)
Fell & Hughes (49)	Mouse spleen cultures	Direct observation	--	8.8--19.5
Brown & Rickless (49)	*Cucurbita pepo*	Phase-timing	25°C	83.3
			20	18.2
			15	9.4
			5	17.9
Brown (51)	*Pisum*	Modified Phase-timing	15	25.55
			20	18.83
			25	15.78
			30	14.39
Wilson *et al*. (59)	*Pisum*	Continuous conchicine	22.5	8 (min.)
Van't Hof *et al*. (60)	*Pisum*	Colchicine tag	22.5	12
Van't Hof & Ying	*Pisum*	Colchicine tag	20	14
Van't Hof (65)	*Pisum*	Colchicine tag	20	14
		Tritiated thymidine	20	18
Howard & Pelc (52)	*Vicia*	Phosphorus-32	19	30
Evans *et al*. (57)	*Vicia*	C-metaphase accumulat'n	19	24.6±1.5
Savage & Evans (59)	*Vicia*	C-metaphase accumulat'n	3	260
			19	25
Evans & Savage (59)	*Vicia*	C-metaphase accumulat'n	3	260
			10	64.2
			19	26.2
			25	22.9

EXPERIMENTAL PROCEDURE AND RESULTS

The meristematic root tip of the garden pea, *Pisum sativum* var. Alaska, was used in this investigation. The peas were furnished by the Ferry-Morse Seed Company and the Vaughn Seed Company. Both agencies stated that the peas were disease-free and had not been treated with any insecticides or herbicides.

The peas were soaked for six hours in glass-distilled water (pH 5.5--5.7) and rolled in moistened Scott 110 paper toweling. Each roll was wrapped in wax paper to prevent excess evaporation, placed in an upright position in a beaker containing about an inch of glass-distilled water, and allowed to germinate 34 hours, at 22.5°C in an incubator.

Seedlings with roots of 15-2cm were collected and suspended on waxed one quarter inch wire mesh grids. Each grid rested on dishes containing 350ml of full strength, modified Hoagland nutrient solution (pH 5.5--5.7). The contents of which are listed in grams per liter of nutrient:

Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$	7.6
Ammonium nitrate	NH_4NO_3	10.32
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	14.4
Potassium monobasic phosphate	KH_2PO_4	10.68
Potassium dibasic phosphate	K_2HPO_4	0.56

To allow for acclimatization, the peas remained in the nutrient for four hours at 22.5°C, and were aerated by a fine stream of filtered air for the entire experiment. All experiments were run in a constant temperature water bath set to the required temperature.

Each experiment consisted of six dishes of seedlings. Two grids of seedlings were transferred to a 1.88×10^{-6}M colchicine in nutrient solution (pH 5.5--5.7), and continuously treated for the remainder of the experiment (Wilson et al., 1959). Another two grids with peas were transferred to a 4.38×10^{-6}M colchicine nutrient solution (pH 5.5--5.7) for thirty minutes, then washed thoroughly with glass-distilled water and returned to the original nutrient solution. This is the tag method of Van't Hof et al., (1960). The remaining two grids with peas were left untreated in nutrient solution as controls.

All colchicine solutions were prepared 15 minutes before using, as colchicine loses its effect when stored in solution (Greenberg, 1962). The colchicine was obtained from Koch-Light Company Ltd., Bucks, Colnbrook, England.

At the end of the second hour of the experiment, half of each group of peas--one control, one continuous, and one tag treatment--were transferred to another constant temperature water bath set to one of the experimental temperatures and remained at that temperature for the rest of the experiment. The experimental temperatures were 17°, 20°, 25°, 27°, and 30°C. A 22.5°C control temperature treatment accompanied each experimental temperature.

Samples of five root tips from each treatment were taken hourly. Each sample was immediately fixed in Pienaar's fixative (Pienaar, 1955) (6:3:2 mixture of absolute methanol, chloroform and propionic acid, respectively), evacuated for ten minutes, corked, and placed in the refrigerator for 24 hours. All samples were coded for identification.

The fixed roots were hydrolized in 1N HCl at 60°C for 18 minutes. The HCl was decanted and leuco-basic fuchsin (Schiff reagent, Lillie, 1951) was poured into each vial containing the root tips. The stained root tips were prepared for analysis by the Feulgen Squash Technique as follows: the highly stained (dark purple) meristematic region was excised and placed in a drop of Acid-Fast Green (0.1% Fast Green and 45% acetic acid) on a glass slide; gently macerated with the flattened end of a solid glass rod and covered with a cover slip; heated gently and pressed between paper toweling to remove excess materials; the slides were coded and placed in a mixture of 90% tertiary-butyl alcohol and 10% ethanol for a minimum of six hours; then made permanent with diaphane. A slide was prepared for each root tip.

The degree of colchicine effect was determined at the second hour, because Van't Hof <u>et al</u>. (1960) has shown that a high colchicine effect at the second hour produced a significant polyploid population. The degree of effect was measured by the method of Hadder and Wilson (1958) as follows:

$$\text{Colchicine Index} = \frac{1\,(\text{No. of scatters}) + 2\,(\text{No. of clumps})}{(\text{No. of normal post prophase}) + (\text{No. of scatters}) + (\text{No. of clumps})}$$

Colchicine Indices for the second hour can be found in Appendix I.

Stage Analysis (Percentage of each mitotic stage/ 200 dividing cells) was done on each control and experimental temperature at the third and sixth hour of the experiment (one and three hours, respectively, at the experimental temperature) to determine if there was a shock effect after the change of temperature, and if prometaphase accumulated as reported by Moh and Alan (1964). Comparison of these analyses seem to indicate that there was no shock effect or pile up of prometaphase after one and three hours at the experimental temperatures. Percentages obtained from the stage analyses can be found in Appendix II.

The appearance of polyploidy in the subsequent division was then used to determine mitotic cycle times (MCT). Both continuous and tag methods were scored for percent polyploids.

The tag method has been used by Van't Hof *et al.* (1960) at Michigan State University and by Van't Hof and Ying (1965) at Brookhaven National Laboratories, and consistently the MCT has been 12 hours at 22.5°C. The continuous treatment has been used more than ten times, and consistently the minimum MCT at 22.5°C has been 10.5 ± 0.5 hours. Both of these methods were used to measure MCT at the above listed temperatures (Figs. 1-6). The cycle times obtained under those temperatures are listed in Appendix III. An increase in temperature decreases MCT. A plot of cycle times against temperature indicates a linear relationship existing between 17°C and 27°C (Fig. 1). However, above 27°C, the linearity breaks down. Facilities were such that it was not possible to determine if and where linearity would break down at a temperature lower than 17°C.

Two series of experiments to measure MCT were conducted. In each series both the continuous treatment and tag method were used. However, in the first series of experiments, only the continuous treatment method produced a scorable polyploid population. Enough polyploid cells were produced with the tag method to give an approximate cycle time for each experimental temperature. The cycle times obtained with the continuous method, in both series of experiments, agreed.

The continuous treatment method has also been used to determine the effects of three antimitotic agents on the MCT. The agents were apholate, an ethyleneimine derivative; two carbamates, ethyl carbamate (urethane) and

Isolan®. Apolate was found to increase the MCT 2.5 hours. Urethane and Isolan® appear to interfere with the colchicine effect, i.e. the production of scatters and clumps, so that by the second hour the colchicine treated seedlings appear almost normal. However, polyploidy was recovered in the subsequent division which indicated the MCT was increased about four hours with urethane and about two hours with Isolan® (Figs. 7 and 8, and Appendix IV). Because these chemicals interfere with the colchicine effect, there is some doubt that these mitotic cycle times are accurate. It can only be concluded that these antimitotic agents increase the mitotic cycle time in peas by no more than the degree indicated.

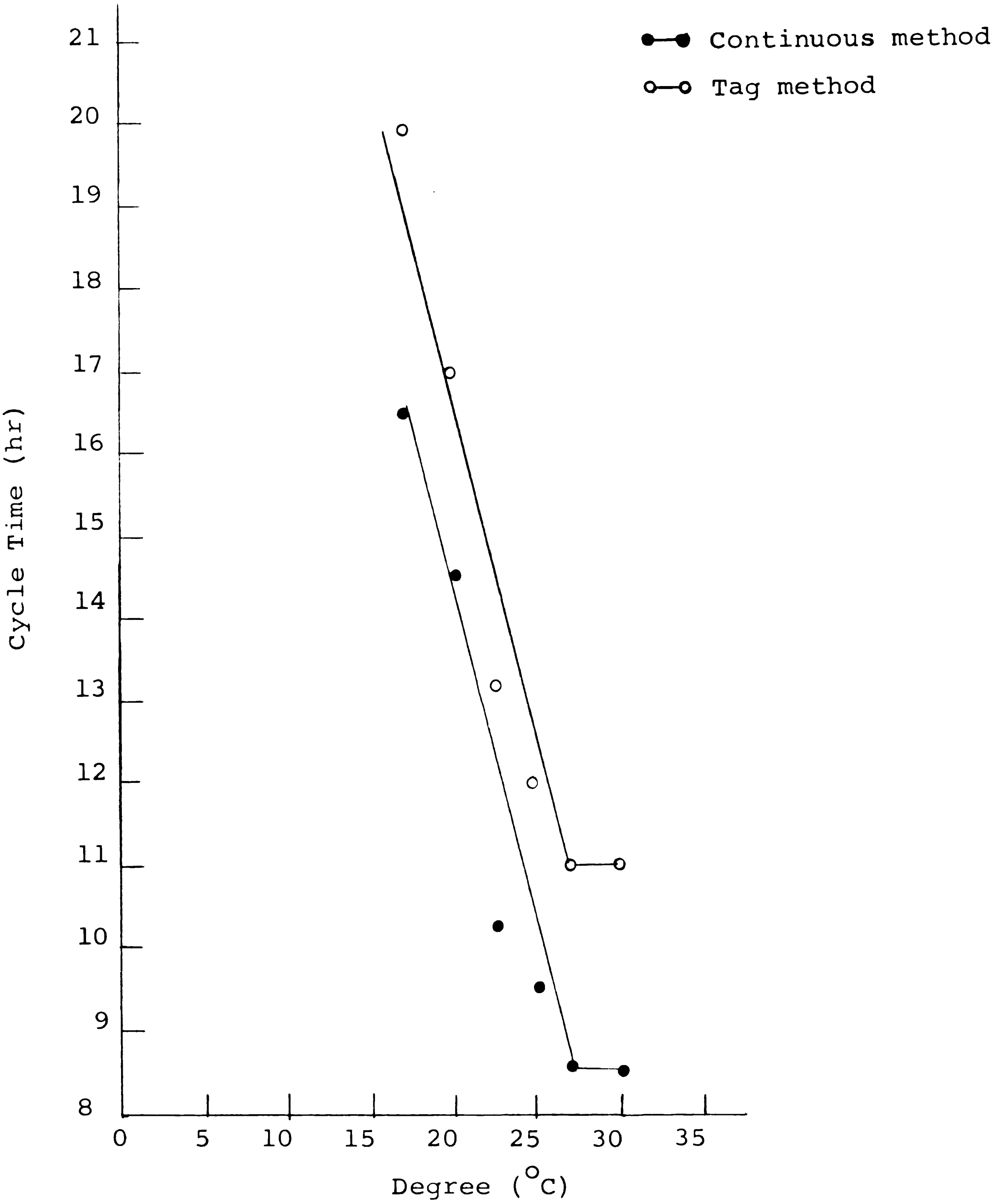
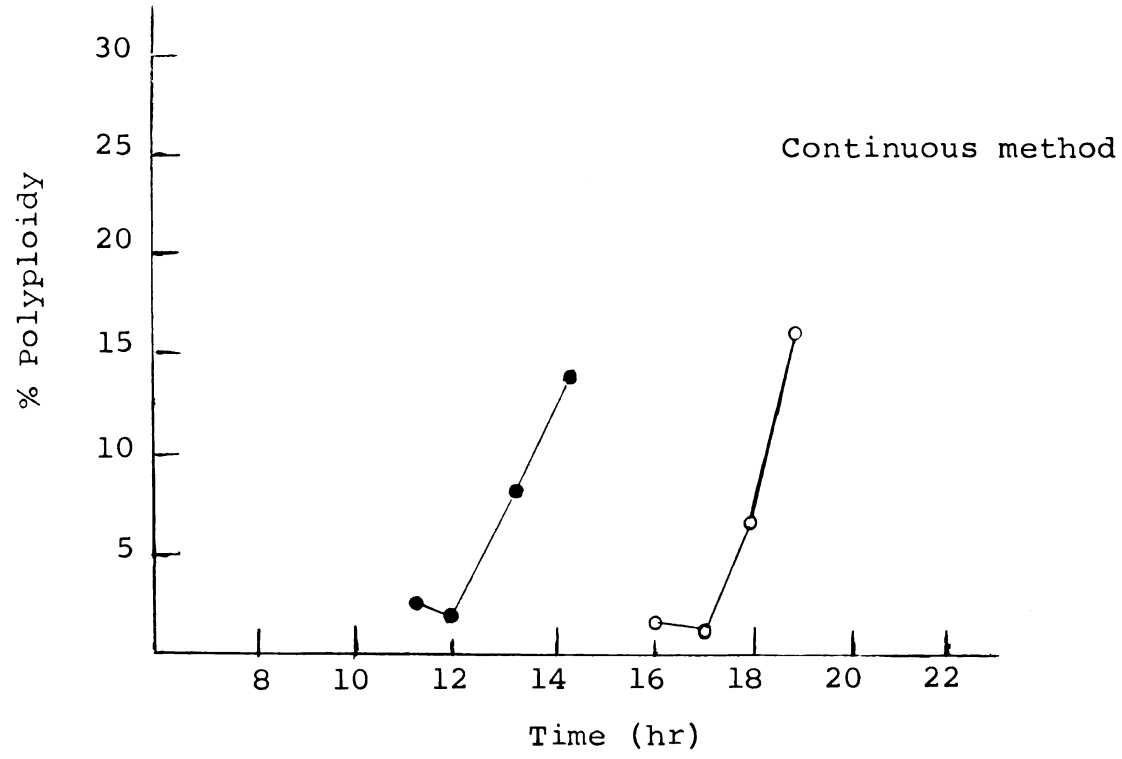


Figure 1.--Cycle time plotted versus degrees.



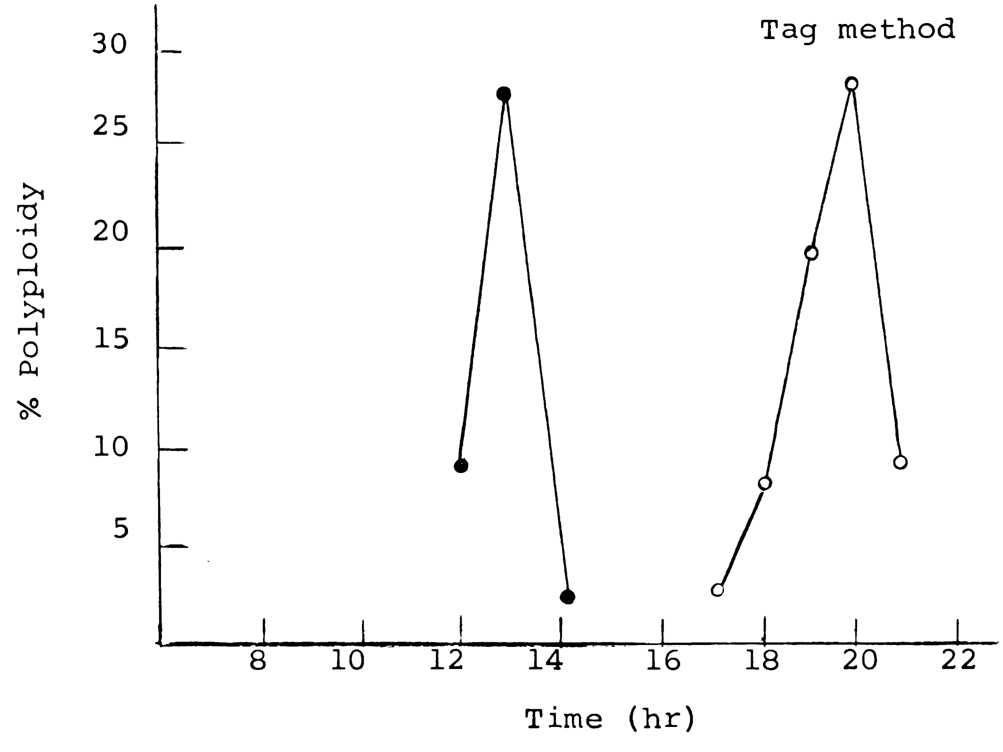


Figure 2.--Percent Polyploidy plotted versus time for $22.5^{\circ}C$ (●—●) and $17^{\circ}C$ (○—○).

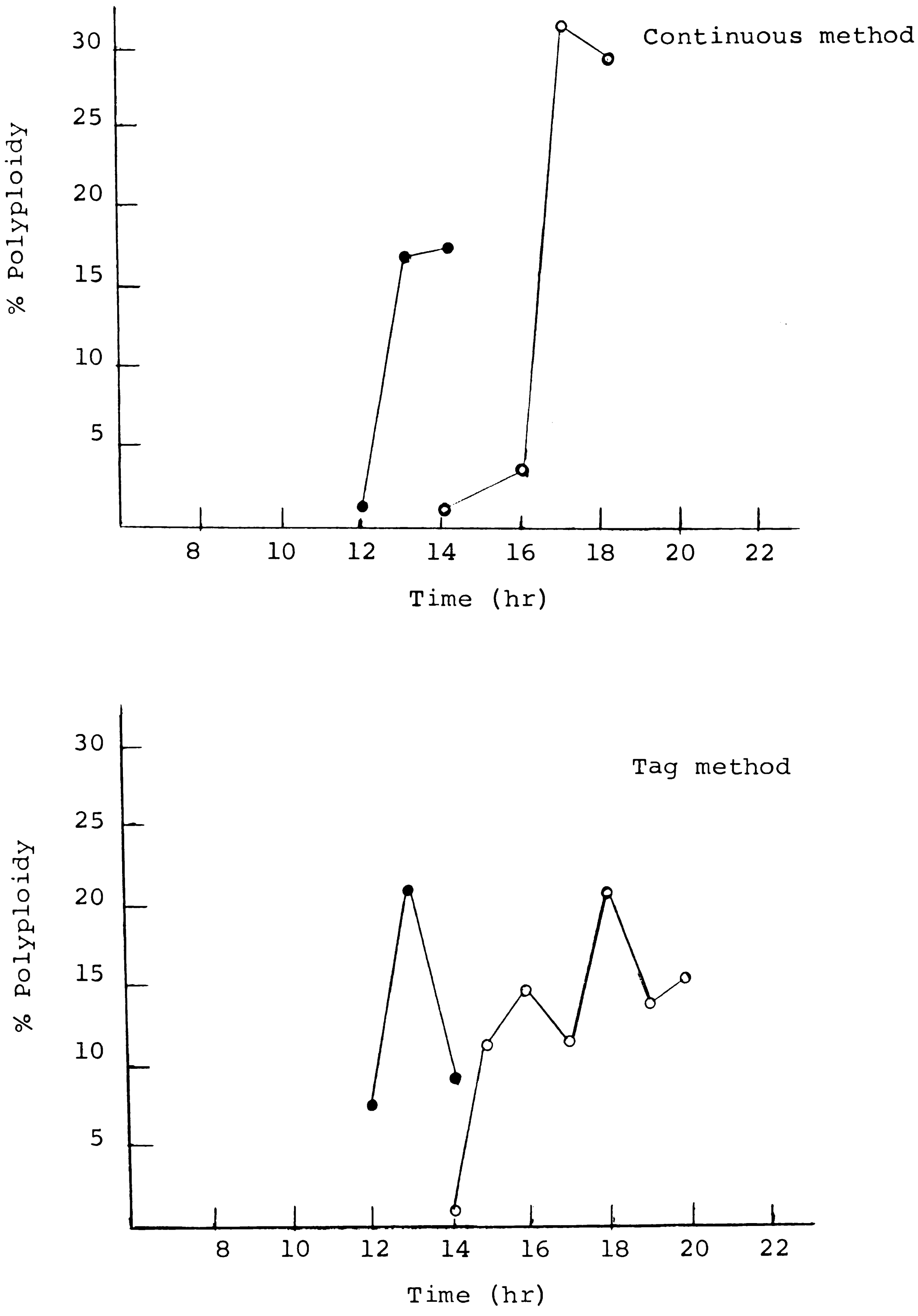


Figure 3.--Percent Polyploidy plotted versus time for 22.5°C (●—●) and 20°C (○—○).

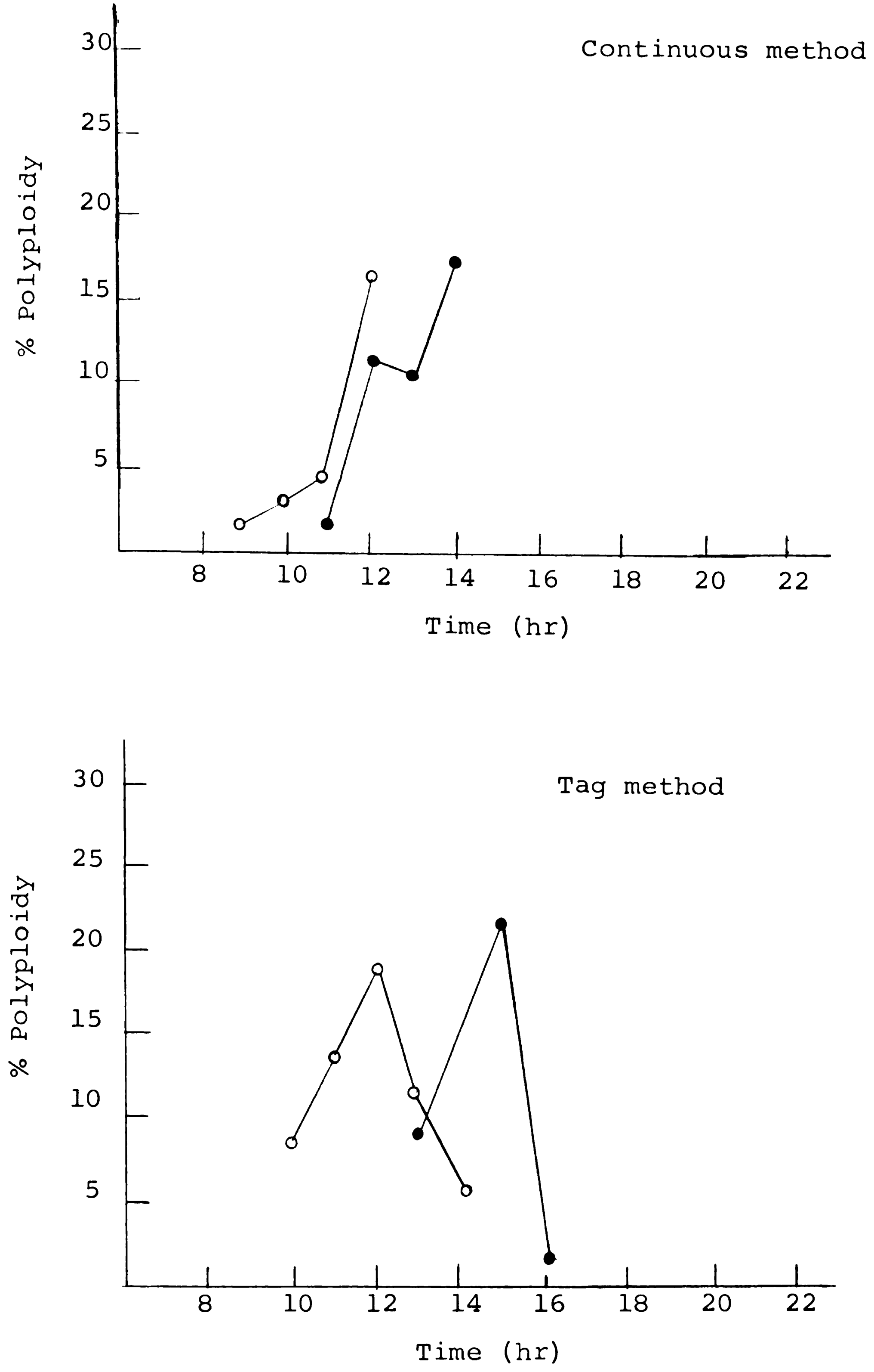


Figure 4.--Percent Polyploidy plotted versus time for 22.5°C (●—●) and 25°C (○—○).

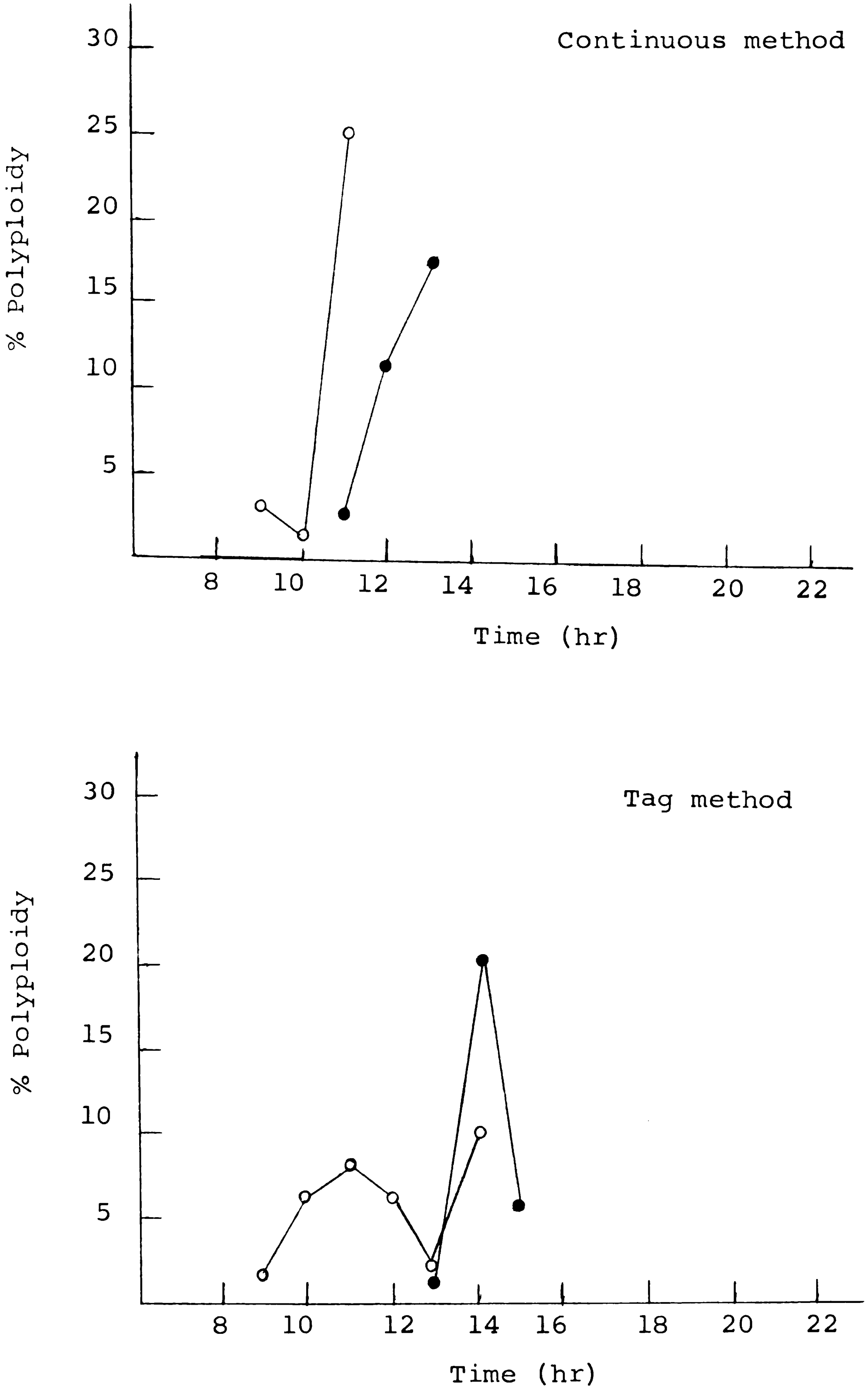
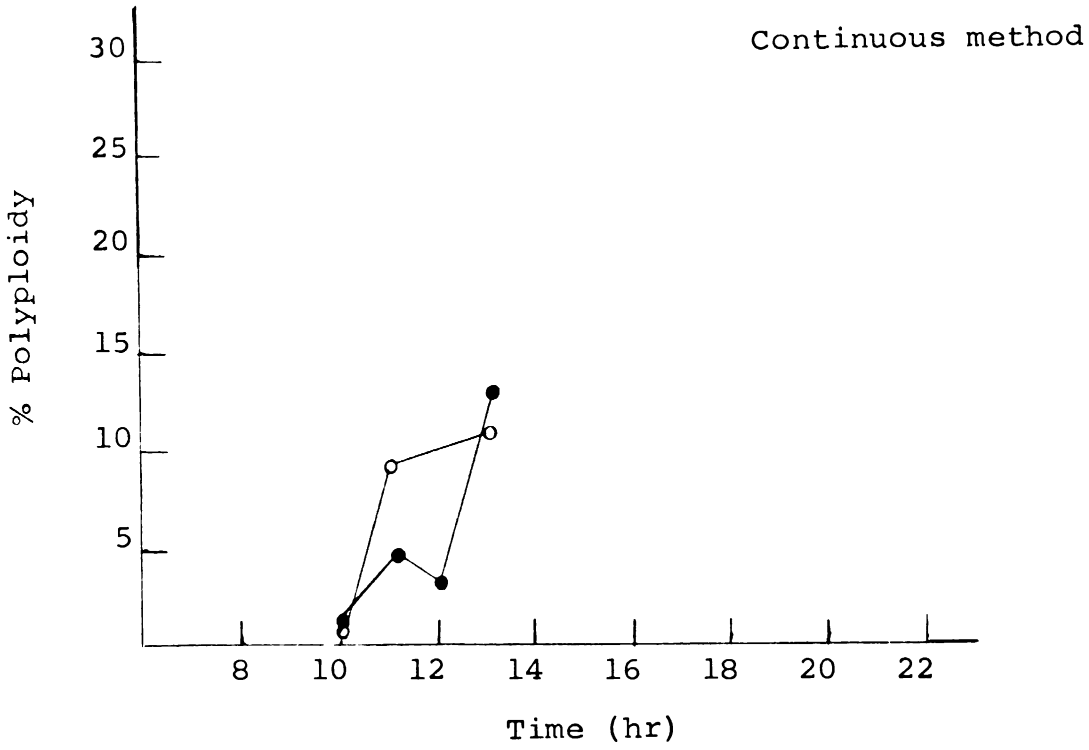


Figure 5.--Percent Polyploidy plotted versus time for 22.5°C (●—●) and 27°C (○—○).



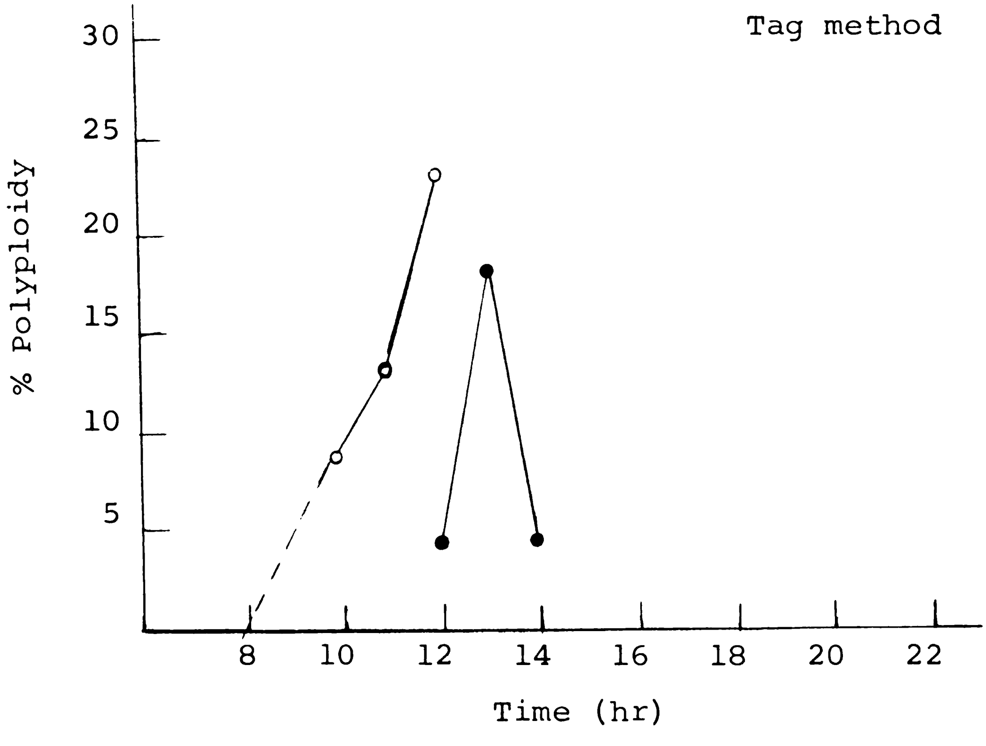
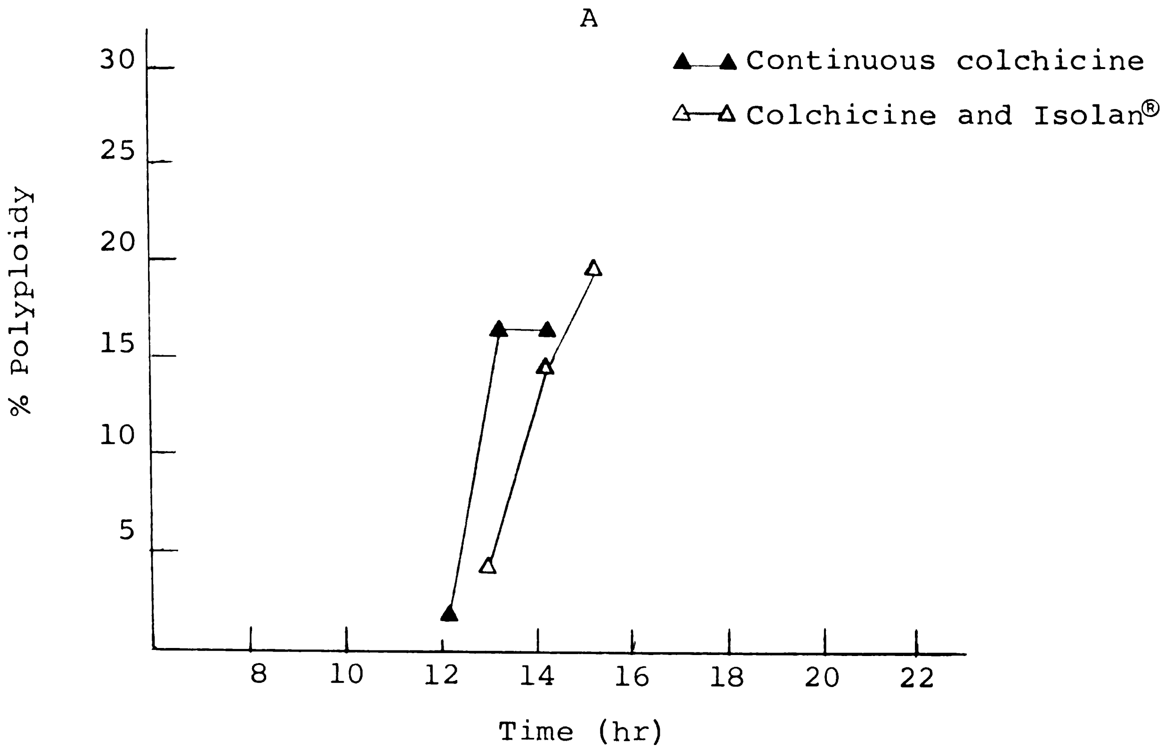


Figure 6.--Percent Polyploidy plotted versus time for 22.5°C (●—●) and 30°C (○—○).



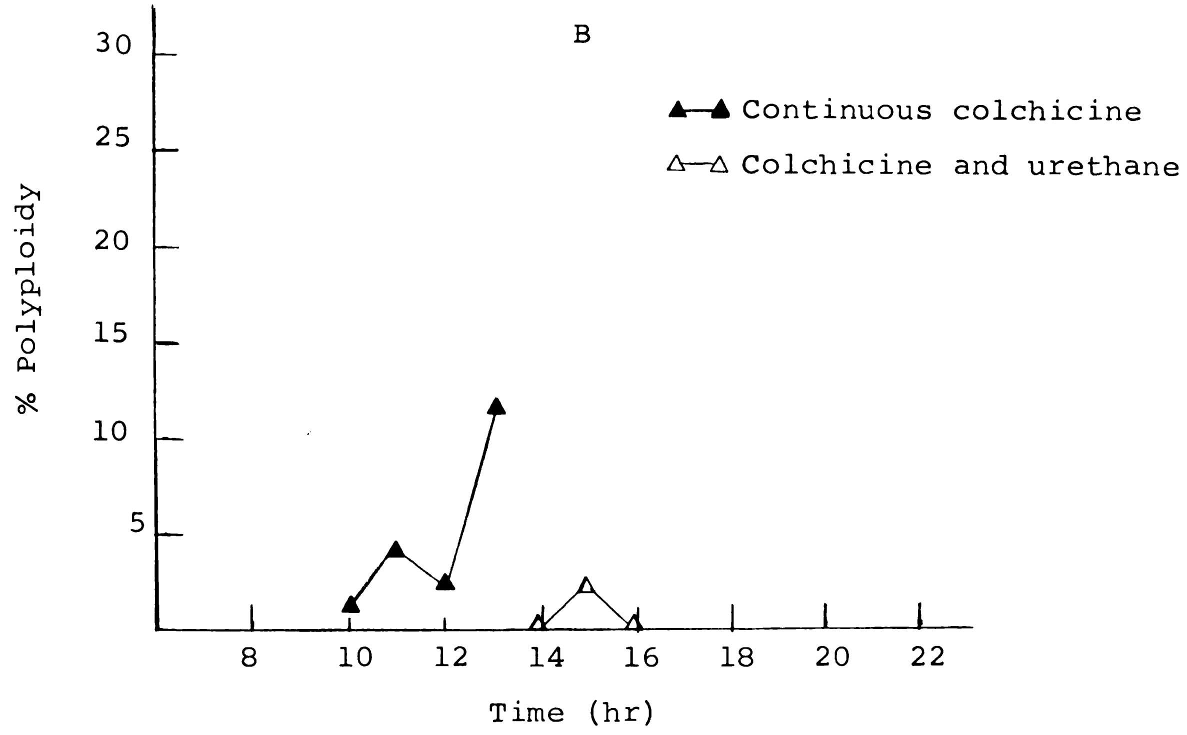


Figure 7.--Percent Polyploidy plotted versus time for (A) continuous colchicine and Isolan®; (B) continuous colchicine and urethane. All at 22.5°C.

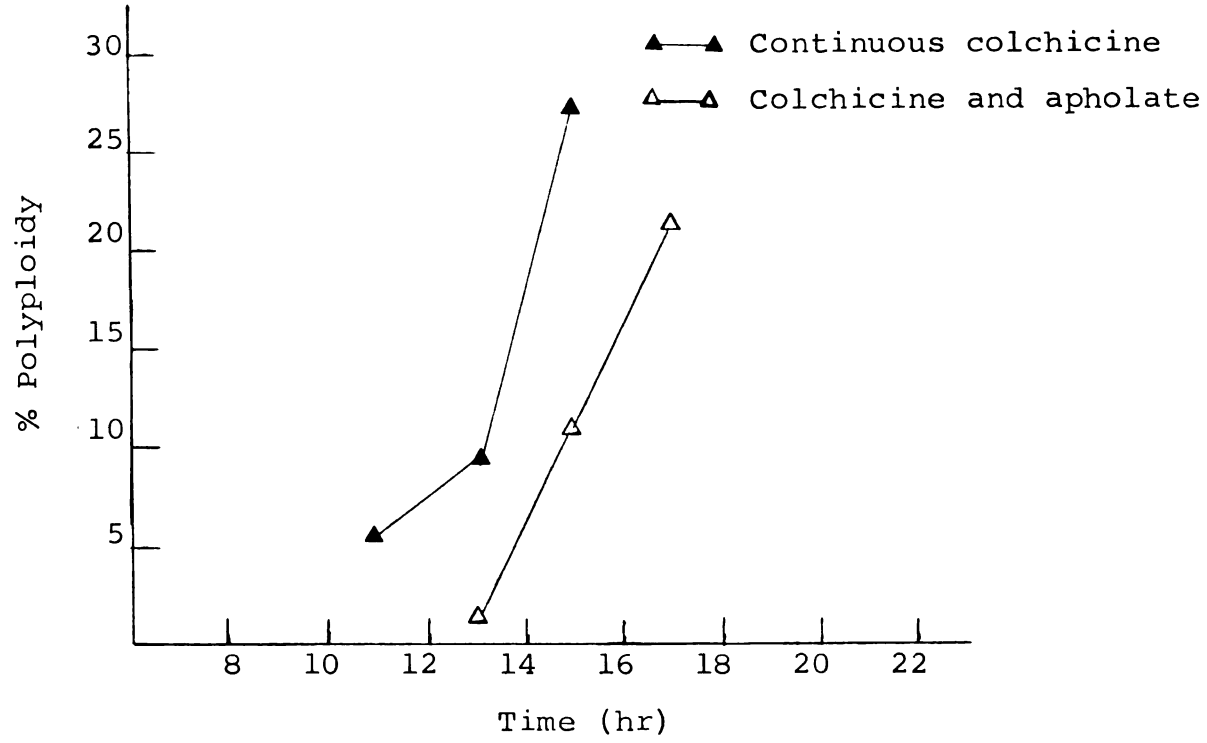


Figure 8.--Percent Polyploidy plotted versus time for continuous colchicine and apholate, both at 22.5°C.

DISCUSSION

In theory, if dividing tissue is continuously treated with the proper concentration of colchicine, the time of appearance of the first significant polyploid cells may be used to estimate mitotic cycle times. This method has been checked for consistency by inducing cycle time changes with different temperatures and several anti-mitotics. At the same time the method was compared with the polyploid tag of Van't Hof _et al_. (1960) and the tritiated thymidime technique. The results reported in this work indicate the method is as reliable as other direct or indirect methods. At 22.5°C, which is the standard temperature for work with the pea root meristem at Michigan State University Cytology Laboratory, some ten repetitions have given a mean mitotic cycle time of 10.5 ± 0.5 hours. The difference may be accounted for by the fact that the continuous treatment method is measuring minimum cycle time, and the tag method is measuring a more average cycle time. Both methods appear more consistent than the tritiated thymidine technique.

It is acknowledged that colchicine may alter the cycle time. Murin (1964), using a 0.1% colchicine solution at 25°C in Vicia, has reported an increase in mitotic cycle time resulting from an increase in c-mitosis and not in interphase. In this regard, several points must be considered: first, the concentration of colchicine used by Murin is sufficiently high to cause toxicity which could result in increased mitosis and cycle time; second, mitosis has been measured in Michigan State University Cytology Laboratory over the past 15 years with some six unrelated mitotic blocking agents--colchicine, actidione, dinitrophenol, iodoacetic acid, and several carbamates--all indicating approximately three hours for mitosis. It has been concluded that "whatever differential stalling occurred was less than the inherent inaccuracies in the tactic" (Wilson, unpublished). For these reasons, the increase in mitosis reported by Murin is probably due to the toxicity of the high colchicine concentration. Other than this, there is no evidence that colchicine alters cycle time as does tritiated thymidine. In any event, the value of the technique is shown by comparing cycle times obtained under contrasting conditions, such as different temperatures.

The change in cycle time per degree change in temperature, is the same for both the continuous and tag methods (Fig. 1). To measure differential effects both methods appear equally valid, and both are more accurate than tritiated thymidine which Van't Hof (1965) has shown decidedly increases mitotic cycle time.

Several drawbacks to the continuous treatment method must be considered in order to evaluate its usefulness. First, it is difficult to determine the beginning of polyploidy, since the colchicine reaction is sigmoidal (Hadder and Wilson, 1958) as is the rate of appearance of polyploidy. However, the minimum cycle time can be estimated by the best straight line drawn along the linear portion of the sigmoid curve. Or, it can be measured from a given level of colchicine reaction to the same level percentage in return of polyploidy (e.g., 2%), in the second cycle. In either case, the estimates are subject to some error and variation. Secondly, the method can only be used for one cycle following treatment. The third drawback is that the continuous treatment method, and probably the tag method, cannot be used with other c-mitotic agents to determine their effect on cycle time.

In the present work, for example, urethane and Isolan® interfered with the colchicine reaction. However, it was still possible to show that these agents did increase the cycle time.

The effects of temperature on mitotic cycle time are not simple. Generally, there appears to be a linear decrease in cycle time from 17°C to 27°C (Fig. 1). Above 27°C, there is no further decrease in cycle time. Over 30°C, mitotic activity is very erratic and the pea root meristem system, in general, appears to be breaking down. A single, well-defined and polyploid peak is characteristic of the tag method at the optimum temperature, i.e. 22.5°C. Above this temperature, the peak becomes less defined and more spread out in time, while at temperatures below 22.5°C, well-defined double peaks are characteristic. If this can be confirmed as characteristic of low temperatures, it would suggest there are different populations of cells present in the meristem which not only have somewhat different cycle times, but may also respond differentially to temperature. If this is true, then temperature could effectively change the kinetics of cell reproduction within a growing meristem. The data presented here suggest further investigations of the reproductive abilities of different parts of the root tip.

The purpose of the work presented here was to determine if a valid measurement of mitotic cycle times could be made by establishing the time of appearance of polyploidy following a continuous treatment of colchicine. It is concluded that with certain limitations this method is as valid as the other methods which have been proposed or used. The major precaution to be considered is that the concentration of colchicine should be sufficiently high to produce a rapid c-mitotic reaction, but low enough to avoid toxicity.

SUMMARY

A method of continuous treatment with a low concentration of colchicine has been shown to be equivalent to the tag method for estimating mitotic cycle times. Both methods are more consistent than the tritiated thymidine technique.

With the continuous treatment method the mitotic cycle time at 22.5°C was 10.5±0.5 hours. An almost linear decrease in cycle time was observed in the temperature range of 17°C to 27°C. At temperatures above and below 22.5°C the mitotic activity becomes erratic. The tag method at low temperatures, e.g. 17°C and 20°C, was characterized by double polyploid peaks. This may indicate cells present in the meristem which have somewhat different cycle times and react differentially to temperature.

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APPENDIX

APPENDIX I

Colchicine Indices of the second hour after treatment

TEMPERATURE	TREATMENT	
	Continuous	Tag
17°C	1.3	1.7
20	1.2	1.6
22.5	1.7	1.7
25	1.9	1.8
27	1.8	1.7
30	1.8	1.8

APPENDIX II

Mitotic Indices and Stage Analyses (%) of the controls at the third and sixth hour

TEMPERATURE	HR	MI	EP	LP	PM	Meta	Ana	Telo
17°C	3	74.2	45.4	15.4	11.9	13.4	6.4	8.5
	6	92.8	46.2	17.7	5.4	15.5	5.9	9.5
20	3	82.8	44.9	15.0	7.2	17.2	9.0	7.5
	8*	80.8	44.7	19.7	4.4	15.5	7.0	8.7
22.5**	3	80.1	45.8	18.9	5.8	15.3	6.6	7.7
	6	78.6	45.9	18.0	6.9	14.4	6.9	8.9
25	3	63.8	45.9	28.7	3.7	13.5	4.4	2.2
	6	42.5	41.5	30.4	2.5	14.9	4.9	6.4
27	3	87.2	44.3	20.5	3.9	16.9	6.5	8.4
	6	86.2	42.8	17.7	6.5	15.2	9.4	9.0
30	3	62.7	45.6	36.8	2.4	6.2	3.4	6.0
	6***	23.5	36.5	36.5	5.5	9.5	7.0	5.0

* Only 8th hour sample was taken.

** All 22.5°C at 3rd and 6th hour were averaged together.

*** Only one slide could be analyzed.

MI = Mitotic Index
EP = early prophase
LP = late prophase
PM = prometaphase
Meta = metaphase
Ana = anaphase
Telo = telophase

APPENDIX III

Percent Polyploidy obtained in the first division with continuous and tag treatments

Temperature		9 hr	10 hr	11 hr	12 hr	13 hr	14 hr	15 hr	16 hr	17 hr	18 hr	19 hr	20 hr
22.5°C	C*	1.2	4.1	2.7	5.7	10.9	--	--	--	--	--	--	--
	T**	--	4.0	8.6	25.8	2.3	--	--	--	--	--	--	--
17	C	--	--	--	--	--	--	--	1.2	0.3	5.8	15.9	--
	T	--	--	--	--	--	--	--	--	2.6	7.2	18.6	24.5†
22.5	C	--	--	--	1.8	17.4	17.0	--	--	--	--	--	--
	T	--	--	--	7.0	21.6	9.1	--	--	--	--	--	--
20	C	--	--	--	--	--	1.6	0.6	3.5	30.6	27.0	--	--
	T	--	--	--	--	--	1.8	14.1	14.1	12.1	21.8	14.5	15.5
22.5	C	--	--	1.9	12.8	9.3	15.1	--	--	--	--	--	--
	T	--	--	--	--	8.5	20.0	1.8	--	--	--	--	--
25	C	0.5	2.2	3.0	15.8	--	--	--	--	--	--	--	--
	T	--	7.7	14.1	18.3	11.6	6.0	--	--	--	--	--	--
22.5	C	--	--	2.6	5.3	16.6	--	--	--	--	--	--	--
	T	--	--	--	0.5	0.3	20.5	6.7	--	--	--	--	--
27	T	1.1	6.5	7.8	6.4	1.0	9.5	--	--	--	--	--	--
22.5	C	--	1.2	4.6	3.1	12.6	--	--	--	--	--	--	--
	T	--	--	4.8	17.7	4.8	--	--	--	--	--	--	--
30	C	14***	7.5	11.7	23.0	--	--	--	--	--	--	--	--
	T	--	0.25	9.3	--	11.25	--	--	--	--	--	--	--

* C = Continuous treatment

** T = Tag treatment

*** = One slide only

† = 17°C T 21 hr. 10.1%; 22 hr. 14.4%; 23 hr. 12.7%.

APPENDIX IV

Percent Polyploidy obtained in the first division with Isolan®, urethane and apholate

TEMPERATURE		10hr	11hr	12hr	13hr	14hr	15hr	16hr	17hr
22.5°C	C	--	--	1.8	17.4	17.0	--	--	--
Isolan®	C	--	--	--	4.3	14.2	18.9	--	--
22.5°C	C	1.2	4.6	3.1	12.6	--	--	--	--
urethane	C	--	--	--	--	0	2.5	--	0
22.5°C	C	--	6.0	--	9.0	--	27.5	--	--
apholate	C	--	--	--	1.0	--	12.0	--	21.0

APPENDIX V

Average Mitotic Indices of controls at the various temperatures

Temp.	0 hr	1 hr	2 hr	3 hr	6 hr	8 hr	10 hr	12 hr	13 hr	14 hr	16 hr	18 hr	20 hr	23 hr
17°C	106.3	93.0	----	74.2	92.8	----	104.3	-----	----	83.3	----	88.0	----	108.3
20	76.0	-----	68.0	82.8	80.8	----	-----	101.0	----	-----	----	90.0	87.0	-----
22.5	80.9	-----	88.3	80.1	78.6	75.4	77.0	-----	93.6	101.7	82.5	----	85.5	-----
25	78.0	89.3	----	63.8	42.5	----	69.3	-----	82.3	-----	----	----	----	-----
27	72.0	101.0	81.3	87.2	86.2	----	-----	89.7	----	73.3	----	----	----	-----
30	63.7	56.0	----	62.7	23.0	----	60.7	71.7	----	-----	----	----	----	-----