

# THE EFFECTS OF VARYING CONCENTRATIONS OF COLCHICINE ON THE PROGRESSION OF GRASSHOPPER NEUROBLASTS INTO METAPHASE

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## ABSTRACT

The effects of four concentrations of colchicine ( $2.5 \times 10^{-7}$ ,  $\times 10^{-5}$ ,  $\times 10^{-3}$ , and  $\times 10^{-2}$  M) on the cell cycle of grasshopper neuroblasts have been determined by direct observations on living cells. The lowest concentration,  $2.5 \times 10^{-7}$  M, does not completely disorganize the spindle but does retard its action. The three higher concentrations disorganize the spindle, so that all cells reaching metaphase are blocked in a *c*-mitotic condition throughout the period of observations (308 min at 38°C, the minimum duration of the cell cycle in untreated neuroblasts). Continuous treatment with all concentrations reduces the rate at which neuroblasts enter metaphase, the extent of the reduction being a function of increasing concentration and time of exposure. After a short exposure to  $2.5 \times 10^{-5}$  M colchicine, the neuroblasts recover from the inhibiting effects on progression through the cycle to metaphase, but they show no recovery from the inhibiting effects on spindle formation for more than 3 hr. Apparent stimulation of progression rate occurs early in exposure to all concentrations and during recovery from a short exposure to  $2.5 \times 10^{-5}$  M. Morphological alterations in the chromatin of telophase, interphase, and prophase cells are induced by the higher concentrations of colchicine. The data indicate that caution should be exercised in the use of colchicine for determining cell cycle duration and/or the effects of physical and chemical agents on the cycle.

## INTRODUCTION

Colchicine and Colcemid (*N*-deacetyl-*N*-methylcolchicine) block dividing cells at metaphase by preventing spindle formation. On the basis of the widely held assumption that this is the only effect of these compounds, they have been used, sometimes in conjunction with tritiated thymidine, to determine the duration of the cell cycle and its phases as well as the effects of radiation and chemicals on the cycle (3, 11, 19, 21, 31, 34, 36, 48). That this assumption is questionable is pointed up by reports of colchicine and Colcemid

effects on prophase progression (17, 20, 24, 43, 44), on the rate at which cells reach metaphase (22, 32, 41), and on macromolecular synthesis (10, 13, 14, 16, 27, 28, 29, 45, 46, 47). In addition, it has been found that colchicine binds to protein at interphase (5, 37) and perhaps to DNA (29). Such data suggest that the effects of colchicine and Colcemid on cell progression may be as universal as their effects on the spindle. Nevertheless, some investigators have reported finding no effect of these two compounds on any phase of

the cycle, except metaphase (30, 36, 42), or on macromolecular synthesis (4, 42).

It is difficult to integrate the positive and negative reports of the effects of colchicine and Colcemid on the cell cycle, because a wide variety of cells, compound concentrations, exposure times, and conditions have been used. Also, with a few exceptions (24, 41, 43) observations on the effects of the compounds on progression have had to be made with cells fixed and stained at intervals during and/or after treatment. Such a method may not permit detection of subtle changes in cell kinetics, especially in cells with long cycles. The present investigation was initiated to determine by direct observations on living grasshopper neuroblasts whether colchicine over a wide range of concentrations alters the rate at which cells enter metaphase. It reveals that colchicine does affect the progression of phases of the cell cycle other than metaphase.

The grasshopper neuroblasts used in this investigation are large cells ( $\approx 25 \mu$  in diameter) that can be easily studied in embryo cultures (7). In the living neuroblast about 20 phases and sub-phases of the cell cycle can be readily identified (6, 9). The neuroblasts, which divide asynchronously, lie in a single layer near the ventral surface and are situated in rows at right angles to the midventral line of the embryo. This arrangement, together with the fact that their relative positions do not change with division, makes it possible to designate ("map") the exact position of given neuroblasts in an embryo and to follow concurrently many individual cells in one or several embryos. With frequent observations, it is possible to make accurate determinations of the time at which each of a large number of neuroblasts reaches metaphase. Such a method reveals small changes in the progression rate of phases prior to metaphase.

Three specific questions were posed in the present study. (a) Does the lowest concentration of colchicine that immediately blocks metaphase affect the rate at which neuroblasts reach metaphase? If so, (b) does the extent of the effect on cell progression vary with concentration of colchicine; and (c) how quickly does the cell recover from a short exposure to colchicine?

#### MATERIALS AND METHODS

Techniques for the preparation of 14-15-day old embryos of the grasshopper *Chortophaga viridifasciata* (De Geer) for hanging drop cultures have been

described in detail by Carlson and Gaulden (7). Only those features pertinent to this study will be described.

#### Preparation of Cultures

A germicidal lamp (predominantly 254 nm ultraviolet radiation) served as the sterilizing agent for instruments, slides, cover glasses, and culture medium. Because ultraviolet radiation alters the colchicine molecule to make it less effective as a mitotic poison (2, 24), the colchicine was not irradiated but was placed in a sterile flask containing either sterilized medium or sterilized water for dilution. The colchicine used in these experiments was purchased from Calbiochem, Los Angeles, Calif. (Lot 800585: grade A quality, 3.65% nitrogen, chromatographically homogeneous). In the first series of experiments a concentration of  $2.5 \times 10^{-5}$  M (0.001% or 10  $\mu$ g/ml) was used because Gaulden and Carlson (24) had shown that this was the lowest concentration that blocked neuroblasts immediately at metaphase. Other concentrations used were  $2.5 \times 10^{-7}$  M (0.00001% or 0.1  $\mu$ g/ml),  $2.5 \times 10^{-3}$  M (0.1% or 1 mg/ml), and  $2.5 \times 10^{-2}$  M (1% or 10 mg/ml).

A hanging-drop embryo culture was prepared as follows. An egg was placed in Shaw's artificial culture medium (39) and the embryo was separated with small knives from the surrounding membranes and yolk. The mouth parts and appendages were cut off close to the body to expose the ventral surface; the neuroblasts lie a few microns beneath this surface. The head and the posterior half of the abdomen were removed to reduce the tissue mass. Thus the remaining piece of embryo consisted of the second and third maxillary segments, three thoracic segments, and the first and second abdominal segments. This piece of embryo was transferred from the dish of dissecting medium (Shaw's 3.3) to a dish of mounting medium (Shaw's 4.5), which contained either the desired amount of colchicine (treated culture) or no colchicine (untreated culture). The piece of embryo was mounted, ventral surface against the cover glass, in a thin film of mounting medium and one-quarter of the yolk in an egg, and the cover glass was sealed over a depression slide with heavy mineral oil. Double depression slides were used so that an untreated and a treated embryo could be placed on the same slide. In one-half of the experiments with a given concentration of colchicine, the control culture was prepared first; 5 min later the treated culture was prepared. In the other half, this procedure was reversed. 20 min after preparation of a slide was completed it was placed on the stage of a microscope enclosed, except for the eyepieces, in a box maintained at 38°C. All observations were made with 10 X eyepieces and a

97 × oil immersion lens with bright-field illumination.

At this point in the procedure the embryos were handled in one of two ways: (a) "Continuous colchicine treatment": the mounting medium was left in the hanging drop for all observations of both treated and untreated cultures. (b) "Short-term colchicine treatment" was used to study recovery from treatment. After 66 min in colchicine, the treated embryos were returned to room temperature for rinsing in medium containing no colchicine. The untreated embryos were similarly handled. After removal from the cover glass, each embryo was given two successive 3-min rinses in dishes of Shaw's 3.3 medium and a third 3 min rinse in Shaw's 4.5 medium. Each embryo was then re-made into a hanging drop preparation and returned to 38°C 2 min before mitotic counting was resumed.

### *Determination of Progression Rate*

To determine the rate at which neuroblasts progress to metaphase in the untreated embryos (continuous treatment experiments), the method described by Carlson and Gaulden (7) was used. This method takes advantage of the fact that the midmitotic period (prometaphase, metaphase, and anaphase) in the neuroblast is of almost constant duration: 22 min at 38°C. Therefore, when the number of cells in midmitosis is counted precisely at 22-min intervals, essentially all cells passing through midmitosis in the observed segments (thoracic) of an embryo will be counted and no cell will be recorded in two successive counts. The number of "counting periods," i.e. 22-min intervals, was limited to 14, which covers 308 min, the minimum duration of the cell cycle of the neuroblast *in vitro*. Thus, no counts were made of cells in the second cell cycle after culture preparation. The first count was made 22 min after the embryo had been placed in the mounting medium. A cell observed in midmitosis is described as having "reached metaphase."

Because colchicine retards or stops cells at metaphase it was necessary to make the mitotic counts for the continuously treated cells as follows. As soon as the slide was placed at 38°C for observations, a "map" was drawn designating the positions of the neuroblasts in the three thoracic segments of an embryo. This operation took 2 min. The first count was made 22 min after the embryo had been placed in the colchicine mounting medium. The number "1" was placed over each cell on the map that was in midmitosis at this first counting period, the number "2" at the second period, etc. Similar counts were made at 22-min intervals for a total of 14 counting periods for all concentrations of colchicine except  $2.5 \times 10^{-2}$  M, which reduced the number of neuroblasts in midmitosis to zero at the fourth

counting period. At the conclusion of the counts the number of cells observed to enter midmitosis at each counting period in each embryo was tallied. This same procedure was used in the short-term treatment experiments except that the cells were mapped in the untreated as well as the treated embryos at room temperature immediately before the cultures were placed at 38°C (2 min before counting began).

It should be emphasized that with this counting method ("neuroblast method") the cumulative number of blocked metaphases is *not* counted. Instead, the number of cells *newly arrived* at metaphase (not at "mitosis") within each 22 min interval is counted.

A total of 88 embryo cultures was used for this study. Previously it had been determined that the number of neuroblasts in 24 untreated and 24 treated segments, i.e. a population of approximately 1500–1800 cells, was sufficient for reliable statistical treatment (25). Therefore, this was the sample size used for each concentration of colchicine. Because of the tedious method required for obtaining data on the colchicine-treated cells, observations had to be limited to one untreated and one treated embryo in a day's experiment (both taken from the same egg case). Data were obtained on a total of 6836 cells that reached metaphase.

### RESULTS

Prior to beginning the present experiments on progression rate we examined the effects of the different colchicine concentrations on the spindle and on chromosome distribution and obtained the same results as were previously reported in detail for living and fixed neuroblasts by Gaulden and Carlson (24). Suffice it to say here that the lowest concentration of colchicine used,  $2.5 \times 10^{-7}$  M, did not completely disorganize the spindle but did retard its action, i.e., cells were temporarily inhibited but not blocked in their progress through metaphase. At this concentration of colchicine cells remained in metaphase from 22 to 242 min; those cells escaping metaphase went into anaphase. The three higher concentrations disorganized the spindle, so that all cells reaching metaphase were blocked in the *c*-mitotic condition throughout the exposure to colchicine; the chromosomes did not show any restitution.

### *Progression Rate*

The raw data obtained on the number of cells progressing to metaphase for each concentration of colchicine and each type of treatment are summarized in Table I.

TABLE I  
Raw Data

Summary of the raw data from which the ratios in Figs. 1-8 and 10-11 were determined. The numerator is the total number of midmitotic cells in colchicine-treated cultures and the denominator is the total number in untreated cultures.

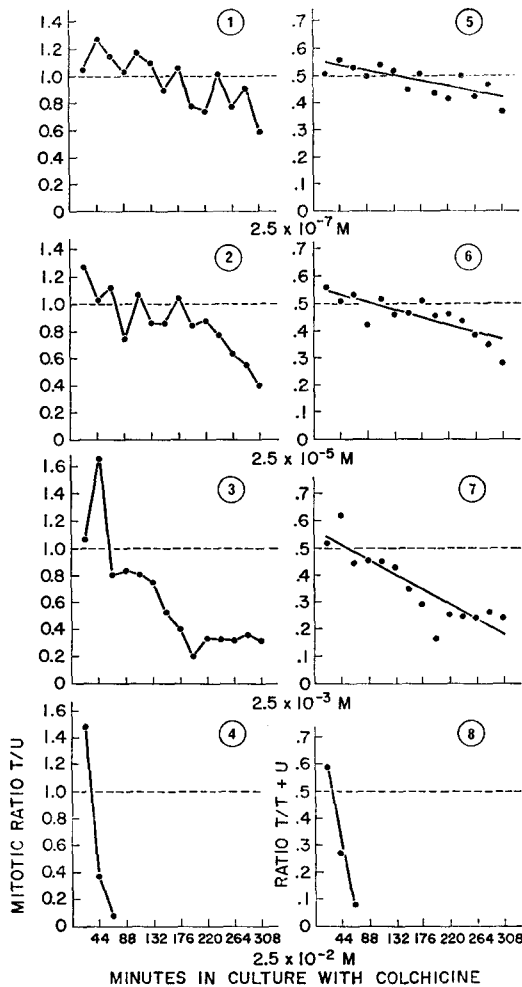
Count	Time in culture	Continuous treatment (Molar concentration)				Short-term treatment (66 min in $2.5 \times 10^{-5}$ M colchicine + 242 min without colchicine)
		$2.5 \times 10^{-7}$	$2.5 \times 10^{-6}$	$2.5 \times 10^{-5}$	$2.5 \times 10^{-2}$	
	<i>min</i>					
1	22	72/68	93/73	99/93	104/70	65/65
2	44	78/61	73/71	125/75	26/69	91/69
3	66	70/61	84/74	55/68	6/70	54/60
4	88	58/56	59/79	56/67	0/64	44/53
5	110	69/58	71/66	57/70		50/34
6	132	62/56	60/69	51/68		49/42
7	154	53/59	53/61	36/69		58/48
8	176	53/50	67/64	28/69		58/48
9	198	44/56	54/64	13/67		60/50
10	220	43/58	60/68	21/62		51/49
11	242	58/57	54/70	23/70		44/47
12	264	39/50	44/69	19/59		47/43
13	286	45/49	40/72	26/73		50/61
14	308	38/63	32/82	21/66		42/43

CONTINUOUS COLCHICINE TREATMENT: Figs. 1-4 show the mitotic ratios obtained for each concentration of colchicine, i.e., the ratio of the number of cells reaching midmitosis in continuously treated embryos to the number in untreated embryos at each counting period. The over-all chi squares for the treated and untreated data at each counting period for each colchicine concentration are given in Table II. These chi squares were partitioned (18) to test whether the data could be fitted to a straight line. In Figs. 5-8 are shown the regression lines fitted to the data by the maximum likelihood method which gave a slightly better fit than the weighted least squares method (1, 12, 33); the points represent the ratio of the number of cells reaching midmitosis in treated cultures to the total number in treated and untreated cultures at each counting period. The chi squares for linear regression were calculated to determine whether the lines have a slope of zero and do not, therefore, differ from the expected constant value of 0.5. In other words, are half of the total number of cells observed to enter midmitosis at each counting period derived from the treated cultures and the other half from the untreated cultures? For

all four concentrations of colchicine the chi squares for regression were highly significant with P values of less than 0.005 (Table II). In all cases the variation (departure) of the data from the regression line was not significant, but the low P values for the three higher concentrations suggest that the departures may represent differential effects of colchicine on parts of the cycle. The reduction in progression rate is reflected in a significant decrease in the total number of cells reaching metaphase over a 5 hr treatment period with all concentrations except the lowest,  $2.5 \times 10^{-7}$  M (Table III). In the latter case, the increased number of treated cells reaching metaphase during the initial exposure period is balanced by the decreased number during the later period (Fig. 1).

Thus, we can conclude that continuous treatment with colchicine reduces the progression rate of neuroblasts at all concentrations from  $2.5 \times 10^{-7}$  M to  $2.5 \times 10^{-2}$  M. When the slope of the regression line is plotted as a function of log molar concentration, it is seen to be a rectangular hyperbola (Fig. 9).

SHORT-TERM COLCHICINE TREATMENT: Because  $2.5 \times 10^{-5}$  M colchicine (0.001%) is the



FIGURES 1-8 show the data obtained from cultures continuously exposed to four concentrations of colchicine. The cultures were placed at 38°C 20 min after preparation, and the first count was made 2 min later.

FIGURES 1-4 Mitotic ratios of the number of neuroblasts reaching metaphase in the treated (*T*) cultures to the number in the untreated (*U*) at 22-min intervals. FIGURES 5-8 Ratios of the number of neuroblasts reaching metaphase in the treated (*T*) cultures to the total number in the treated and untreated (*U*) cultures at each counting period. The solid lines are regression lines fitted to the data by the maximum likelihood method.

lowest concentration that causes immediate disorganization of the spindle in the neuroblast, it was selected for determining the extent of recovery of cells from treatment. Neuroblasts were exposed to colchicine for 66 min, that is, for three counting periods or 18% of the average cell cycle time. The

“recovery period” has been designated as that time from 110 to 308 min in culture (Fig. 10). The fourth count (88 min) was not included because the cultures were being rinsed at room temperature during the 20 min immediately preceding this count.

Fig. 10 shows that during the first part of the recovery period the mitotic ratios increased considerably and then decreased. In Fig. 11 is shown the regression line fitted, as described above, to the data for the recovery period; the chi square for the regression line has a *P* value between 0.05 and 0.025 (Table II). Thus, when the external source of colchicine is removed, neuroblasts proceed into metaphase at a faster rate in the first part of the recovery period than do untreated cells. A similar but shorter lived “stimulation” of progression observed following long, low dose rate exposure of neuroblasts to gamma radiation has been shown to result from the simultaneous recovery of cells retarded in mid- and late prophase by the radiation (8). That this explanation is probably applicable to part of the cells recovering from colchicine is suggested by the fact there appeared to be more cells in mid- and late prophase at the 88 min count in the treated than in the untreated cultures (actual counts were not made). Further, Gaulden and Carlson (24) have reported that  $2.5 \times 10^{-6}$  m colchicine inhibits neuroblasts when they reach very late prophase, evidently by retarding breakdown of the nuclear membrane. There may, however, be some real acceleration of progression involved during the recovery period (see below).

Although removal of the colchicine resulted in a rapid recovery from inhibition of progression rate, there was no recovery from the spindle-inhibiting effects. A *c*-mitotic condition occurred and persisted in all cells reaching metaphase throughout the recovery period. Evidently enough colchicine remained in the cells to prevent spindle formation for at least 3 hr after rinsing.

#### Possible Stimulation of Progression

One interesting aspect of the data in Figs. 1-4 and Fig. 10 is that, for all concentrations of colchicine, the mitotic ratios increase before they begin to decrease. The maximum ratio was observed at either 22 or 44 min of exposure and was greater for the two higher than for the two lower concentrations. In Figs. 5-8 all the regression lines begin above 0.5. In other words, there appears to be a

TABLE II  
Results of Statistical Analysis

Concentration of colchicine	Source of variation	Degrees of freedom	Chi square	P value
<b>I. Continuous</b>				
$2.5 \times 10^{-7}$ M	Over-all value	13	15.434	
	Due to linear regression	1	9.303	0.005-0.001
	Departure from regression line (by subtraction)	12	6.131	0.95-0.90
$2.5 \times 10^{-5}$ M	Over-all value	13	39.206	
	Due to linear regression	1	21.651	<0.001
	Departure from regression line (by subtraction)	12	17.555	0.20-0.10
$2.5 \times 10^{-3}$ M	Over-all value	13	112.830	
	Due to linear regression	1	98.930	<0.001
	Departure from regression line (by subtraction)	12	13.900	0.40-0.30
$2.5 \times 10^{-2}$ M	Over-all value	2	74.689	
	Due to linear regression	1	73.203	<0.001
	Departure from regression line (by subtraction)	1	1.486	0.30-0.20
<b>II. Short term</b>				
$2.5 \times 10^{-5}$ M (counts 5-14)	Over-all value	9	5.837	
	Due to linear regression	1	4.189	0.05-0.025
	Departure from regression line (by subtraction)	8	0.648	0.99

TABLE III  
Total Number of Cells Reaching Metaphase in Colchicine-Treated and Untreated Embryos

Concentration	Time in colchicine <i>min</i>	Number of cells		Chi square*	P value
		Treated cultures	Untreated cultures		
<b>I. Continuous</b>					
$2.5 \times 10^{-7}$ M	308	782	802 (80%) ‡	0.5	0.50-0.40
$2.5 \times 10^{-5}$ M	308	844	982 (99%)	19.39	<0.001
$2.5 \times 10^{-3}$ M	308	630	976 (98%)	122.65	<0.001
$2.5 \times 10^{-2}$ M	110	136	209	25.49	<0.001
<b>II. Short term</b>					
$2.5 \times 10^{-5}$ M	66 §	509	465	4.16	0.05-0.025

\* One degree of freedom.

‡ Per cent of untreated cell population that reached midmitosis in 308 min.

§ At the end of 66 min the colchicine medium in the hanging drop was replaced with culture medium containing no colchicine, and observations were continued for 11 more counting periods to give a total observation time of 308 min.

|| The number of cells reaching midmitosis during the recovery period (110-308 min).

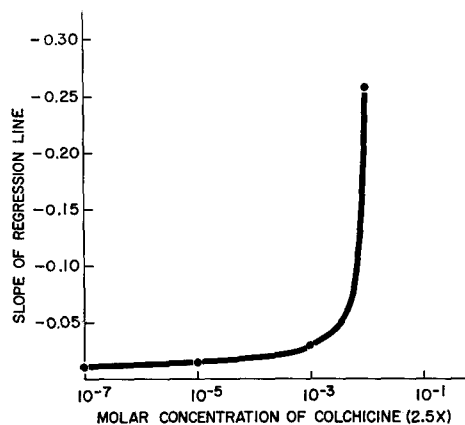


FIGURE 9 The slopes of the regression lines in Figs. 5-8 are plotted against the logarithm of colchicine concentration. A hyperbola was fitted to the points by weighted least squares as used by Mounter and Turner (35) with a correlation coefficient in excess of 0.999. Slope =  $A_1/(y + A_2)$  where  $A_1$  and  $A_2$  are constants and  $y = \log 2.5 - \log(\text{colchicine concentration})$ .

brief initial period of mitotic stimulation followed by a period of inhibition.

During the recovery period after a short exposure to colchicine (Figs. 10-11), more neuroblasts reached metaphase in the treated than in the untreated embryos (Table III). Part of this can be undoubtedly explained as a release of prophase cells that had piled up during treatment (see above), but part of it may represent a real acceleration of some phases of the cycle, because more cells reached metaphase in the treated than in the untreated cultures during the whole 308 min (one cell cycle) of observations.

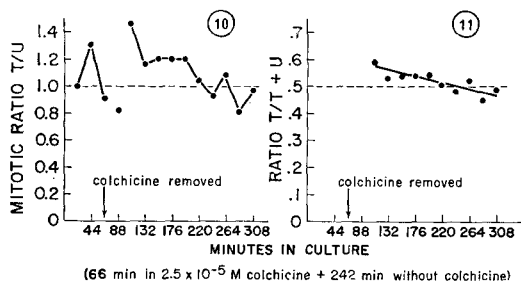
### Effects on Phases of the Cycle

For interpretation of the mitotic ratios observed above, a study is presently underway to determine what phases of the neuroblast cycle are affected by colchicine. Individual neuroblasts in all phases are mapped in an embryo culture. Colchicine medium is then added to the culture and frequent observations are made on each cell for 1.5-5 hr, depending on the concentration of colchicine. The effects of two concentrations have been studied so far:  $2.5 \times 10^{-2}$  and  $2.5 \times 10^{-3}$  M. The details, which will be reported elsewhere along with the effects of  $2.5 \times 10^{-5}$  and  $2.5 \times 10^{-7}$  M, can be summarized as follows:

During the first hour in the highest concentra-

tion, the chromosomes in all prophase cells, except late prophase, underwent a partial decondensation so that superficially they resembled untreated chromosomes at late telophase. On the other hand, the chromatin of cells initially treated in telophase and interphase became partially condensed. Therefore, the chromatin of all phases of the cell cycle, except metaphase, was at the end of 1 hr in a partially extended and partially condensed condition. Thus, the drastic mitotic inhibition induced in neuroblasts by  $2.5 \times 10^{-2}$  M colchicine (Fig. 4) resulted from effects of the compound on all phases of the cell cycle.

Exposure to  $2.5 \times 10^{-3}$  M caused several effects on prophase cells: some cells were not retarded, some were retarded with no morphological changes, and some were "reverted" and thereby were greatly retarded. This variation in response is reflected in a much slower decline in mitotic ratio (Fig. 3) than was observed with  $2.5 \times 10^{-2}$  M. The chromosomes of interphase and telophase cells underwent erratic condensation during the first hour of treatment so that each chromosome had at random throughout its length a variety of diameters ranging from that of a fine thread to that typical of the highly condensed chromosomes



FIGURES 10 and 11 show data obtained from short-term colchicine-treated embryos (exposed for 66 min). The cultures were placed at  $38^\circ\text{C}$  20 min after preparation, and the first count was made 2 min later. They were returned to room temperature between the 66- and 88-min counts for rinsing in medium without colchicine. The "recovery period" is designated as that period from the 110- through the 308-min counts.

FIGURE 10 Mitotic ratios of the number of neuroblasts reaching metaphase in the treated ( $T$ ) cultures to the number in the untreated ( $U$ ) at 22-min intervals. FIGURE 11 Ratios of the number of neuroblasts reaching midmitosis in the treated ( $T$ ) cultures to the total number in the treated and untreated ( $U$ ) cultures during the recovery period. The solid line is the regression line fitted to the data by the maximum likelihood method.

of very late prophase. These interphase and telophase cells did not show progression and did not, therefore, enter metaphase during the 5 hr observation period.

A concentration of  $2.5 \times 10^{-5}$  M colchicine was reported by Gaulden and Carlson (24) to cause a temporary reversion of the chromosomes of some mid- and late prophase cells. The reversion was not so extensive as that described above for  $2.5 \times 10^{-2}$  M, in that the chromatin resumed the appearance characteristic of that of early prophase nuclei and then moved through prophase a second time to reach metaphase about 3 hr after initiation of treatment. Untreated cells in these phases reach metaphase in 30–60 min.

We can conclude that colchicine at the higher concentrations causes morphological alterations in the chromatin of telophase, interphase, and prophase cells. The extent of the changes is related to concentration of colchicine. The changes observed so far have resulted in retardation or blockage of progression. Retardation is, however, not always accompanied by detectable chromatin alterations. Colchicine also causes the chromosomes in cells first treated while in late prophase and some in midprophase to condense more rapidly than normal—this may cause the cells to move into metaphase at an accelerated rate.

#### DISCUSSION

The mitotic ratio data presented here show that colchicine over a  $10^4$ -fold range of concentrations affects the progression rate of cells in portions of the grasshopper neuroblast division cycle in addition to metaphase. Observations on individual cells treated at various phases of the cycle confirm this conclusion and reveal that all phases of the cycle are subject to colchicine effects, at least at the two higher concentrations. The sensitivity of the neuroblast-counting method permits detection of subtle changes in progression rate that may not be revealed by fixing cells at intervals during and/or after colchicine exposure. The latter method only measures the progressive accumulation of blocked metaphases.

To compare the results obtained with the neuroblasts to those obtained with the fixed cell method by other workers, the data in Table I were used to calculate cumulative midmitotic counts with time in colchicine at the three lower concentrations. In Figs. 12–14 these cumulative counts have been plotted as Puck (36) did for mammalian cells

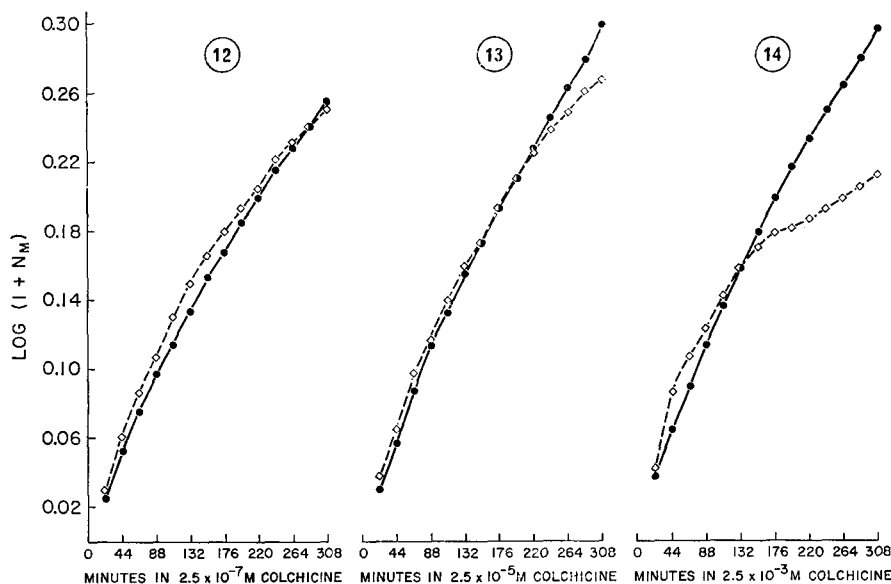
in vitro. Two points are of interest. First, the accumulation rate for the untreated neuroblasts is not a straight line function, i.e., without colchicine the progression rate of neuroblasts gradually slows down with time in culture. This presents no difficulty in interpretation when mitotic ratios in treated/untreated cultures are used. Unlike the mammalian cells, the neuroblasts had recently been removed from their in vivo environment and were not, therefore, adapted to in vitro conditions. Second, the increased number of treated neuroblasts reaching metaphase during the initial period of exposure masks, especially in the case of  $2.5 \times 10^{-7}$  M, the decreased numbers of the later period. Thus, the limits of the mitotic “stimulation” effects and the full extent of the inhibiting effects of colchicine are not revealed as clearly by the cumulative count method as by the neuroblast mitotic ratio method, in which the calculations for each point are independent of the previous ones. The latter method detects every cell that reaches metaphase over a given period so that an increase or decrease in mitotic ratio is known to reflect a change in the rate at which cells progress to this point in the cycle. With the cumulative method, on the other hand, it is difficult, if not impossible in some cases, to determine whether a drop in accumulation rate is caused by the entrance of fewer cells into metaphase or by the escape of cells from blocked metaphase.

With the neuroblast data in mind, let us review three reports of no Colcemid or colchicine effects on premetaphase progression (30, 36, 42), two of inhibiting effects (22, 41), and one of stimulation (45).

The straight-line function of blocked “mitotic” cell accumulation obtained by Puck (36) for Chinese hamster and S3 HeLa cells indicates that Colcemid did not selectively affect any stage of the cycle except mitosis. However, this held only for low concentrations of Colcemid, namely, 0.05  $\mu$ g/ml. Under the term “mitotic” were evidently included prophase cells, so that this stage as well as metaphase may have been blocked. Prophase, as usually defined in mammalian cells, is comparable to the latter part of middle and all of late prophase as defined in the neuroblast (6), both of which are affected by colchicine.

Taylor (42) exposed human cells, strain K.B., in vitro to several concentrations of colchicine. On the basis of the rate of increase in the per cent of fixed cells in mitosis with time in  $10^{-7}$  M colchi-





FIGURES 12-14 The effects of colchicine on the progression rate of neuroblasts as revealed by cumulative counts plotted as  $\text{Log} (1 + N_M)$ , where  $N_M$  is the fraction of the cell population in midmitosis (prometaphase, metaphase and anaphase, or blocked metaphase), at intervals equal to the duration of the midmitotic period.  $\bullet - \bullet$ , untreated;  $\diamond - \diamond$ , treated.

cine, he concluded that colchicine had no effect for 11 hr on the movement of cells through the cycle, exclusive of the mitotic period (doubling time: 24 hr). Under the category of "mitosis" were included cells in normal prophase, metaphase, anaphase, and blocked metaphase. When only the accumulation of blocked metaphases was plotted against time in  $5 \times 10^{-7}$  M colchicine (his Fig. 6), a break occurs in the curve after only 30 min exposure, which indicates a change in the rate at which cells are entering metaphase. This concentration blocks all metaphases within 5 min of the beginning of treatment. A less abrupt break is observed after 90 min exposure to  $2 \times 10^{-7}$  M, which did not block all metaphases until 50 min. When the present data on the neuroblast are plotted as cumulative per cent of cells in midmitosis with time in colchicine, the decreased numbers of treated cells reaching metaphase are reflected in breaks in the curves, but they occur at much later times than are revealed by plotting the mitotic ratios. Taylor could not determine whether the break was caused by degeneration of blocked cells, by restitution of blocked cells (reformation of interphase nuclei), or by inhibition of earlier phases. In the neuroblast there is no question that the break is caused by inhibition of earlier phases.

Kleinfeld and Sisken (30) interpret their data on the rate of increase of per cent of HeLa cells in mitosis with time in  $10 \mu\text{g/ml}$  Colcemid and colchicine as showing that there is no effect on progress into metaphase. Cumulative counts of fixed blocked metaphases decreased at  $1\frac{1}{4}$  hr of treatment with colchicine and then increased at  $2\frac{1}{2}$  hr (their Fig. 3). They observed a lag period of 1 hr in colchicine before cells became blocked at metaphase. Therefore, if colchicine does not alter the rate of entrance of cells into metaphase, the per cent of mitoses should not drop at  $1\frac{1}{4}$  hr but should at least remain at the untreated level or show some increase. The decrease at  $1\frac{1}{4}$  hr suggests that there may be some initial inhibiting effect of colchicine on these cells.

Retardation of the cell cycle by both Colcemid and colchicine has been reported for two mammalian cell types. From time-lapse film records, Stubblefield (41) analyzed the effects of  $1.6 \times 10^{-7}$  M Colcemid on "Don" strain Chinese hamster cells. He found that the number of blocked metaphases increased at a logarithmic rate for 6-7 hr, after which the accumulation rate decreased considerably, so that the generation time was increased to 20 hr as compared to 12 hr for untreated cells. He suggests that the break in the

accumulation curve is caused by some unspecified effect on the early stages of DNA synthesis. Fitzgerald and Brehaut (22) showed that exposure of phytohemagglutinin (PHA)-stimulated human lymphocytes to 10 or 15  $\mu\text{g}/\text{ml}$  colchicine for 1–6 hr caused a decreased rate of mitotic accumulation that was not seen at lower concentrations. This decrease was associated with a decrease in the relative numbers of cells in S (tritiated thymidine) and  $G_2$  and an increase in the numbers of cells in  $G_1$  and M. They conclude that colchicine at the higher concentrations depresses DNA synthesis of cells already in S and inhibits the entry of  $G_1$  cells into S, thereby altering progression rate. In the grasshopper neuroblast colchicine retards or blocks, depending on concentration, cells in S. A colchicine-induced reduction or blockage of labeled DNA-precursor uptake has been reported for other cell types (16, 27, 28, 29, 47).

In contrast to the inhibiting effects of colchicine on premetaphase portions of the cycle is the stimulating effect reported by Davidson and co-workers (15, 32). Webster and Davidson (45) have made a detailed study of the effects of 0.025% colchicine on *Vicia faba* root tip cells fixed at intervals during recovery from a 3 hr treatment (21% of cycle time). In addition to an elevated mitotic index for 21 hr, there is, at the 9- and 12-hr intervals, an increased number of cells in prophase as well as in metaphase. Deysson (17) has interpreted the increased numbers of prophase cells in fixed onion meristems as indicating a prolongation of this phase. He points out that the increase is observed even at concentrations that do not completely block cells at metaphase. Wada (44) has observed a lengthening of prophase in living *Tradescantia* stamen hairs exposed to 0.05 and 0.1% colchicine, and a similar response of some prophase cells occurs in grasshopper neuroblasts in  $2.5 \times 10^{-2}$  and  $2.5 \times 10^{-3}$  M colchicine (see above) as well as in  $2.5 \times 10^{-5}$  and  $2.5 \times 10^{-6}$  M (24). That the "stimulation" reported by Webster and Davidson may be real is suggested by the fact that they found significantly higher grain counts over tritiated thymidine-labeled, treated cells than over untreated cells. This evidence for an increased rate of DNA synthesis strengthens their conclusion that colchicine causes cells to complete interphase faster than untreated cells, so that they move into prophase at a more rapid rate. An enhancement of macromolecular synthesis by

colchicine in several cell types has been found by other workers (10, 29, 46).

In the present study an apparent acceleration or stimulation of progression rate was observed at two points: early in exposure to all concentrations of colchicine and during the recovery from a short exposure to  $2.5 \times 10^{-5}$  M (18% of cycle time). The first instance probably resulted from the accelerated condensation induced by colchicine in some mid- and late prophase chromosomes. This phenomenon has also been observed in neuroblasts exposed to slightly hypertonic culture medium, which has been shown to accelerate the rate at which cells reach metaphase (23). In the second instance, the increased number of cells reaching metaphase during recovery from colchicine probably represents in part a release from prophase inhibition and in part a real acceleration. Further observations on cells treated at all phases of the cycle are needed for clarification.

The slow recovery of the spindle from colchicine treatment observed in neuroblasts has also been reported for other cells (30, 42, 45) and can probably be explained by data obtained with tritiated colchicine (5, 37, 40, 42). Taylor (42) showed that, in human cells (strain K. B.), tritiated colchicine was bound to a cell site, and that this bound form was only slowly lost when cells were resuspended in colchicine-free medium. He concluded that if only 3–5% of the sites are complexed with colchicine, the cell is unable to form a functional spindle. Borisky and Taylor (5) later showed that the binding site was a 6S protein, and they suggested that it is a subunit of spindle microtubules. On the basis of the fact that a 6–8 hr exposure to  $10^{-7}$  M colchicine resulted in blocking of metaphase cells for 24 hr, Taylor (42) felt that colchicine was bound in interphase cells. Robbins and Shelanski (37) recently reported that tritiated colchicine in HeLa cells binds to both 2–3S and 6S proteins, which they view as interconvertible and as subunits of microtubule protein, and that these proteins are continuously synthesized throughout interphase. Therefore, removal of colchicine from culture medium would not result in recovery of spindle action for a period at least equal to the interval from interphase to metaphase. In the neuroblast this interval includes the telophase immediately preceding interphase.

The cell site(s) affected by colchicine or the nature of the effects that change cell progression rate are not known. The fact that these effects in

the neuroblast are accompanied by visible alterations in the physical state of chromatin leads to the speculation that some kind of interaction of colchicine with the chromosomes may be involved. Ilan and Quastel (29) have reported that colchicine alters the optical rotation of DNA in vitro and have suggested that it may combine or complex with DNA. Such a complexing in the cell might account for the chromosomal alterations observed in the neuroblast. The induction of visible changes in the chromosomes by colchicine also raises the possibility that at certain concentrations it may alter chromosomal response to radiation. Sharp et al. (38) showed that X-irradiated human lymphocytes had the same frequency of dicentric chromosomes when exposed to an unspecified concentration of colchicine for 4 as for 24 hr. Whether the same frequency would be found with no colchicine treatment remains to be determined.

#### CONCLUSIONS

Data on the grasshopper neuroblast and other cells demonstrate that the effectiveness of colchicine in altering progression through the premetaphase portions of the cycle varies with concentration, with exposure time, and with different types of cells, even with different strains from the same organism. Thus, in the absence of conclusive evidence to the contrary, it cannot be assumed for a given cell type that any concentration of colchicine will only affect the metaphase or the longer so-called mitosis portion of the cell cycle. Although Colcemid is reported to be less "toxic" than colchicine (26, 28, 30), it has been shown to affect the progression of at least one cell type at a relatively low concentration (41).

Within the  $10^4$ -fold range of concentrations used in the present study, colchicine induced not only inhibition but some stimulation that occurred during the first hour of treatment. There was also some apparent stimulation during recovery from a short exposure. Differential stimulation, like inhibition, of some phases could cause a *mixing of the normal order in which cells reach metaphase*, a factor that may be of importance, for example, in determining the frequencies of chromosome aberrations induced at the various phases of the cycle by radiation. A brief exposure to colchicine cannot be assumed to be without effects on premetaphase progression.

Therefore, we join others (22, 45) in cautioning

that the effects of colchicine on the cell are not always limited to the metaphase spindle.

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