5-AMINOURACIL TREATMENT

A Method for Estimating G_2

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ABSTRACT

Treatment of *Vicia faba* lateral roots with a range of concentrations of 5-aminouracil (5-AU) indicate that cells are stopped at a particular point in interphase. The timing of the fall in mitotic index suggests that cells are held at the S − G_2 transition. When cells are held at this point, treatments with 5-AU can be used to estimate the duration of G_2 + mitosis/2 of proliferating cells. Treatment with 5-AU can also be used to demonstrate the presence of subpopulations of dividing cells that differ in their G_2 duration. Using this method, 5-AU–induced inhibition, we have confirmed that in *V. faba* lateral roots there are two populations of dividing cells: (*a*) a fast-dividing population, which makes up ≈85% of the proliferating cell population and has a G_2 + mitosis/2 duration of 3.3 hr, and (*b*) a slow-dividing population, which makes up ≈15% of dividing cells and has a G_2 duration in excess of 12 hr. These estimates are similar to those obtained from percentage labeled mitosis (PLM) curves after incorporation of thymidine-^{3}H.

INTRODUCTION

5-aminouracil (5-AU), an analogue of both thymine and uracil, has been shown to (*a*) reduce the rates of synthesis of DNA and RNA, (*b*) reduce the mitotic index, and (*c*) result in waves of partially synchronized mitoses. Jakob and Trosko (1) have shown that DNA synthesis occurs at a reduced rate in roots of *Vicia faba* treated with 5-AU, but it is difficult to estimate accurately the extent to which synthesis is slowed down. The important point is that DNA synthesis does continue during treatment. Since cells continue to enter (3, 6) and leave S (1, 4), even in the presence of 5-AU, it does not seem that a slowing down of DNA synthesis is completely responsible for either the fall in mitotic index observed initially or the subsequent synchronization of cell division. The effect of 5-AU on levels of mitotic activity must be due to a block in the cell other than in S itself.

It has been suggested that cells are held in G_2 (4).[1] In the study reported here, (*a*) the regularity in the pattern of the drop in mitotic activity and (*b*) the timing of the fall in mitotic index suggest that cells in G_2 at the beginning of treatment are not blocked by 5-AU but proceed through to mitosis and into G_1 at normal rates. The timing of the initial fall in mitotic index indicates that cells are held at the S − G_2 transition. The regularity of the effects induced by 5-AU provide a method for (*a*) estimating the duration of G_2 + mitosis/2 of the dividing population and (*b*) demonstrating the

[1] S. Wolff and H. Luippold. 1964. Unpublished (quoted by Mattingly, 1966).

presence in a growing tissue of subpopulations of cells that differ in the duration of their G_2.

MATERIALS AND METHODS

Seeds of *V. faba* L. were soaked in distilled water for 24 hr. The testae were removed and the seeds were placed in moist sand. The beans were grown in sand until the primary roots were about 6 cm long. The beans were then washed and suspended over tanks containing half-strength Hoagland's solution. The beans were grown in the dark and maintained at 20 ± 1°C. The culture solution was continuously aerated and changed every 24 hr.

When lateral roots had been produced, the whole root systems were treated with 5-AU (Nutritional Biochemicals Corp., Cleveland, Ohio). The concentrations of 5-AU used were: 75 ppm (5.90×10^{-4} M), 500 ppm (3.93×10^{-3} M), and 1500 ppm (1.18×10^{-2} M). In experiment A, roots were incubated in 500 ppm 5-AU for up to 24 hr; fixations were made after 6, 12, 18, and 24 hr of continuous treatment. In experiment B, roots were treated with 75 ppm, 500 ppm, and 1500 ppm 5-AU for up to 6 hr. Roots were fixed at hourly intervals from 2 to 6 hr.

Roots were fixed in a chilled mixture of 3 parts absolute alcohol to 1 part glacial acetic acid, containing a few drops of formalin. These fixed roots were then washed, hydrolyzed in 1 N HCl at 60°C for 9 min, stained by the Feulgen method, and prepared as permanent squash preparations.

RESULTS

Experiment A: Treatment for up to 24 hr

In meristems of lateral roots treated with 500 ppm 5-AU for up to 24 hr, there are, first, an inhibition of mitosis and, subsequently, a recovery of mitotic activity (Table I). From percentage labeled mitosis (PLM) curves it was estimated that G_2 + mitosis lasts 5 hr in the fast-dividing subpopulation of cells in *V. faba* lateral root meristems, and also that about 20–25% of the dividing cells in the meristem are slow dividing (2, 9). After a 6 hr exposure to 5-AU, which is approximately the expected duration of G_2 + mitosis of the fast dividing cells, the mitotic index (MI) is 28.8% of the control value (Table I). Therefore, in a period equal to G_2 + mitosis the inhibitory effect of 5-AU has reduced the MI to the level that would be expected if the only cells in division were those of the slow-dividing population.

TABLE I

Mitotic Index in Roots Treated with 500 ppm 5-AU and in Control Roots

t	5-AU	Control
6	2.08 ± 0.85	7.23 ± 2.09
12	0.80 ± 0.59	5.33 ± 3.35
18	0	—*
24	3.40 ± 1.64	6.40 ± 1.86

* Not determined.

Time (t) was measured, in hours, from the beginning of 5-AU treatment.

Values are based on samples of 3000–6000 cells.

A further reduction in mitotic activity is seen after a 12 hr 5-AU treatment. Although mitoses are not frequent (MI = 0.80), they are still present. These should represent cells of the slow-dividing population. 18 hr after the beginning of treatment, a complete suppression of mitosis is observed. It has been shown that some cells of the slow-dividing population in *V. faba* roots have a G_2 duration of about 18 hr (9). G_2 cells of both the fast- and slow-dividing subpopulations appear to be unaffected by 5-AU. From these results it is suggested that 5-AU does not affect the progression of cells in G_2 through G_2 and mitosis.

Experiment B: Treatment for up to 6 hr

In order to test this hypothesis, roots were exposed to a range of concentrations of 5-AU and the MI was determined every hour. This enabled us to determine (*a*) the pattern of the fall in MI and (*b*) whether the fall in MI was faster with higher concentrations of 5-AU, which would indicate whether 5-AU does have an effect in G_2.

Since the progression of cells through mitosis follows the known sequence of prophase, metaphase, anaphase, and telophase, an analysis of the disappearance of cells from mitosis can be used to determine whether cells are stopped at a particular point in the cell cycle. Thus, if the disappearance of cells from the various stages of mitosis shows a regular progression, the time period over which the drop in MI occurs can be used to estimate the point in the cycle where cells are blocked.

Both the timing and the pattern of the drop in the frequency of mitoses are similar in lateral roots treated with 75 ppm, 500 ppm, and 1500 ppm 5-AU (Fig. 1). The drop in MI is linear from 2 to 4 hr and shows the beginning of a plateau from 4 to 6 hr.

The disappearance of cells from the different stages of mitosis is regular with time (Table II).

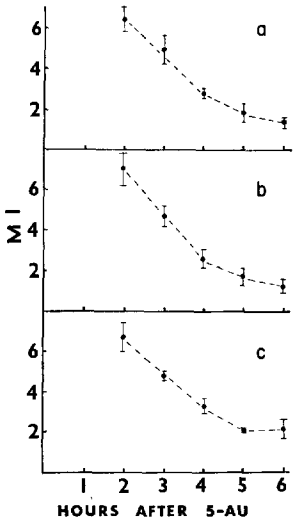

FIGURE 1 Mitotic index (*MI*) in lateral roots of *V. faba* treated for up to 6 hr with 5-aminouracil. *a*, 500 ppm; *b*, 1500 ppm; *c*, 75 ppm.

This would be expected if the progression of cells through mitosis is unchanged and if the block induced by 5-AU occurs at a particular point in the cell cycle prior to mitosis. For the first 3 hr of 5-AU treatment, the fall in MI is due almost entirely to a reduction in the frequency of cells in prophase. At 4 hr, a real drop is observed in the frequency of cells in metaphase and anaphase. It is not until the end of a 5 hr treatment that a real reduction in the number of cells in telophase is observed. It must be noted that from 4 to 6 hr the number of cells in prophase remains constant and that from 5 to 6 hr the number of cells in metaphase remains constant. Essentially the same pattern and timing of the disappearance of cells from the different stages of mitosis were observed in roots treated with 75 ppm and 1500 ppm as with 500 ppm 5-AU (Table II).

Since (*a*) the frequency of cells in prophase remains constant from 4 to 6 hr, (*b*) the percentage of cells in prophase at 4, 5, and 6 hr is about 20% of the control frequency, that is, equal to the number of cells of the slow-dividing population that should be in division at any one time, and (*c*) the duration of G_2 + prophase of the fast-dividing population of cells in lateral of *V. faba* is about 4 hr (9), it appears that 5-AU does not inhibit the progression of cells in G_2 at the beginning of treatment through G_2 and mitosis. Furthermore, cells

TABLE II

Mitotic Index and Frequencies of Cells, per 5000 Cells Scored, in the Various Stages of Mitosis in (A) Roots Treated with 500 ppm 5-AU, and (B) in Untreated Roots

t	P	M	A	T	MI
A					
2	220	31	49	50	7.00 ± 0.64
3	119	24	58	59	5.00 ± 0.76
4	51	10	31	62	2.88 ± 0.23
5	49	5	13	25	1.84 ± 0.50
6	49	4.5	9	10	1.45 ± 0.28
B					
2–6	272.8	27.8	60.4	57.4	8.37

At least 5000 cells were scored at each fixation.
Time (t) was measured, in hours, from the beginning of treatment with 5-AU.
P, prophase; M, metaphase; A, anaphase; T, telophase.

enter and leave S in the presence of 5-AU (1, 3, 4, 6); the treatment reduces the rate at which the cells progress through S (1). The initial drop in MI must be due, therefore, to cells being held at a particular point in the cell cycle between S and G_2. It is suggested that cells are held at the S – G_2 transition point.

If cells are initially held by 5-AU at this point in the cycle, short treatments with this analogue can be used to estimate (*a*) the duration of G_2 + mitosis/2 and (*b*) the relative proportions of slow- and fast-dividing cells in a proliferating population if the difference in cycle time is due to a longer G_2 duration in the slow-dividing population. These parameters can be estimated from the initial drop in MI observed during 5-AU treatment. The fall in MI should be followed until the MI remains constant, that is, at zero, if there is heterogeneity in G_2. Values that remain constant over 3 hourly fixations should be sufficient.

DETERMINATION OF G_2 + MITOSIS/2: The calculation of this parameter is similar to the calculation of G_2 + mitosis/2 using the percentage labeled mitosis method. The time interval between the beginning of 5-AU treatment and the time at which the MI falls to 50% of the control value represents the mean duration of G_2 + mitosis/2. This interval is the sum of the mean duration of G_2 and of half the mean duration of mitosis for the population of cells contributing to the drop in MI. In the case of *V. faba* lateral roots, the popula-

tion of cells contributing to the drop in MI during 5-AU treatment is the fast-dividing population. The standard deviation of this phase can be determined with the use of the 32% and 68% intercepts (5). With this method, the estimates of the duration of G_2 + mitosis/2 are:

(*a*) 75 ppm 5-AU, 3.40 ± 1.70 hr
(*b*) 500 ppm 5-AU, 3.35 ± 1.45 hr
(*c*) 1500 ppm 5-AU, 3.30 ± 1.40 hr.

From these estimates it appears that 1500 ppm 5-AU has no greater effect than 75 ppm 5-AU. The mean duration of G_2 + mitosis/2 is 3.35 hr.

DETERMINATION OF THE DEGREE OF POPULATION HETEROGENEITY: The percentage of cells that are slow dividing can be found by expressing the MI at the end of a 5-AU treatment as a percentage of the control MI. In the present study the numbers of prophases and metaphases were used for this determination, since their values had stabilized by the end of a 6 hr treatment. The percentage calculated in this way was used as a measure of the proportion of slow-dividing cells, and not only as a measure of slow-dividing cells in prophase and metaphase. It appears that the distribution of cells in the different stages of mitosis is the same for both dividing populations since (*a*) the prophase:metaphase ratio is the same in untreated roots and in roots sampled after a 6 hr 5-AU treatment, that is, when only slow-dividing cells are in prophase and metaphase, and (*b*) Webster (8) has shown that the prophase:metaphase:anaphase and teleophase ratios are the same in both the fast- and slow-dividing subpopulations in *V. faba* lateral roots. With use of the 5-AU method, the following percentages of slow dividing cells are found:

(*a*) 75 ppm 5-AU, 17.4%
(*b*) 500 ppm 5-AU, 17.8%
(*c*) 1500 ppm 5-AU, 16.0%.

The mean percentage of slow-dividing cells in lateral roots is 17.1%; this value is similar to the estimate of 20–25% obtained with thymidine-^{3}H (9).

DISCUSSION

Treatment with 5-AU is a technique for marking cell populations. Cells affected by 5-AU do not proceed to mitosis. Only cells in G_2, i.e. cells that have passed through the S – G_2 transition, are able to complete interphase unimpeded and then divide. It is the average duration of G_2 + mitosis/2 of the cells not stopped by 5-AU that is measured.

For the fast-dividing cells of lateral roots this value is estimated to be 3.35 hr. It is somewhat less than the value of 4 hr obtained for similar cells by use of the percentage labeled mitosis method (2, 9). The two methods differ, however; the estimate obtained with the use of 5-AU is only of cells already in G_2 at the beginning of treatment, while the PLM curve gives an estimate of G_2 + mitosis/2 in cells in S at the time of thymidine-^{3}H labeling. The two methods measure the duration of G_2 + mitosis/2 in two samples of the fast-dividing subpopulation that cover different periods of interphase. The 5-AU inhibition method has been used to estimate the duration of G_2 + mitosis/2 in primary roots of *Pisum sativum*.[2] The agreement with the published values (7) indicates that the effects of 5-AU are not confined to *V. faba* and that the method could be used for other systems.

Either a synthetic or a physical change could be blocked by 5-AU. The S – G_2 transition cannot yet be defined in any way other than that it represents the end of DNA synthesis; it would be difficult to identify a cell as being at the transition. This restricts an analysis of the time when 5-AU acts. We are forced, therefore, to rely on the evidence from the timing of the fall in MI. That fall indicates that cells in G_2 are not inhibited by 5-AU. Since (*a*) cells treated while in S continue to undergo DNA synthesis and (*b*) cells eventually overcome the 5-AU block, even during treatment, the fall in MI and the continued low MI seen for several hours (Table I) show that cells must be blocked at some point that is not actually in S and yet is not in G_2. We suggest that this block occurs at the S – G_2 transition.

One consequence of such a block could be induced synchrony. We did not observe sharp peaks of synchronized mitoses; the highest values for MI were about 25, and these were, at best, only partially synchronous waves of division (data in preparation for publication). It appears that the block induced by 5-AU is not reversed simultaneously in all affected cells.

The method described here is a dependable

[2] D. Davidson and K. Simms. 1969. Unpublished.

way of determining the duration of G_2 + mitosis/2 and the proportions of fast- and slow-dividing cells. It has obvious uses in systems that do not label with thymidine-^{3}H. 5-AU may also prove to be useful in studies of events occurring in G_2; initially, the only cells entering mitosis will be those that were in G_2 at the beginning of the treatment, and later, as cells recover, there will be a partially synchronized wave of cells entering G_2, and later, mitosis. 5-AU treatments should prove useful in both heterogeneous populations and those populations in which there is already some degree of synchrony in interphase.

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