THE MECHANISM OF 5-AMINOURACIL-INDUCED
SYNCHRONY OF CELL DIVISION
IN VICTA FABA ROOT MERISTEMS

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ABSTRACT

Cessation of mitosis was brought about in Vicia faba roots incubated for 24 hours in the thymine analogue, 5-aminouracil. Recovery of mitotic activity began 8 hours after removal from 5-aminouracil and reached a peak at 15 hours. If colchicine was added 4 hours before the peak of mitoses, up to 80 per cent of all cells accumulated in mitotic division stages. By use of single and double labeling techniques, it was shown that synchrony of cell divisions resulted from depression in the rate of DNA synthesis by 5-aminouracil, which brought about an accumulation of cells in the S phase of the cell cycle. Treatment with 5-aminouracil may have also caused a delay in the rate of exit of cells from the G1 period. It appeared to have no effect on the duration of the G1 period. When roots were removed from 5-aminouracil, DNA synthesis resumed in all cells in the S phase. Although thymidine antagonized the effects of 5-aminouracil, an exogenous supply of it was not necessary for the resumption of DNA synthesis, as shown by incorporation studies with tritiated deoxycytidine.

INTRODUCTION

Smith, Fussell, and Kugelman (10) have reported partial synchronization of cell division in bean root meristems during recovery of mitotic activity following incubation in 5-aminouracil (5-AU). Synchronization of cell division in higher plants normally occurs only in processes associated with gametogenesis. This phenomenon has been utilized extensively by Hotta and Stern (3) to describe biochemical events associated with gametogenesis, and by Sparrow (9) to correlate radiosensitivity with various stages of the meiotic division cycle in Trillium. The partial synchronization of mitoses obtained by pretreatment with 5-AU has been utilized by Prensky and Smith (8) to study chromosomal proteins through successive cell division cycles in Vicia faba. Both the intrinsic value in understanding the effects of a chemical synchronizing agent and the practical value of utilizing such information for other studies of cellular metabolism have prompted us to investigate further the effects of 5-AU on cellular activity in bean root meristems.

Woods and Duncan (16) and Duncan and Woods (1) have shown that 5-AU depresses mitotic activity in root meristems of Allium cepa. They also observed a decline in the number of nuclei with a 4C content of DNA after a 24-hour treatment with 5-AU. From their data they implied that 5-AU interfered with normal DNA metabolism. The present study was designed to extend previous studies on the effects of 5-AU on processes associated with cell division in Vicia faba.
and to correlate the effects of 5-AU on DNA synthesis with the decline and recovery of mitotic activity.

**Materials and Methods**

*Vicia faba* Cultures

Seeds were washed for 15 minutes in 5 per cent Clorox and detergent, soaked for 24 hours in running tap water (approximately 16°C), again disinfected in Clorox, and finally rinsed in tap water. Germination was then continued in vermiculite at 24 to 25°C, a relatively high temperature which gave smaller losses of seedlings due to fungal infection. After 6 days, seedlings with primary roots 5 to 8 cm long were transferred to ½ strength Hoagland’s mineral nutrient solution. These cultures consisted of about 10 seedlings per liter of medium, and were placed in a dark chamber at 19°C. If secondary roots were desired, the tip on the primary root was broken off upon transfer to liquid culture. On the other hand, secondary roots were removed daily if meristems of the primary roots were to be examined.

5-Aminouracil Treatments

Studies were first made on the concentration of 5-AU which would result in complete suppression of mitosis after a 24-hour incubation and subsequent optimum synchrony of cell division when the seedlings were reincubated in nutrient solution. Concentrations of 5-AU from 100 through 800 ppm were tested, and all concentrations resulted in a mitotic index of no more than 0.5 per cent, compared with control values of from 5 to 12 per cent. A concentration of 500 ppm was the lowest to yield satisfactory synchronization of cell divisions in both primary and secondary root meristems recovering from the effects of 5-AU. Maximum recovery at 19°C was obtained 15 hours after removal from the 24 hour incubation period in 5-AU.

Stocks of 5-AU were obtained from Calbiochem Co. (Los Angeles, California) or from Eastman Kodak Co. (Rochester, New York). Solutions of the compound varied in color from lot to lot from colorless to a deep yellow. The 5-AU was therefore purified by recrystallization from cold water after dissolving to near saturation levels at 85 to 90°C. Successive recrystallizations resulted in progressively less colored 5-AU preparations. Crude and recrystallized 5-AU samples were compared for the effect on mitotic index depression and on mitotic peak at +15 hours (in the text and tables, “+” preceding “hours” signifies time after a 24-hour incubation period in 5-AU; “hours” without a plus sign refers to time during the 5-AU incubation period). No difference between the crude material and samples recrystal-}

lized up to 3 times could be observed on the basis of the mitotic index at 24 or +15 hours. For all labeling experiments 5-AU, obtained from Eastman Kodak and purified once by recrystallization, was dissolved in distilled-deionized water to yield a concentration of 500 ppm (≈ 4 × 10⁻³ M).

**Isotope Incorporation Studies**

Thymidine-³H² (TDR-³H²) and thymidine-¹⁴C (TDR-¹⁴C) were obtained from New England Nuclear Corp., Boston, at specific activities of 3,200 and 25 mCi/mM, respectively. TDR-¹⁴C was used at a concentration of 1 µCi/ml. The concentration of TDR-³H² varied according to the purpose of the experiment. If root tips were to be fixed immediately after labeling, solution concentrations up to 10 µCi/ml were used to obtain short exposure autoradiographs. When root tips were to be fixed a considerable length of time after the labeling period, i.e., sufficient for the incorporated tritium to affect the cell cycle (6, 11), 0.5 to 3 µCi/ml was used. Double labeling with TDR-³H² and TDR-¹⁴C, to study the rate of exit of cells from the DNA synthetic (S) phase, was done on the basis of premises outlined by Wimber and Quastler (15).

Since 5-AU is an analogue of thymine, it was considered advisable to try a labeled precursor other than thymidine for the determination of the rates of DNA synthesis in 5-AU treated material. Roots of *V. faba* seedlings were incubated in concentrations ranging from 2 to 5 µCi/ml of deoxycytidine-³H² (CDR-³H²) purchased from Schwarz BioResearch, Inc., Orangeburg, New York (specific activity 1,100 mCi/ml).

Roots were grown in dilutions of the labeled precursor in distilled-deionized water or in 500 ppm of 5-AU. They were fixed in alcohol-acetic acid (3:1), and autoradiographs were prepared from Feulgen-stained squashes of the terminal 1.5 mm of the roots. Slides were dipped in Kodak NTB liquid emulsion, and processed in the usual manner (D19 developer, 5 minutes, at 18°C). When a thick emulsion was desired, to differentiate between C¹⁴ and H³ labeled cells, one coating of NTB was found adequate when the slides were dried in a horizontal position.

The autoradiographs were mounted in Euparol, and finished preparations of each root tip were examined for nuclear labeling with a Zeiss 100 × oil immersion lens at a total magnification of 1,280.

**Results**

Partial Synchronization of Cell Division

In agreement with previous work (1, 10), 24 hours incubation of *V. faba* seedlings in 700 ppm aqueous solution of 5-AU decreased the mitotic
index in root meristem cells from a control value of about 10 per cent to an index of 0.0 to 0.5 per cent (Figs. 1 and 2). This result was obtained with Tradescantia paludosa and Allium cepa root meristems as well. However, in Tradescantia (tested at 50, 100, 250, and 700 ppm of 5-AU), recovery of mitotic activity was highly erratic, and work was discontinued with this species. Onion root meristems, treated with 250 ppm of 5-AU, recovered their mitotic activity at a rate analogous to that of V. faba; the mitotic index at +14 hours was about 48 per cent.

Observations of primary root meristems treated with 500 ppm confirmed the data on secondary root meristems presented by Smith et al. (10). Reduction in the number of mitoses after a 24 hour 5-AU treatment was not dependent on concentration in the range of 100 to 700 ppm of 5-AU. Partial cell division synchrony at +15 hours was not dependent on concentration from 400 to 700 ppm of 5-AU, when incubated at 19°C.

The rate of recovery from the effects of 5-AU is highly dependent on temperature. Limited observations indicate that following treatment with 5-AU at 19°C, with a recovery period temperature of 12°C, the peak of mitotic activity is reached at about +30 hours. At this temperature a large number of prophase figures was often observed at +28 hours (Fig. 3).

Fig. 4 shows the appearance of a partially synchronized root tip at +15 hours, recovered at a temperature of 19°C. At this temperature all division stages are present, and an accumulation of a particular stage has not been observed.

Since cell divisions commence at +8 hours and reach a peak at +15 hours, we tried to accumulate mitotic figures by the colchicine method with results as shown in Fig. 5. About 70 to 80...
per cent of the cells are in mitosis, which appears to be the maximal frequency of mitosis one can obtain with this experimental system.

**Reversal of 5-Aminouracil Effects**

5-AU is structurally related to thymine through the replacement of the 5-methyl group of thymine with a 5-amino group. It is therefore of interest to see whether the effects of 5-AU can be reversed by uracil or other pyrimidine-containing compounds. Seedlings of *V. faba* were incubated in 200 ppm 5-AU which completely suppressed mitotic activity after 24 hours. Uracil, thymidine, thymidylic acid, and cytidylic acid were added in equimolar concentrations to the 5-AU. Meristems from four primary roots were examined for frequency of cell divisions after 24 hours of growth in each of the treatments. Only general observations on the material were made, as shown in Table I. Both thymidine and thymidylic acid completely antagonized 5-AU effects; in fact, the frequency of cell division was about 1.5 × the control value. Cytidylic acid evidently killed most of the root tips, which became leathery and unsatisfactory for cytological observation. Uracil had no apparent effect on the response to 5-AU.

**DNA Synthesis in 5-AU Treated Root Meristems**

Since thymidine antagonized the effect of 5-AU, labeling with TDR-H could in itself affect DNA synthesis and obscure results with 5-AU. We therefore investigated the incorporation of CDR-H into root meristem nuclei. Secondary root meristems were pulse-labeled for 30 minutes (2 μCi/ml) during the middle or end of the 5-AU treatment period, and root tips were fixed immediately and +15 hours for each labeling time. A control lot consisted of pulse-labeled roots which had not been grown in 5-AU, and which were fixed immediately after labeling. The results of this experiment are presented in Table II; the
appearance of labeled cells at +15 hours is shown in Fig. 6.

The labeling index found immediately after either pulse label did not account for the high labeling index of dividing and interphase cells in roots fixed at +15 hours. Furthermore, there was a large difference between the labeling index of roots grown in 5-AU and the controls. The latter had a labeling index consistent with our experience with TDR-H3 labeling. The comparison between the 5-AU treated roots and the controls suggests an inhibition of CDR-H3 incorporation by 5-AU. A comparison between the labeling indices in roots fixed immediately after labeling and at +15 hours suggests that CDR-H3 enters a precursor pool from which it continues to label DNA for extended periods of time. The following experiment was designed to test whether the continuous labeling characteristics of CDR-H3 are restricted to 5-AU treated material.

A longitudinal cut, 3 to 4 mm in length, was made in each of four primary root tips, which were then labeled with 5 μc/ml of CDR-H3 for 30 minutes. One half of each root tip was fixed immediately after labeling, the other half was fixed after growing for 8 hours in the absence of exogenous CDR-H3. The labeling intensity of the two lots is presented in Table III. Cell division occurring during the 8 hour interval would be expected to increase the labeling index because of the proliferation of labeled cells. However, if CDR-H3 were available for a short period of time, i.e. if it behaved as a pulse label, an increase in the labeling index would be accompanied by a reduction in the average number of grains per labeled nucleus. On the other hand, if the label is available for a considerable amount of time, and in sufficient quantity, then new cells will be labeled as they come into the S phase. The product of the labeling index and the grain count would not be expected to increase with time unless there was either further incorporation into already labeled cells, incorporation into new cells, or both. The results shown in the last column of Table III are interpreted to mean that CDR-H3 continued to label nuclei after the initial treatment period.

Figure 3  Synchronization of cell division after 5-aminouracil treatment. Root meristem fixed at +88 hours, recovery temperature 12°C. Large numbers of prophases are evident. × 740.
Fig. 7 compares the pulse labeling characteristics of TDR-H\(^3\) and CDR-H\(^3\) after varying periods of incubation in 300 ppm of 5-AU. The number of cells incorporating TDR-H\(^3\) almost doubles after a 20 hour incubation period in 5-AU. In agreement with the experiments discussed above, the number of cells incorporating CDR-H\(^3\) decreases with time of incubation in 5-AU. Since TDR-H\(^3\) appears to be incorporated by all cells in the S phase, the effect of thymidine in antagonizing 5-AU is postulated as due to its supplying a precursor for DNA synthesis, the supply of which is inhibited by 5-AU. CDR-H\(^3\) apparently does not satisfy a limiting precursor requirement, and the reduced labeling indices and grain numbers per labeled nucleus reflect a slower rate of DNA synthesis in 5-AU treated roots. Consequently, labeling with CDR-H\(^3\) is a better way of measuring rate of DNA synthesis in the presence of 5-AU than labeling with TDR-H\(^3\). Both incorporation per cell and fraction of cells incorporating measurable amounts of CDR-H\(^3\) decrease with time of 5-AU incubation. On the other hand, the labeling index obtained with TDR-H\(^3\) indicates the proportion of cells that reach the S phase of the cycle during the 5-AU incubation period. It may be concluded that treatment with 5-AU accumulates cells in the S phase because of an inhibition of DNA synthesis due to 5-AU preventing cells from completing DNA replication. Since the pool of cells which can enter the S phase must diminish with time, the relatively largest accumulation occurs in the first 8 hours of the 5-AU treatment period. Experiments with TDR-C\(^4\), which were comparable in protocol with the experiments

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TABLE I
Reversal of the Antimitotic Activity of 5-Aminouracil by the Addition of Other Compounds

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitotic index after 24 hrs (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H₂O)</td>
<td>7-10</td>
</tr>
<tr>
<td>5-AU</td>
<td>0</td>
</tr>
<tr>
<td>5-AU + Uracil</td>
<td>0</td>
</tr>
<tr>
<td>5-AU + Cytidylic acid</td>
<td>killed</td>
</tr>
<tr>
<td>5-AU + Thymidine</td>
<td>10-15</td>
</tr>
<tr>
<td>5-AU + Thymidylic acid</td>
<td>10-15</td>
</tr>
</tbody>
</table>

* All concentrations at 1.6 X 10⁻³ M (= 200 ppm of 5-AU) in distilled-deionized water.
† Five primary root meristems examined per treatment.

above confirmed the data obtained from tritium labeling (Fig. 7).

The next question is whether cells leave the S phase during the 5-AU incubation period. Wimber and Quastler (15) have shown that when cells are labeled with H³ and C¹⁴, with an appropriate interval between labels, two classes of cells can be distinguished microscopically, those labeled with H³ alone, and those labeled with C¹⁴ with or without H³. When thymidine is the isotope carrier, the number of cells labeled only with H³ is a direct measure of the number of cells which were not in the S phase when the TDR-C¹⁴ was made available. We therefore labeled separate lots of root meristems with TDR-H³ for 30 minutes before and during the 5-AU treatment period. After a 24 hour incubation period in 5-AU, all roots were labeled with TDR-C¹⁴. The results of this study are presented in Table IV. After the

Figure 5  Chromosome preparation after arrest of mitosis with colchicine. Root transferred to colchicine
at +11 and fixed at +16 hours. X 340.
### TABLE II

**Labeling Index of 5-Aminouracil Treated Root Meristems when Pulse Labeled with CDR-H\(^3\)**

<table>
<thead>
<tr>
<th>Time of Labeling*</th>
<th>Interphase cells</th>
<th>Dividing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before 5-AU (Control)</strong></td>
<td>36.4 ± 2.9</td>
<td>—</td>
</tr>
<tr>
<td>10.5 w.o. (middle of 5-AU period)</td>
<td>18.8 ± 4.7</td>
<td>—</td>
</tr>
<tr>
<td>Fixed at 11 hours</td>
<td>74.3 ± 2.3</td>
<td>97.9 ± 0.3</td>
</tr>
<tr>
<td>Fixed at +15 hours</td>
<td>7.7 ± 5.2</td>
<td>94.2 ± 2.4</td>
</tr>
<tr>
<td>23.5 w.o. (end of 5-AU period)</td>
<td>81.7 ± 2.4</td>
<td>—</td>
</tr>
<tr>
<td>Fixed at 24 hours</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fixed at +15 hours</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Time with respect to the start of a 24 hour incubation period in 5-AU; 2 µc/ml of CDR-H\(^3\) for 30 minutes, 4 secondary root tips/treatment; approximately 250 cells/root were examined.

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**Figure 6** Nuclear labeling with CDR-H\(^3\). Isotope applied during 5-aminouracil treatment period and fixed at +15 hours. × 840.

The entire 24-hour period in 5-AU only 7.31 per cent of the cells do not incorporate C\(^3\). Half of these cells probably completed DNA synthesis within the H\(^3\) treatment period, and, in fact, most of the cells labeled with tritium only were rather weakly labeled. The values obtained at 16 and 20 hours probably reflect the number of cells which may have completed DNA synthesis because of the thymidine treatment itself. All cells labeled only with H\(^3\) were weakly labeled, which indicates that
TABLE III
Labeling Intensity in Root Tip Halves
After a Pulse Label with CDR-H3

<table>
<thead>
<tr>
<th>Half root tips</th>
<th>A</th>
<th>B</th>
<th>A × B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed immediately</td>
<td>29.3</td>
<td>13.96</td>
<td>411.82</td>
</tr>
<tr>
<td>Fixed 8 hours later</td>
<td>48.8</td>
<td>17.03</td>
<td>831.06</td>
</tr>
<tr>
<td>Difference</td>
<td>19.3</td>
<td>3.07</td>
<td></td>
</tr>
<tr>
<td>se of difference</td>
<td>5.31†</td>
<td>0.2619‡</td>
<td></td>
</tr>
</tbody>
</table>

* Labeled with 5 μc/ml; data from 4 primary root meristems split longitudinally before labeling. Averages based on grain counts of 150 cells per root tip half. † Significant at the 0.01 level.

C14 resulted in an H2:C14 ratio of 0.412 (leading to an estimate of 9.7 hours for the average duration of S. In the 5-AU treated material the 4.5 hour interval (line 3, Table IV) yielded an H2:C14 ratio of 0.030. At this rate of exit from the S phase, one cycle of DNA replication would presumably last about 150 hours \( \left( \frac{1}{0.030} \times 4.5 \right) \).

DNA Synthesis Following Incubation in 5-AU

In the previous section we have shown that incubation of roots in 5-AU causes an inhibition and eventual suppression of DNA synthesis. The observed synchrony of cell division must therefore rest on the ability of the cells to resume synthesis of DNA when the 5-AU titer is reduced by washing and incubation in normal growth medium. With about 80 per cent of the cells labeled in all treatments, the C14 labeling index in Table IV is an indication of the resumption of DNA synthesis and we have, in fact, obtained similar values with a pulse label of TDR-H3 following a 24-hour incubation period in 5-AU.

Since Littlefield (7) showed that exogenous TDR was necessary to overcome the inhibitory effects of 5-fluorodeoxyuridine (FUDR), we tested the incorporation of CDR-H3 following an 8 hour
TABLE IV
Results of Double-labeling to Test Completion of S During 5-Aminouracil Treatment

| Treatment No. | Time of labeling with H\(^3\) and C\(^{14}\) hours | Interval between H\(^3\) and C\(^{14}\) hours | Labeling index
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.0</td>
<td>1/2</td>
<td>7.31 ± 1.13</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>16</td>
<td>2.38 ± 0.47</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>20</td>
<td>2.45 ± 1.02</td>
</tr>
</tbody>
</table>

* One half hour with TDR-H\(^3\), 5 μg/ml in water for treatment No. 1, 10 μg/ml in 500 ppm 5-AU for treatments 2 and 3. TDR-C\(^{14}\), 1.5 μg/ml administered in three 15 minute pulses at +1, +2, and +3 hours after a 24-hour growth period in 5-AU.

incubation period in FUDR and 5-AU, without the addition of TDR. The FUDR-treated root tips did not incorporate any CDR-H\(^3\) (Table V). The 5-AU treated root tips had a higher labeling index than the controls, reflecting the accumulation of cells in the S phase. They also incorporated more tritium per labeled cell, reflecting perhaps an accumulation of a precursor pool which is utilized when the block induced by 5-AU is released. We can conclude, therefore, that an exogenous supply of TDR is not necessary for cells to resume DNA synthesis after removal of the seedlings from the 5-AU solution.

Another problem is the frequency with which cells that will undergo mitosis at +15 hours can be labeled beforehand in the period of recently resumed DNA synthesis. To investigate this we labeled 5-AU treated root tips for 6 hours following removal from a 24-hour incubation in 5-AU. The root tips were fixed 9 hours after labeling ceased, which corresponds to +15 hours, a time when the mitotic index is maximal. With this protocol, almost all the cell divisions in the control lot (no 5-AU treatment) should be labeled, assuming a 4.7 hour G\(_2\) and an 8 hour S period (2, 4, 5). The results are presented in Table VI. The mitotic labeling index of the 5-AU treated root tips was lower than in the control, which could come about if the duration of the G\(_2\) period for some cells is longer in the 5-AU treated root tips.

**Effect of 5-aminouracil on the G\(_2\) Period**

As noted in the preceding section, it appeared that during recovery, the G\(_2\) period is of somewhat longer duration than normal. The following experiment was designed to test for possible direct effects of 5-AU on the progress of cells through the G\(_2\) phase of the cell cycle.

TABLE V
Recovery of DNA Synthesis Without Exogenous Thymidine in 5-Aminouracil Treated Root Tips

| Treatment before labeling | Labeling index | Grain/labeled nucleus
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.8 ± 2.3</td>
<td>14.4 ± 2.3</td>
</tr>
<tr>
<td>5-AU, 500 ppm, 8 hours</td>
<td>62.0 ± 8.7</td>
<td>22.2 ± 4.1</td>
</tr>
<tr>
<td>FUDR, 10(^{-7}) M, 8 hours</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Labeled with 4 μg/ml of CDR-H\(^3\), 4 root tips in each treatment 1 hour.
† Examined 150 nuclei in each root tip.

TABLE VI
Labeling Index of Dividing Cells when Labeled after 5-Aminouracil Incubation Period

| Treatment before labeling | Labeled mitoses
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.9 ± 1.36</td>
</tr>
<tr>
<td>5-AU, 500 ppm, 24 hours</td>
<td>81.8 ± 2.13</td>
</tr>
</tbody>
</table>

* Continuously labeled in 0.5 μg/ml of TDR-H\(^3\), for 6 hours, 7 root tips in each treatment, and fixed 9 hours after labeling (+15 hours).

Seedlings with many secondary roots were labeled for 20 minutes in 3 μg/ml of TDR-H\(^3\), after which they were rinsed for 10 minutes in distilled-deionized water, and returned to the normal growth medium. After postlabeling intervals of 0.5, 1, 2, and 4 hours, seedlings were transferred to 5-AU. Two sets of controls were employed, of which one was an unlabeled set.
placed in 5-AU, and the other was labeled but remained in the normal growth medium. Root tips were fixed at hourly intervals up to 10 hours after labeling, as shown in Table VII.

A recently published estimate for the average duration of the G2 period is about 4 to 5 hours (2). It was expected, therefore, that in treatments with 2 and 4 hour intervals between labeling and incubation in 5-AU, an appreciable number of G2 cells would be labeled at the start of the 5-AU incubation period (about 9 and 18 per cent of all cells, respectively, based on a 36 per cent labeling index and an average duration of S of 8 hours). If passage through the G2 period, or its terminal portion, is not delayed by 5-AU, dividing cells should be labeled with a frequency comparable with those controls which were not placed in 5-AU. If the TDR-H3 labeling interfered with the 5-AU treatment, the non-labeled controls should have a mitotic index different from the experimental group.

Autoradiographs of all TDR-H3 labeled root tips were examined for frequency of labeled mitoses. These observations are recorded in Table VII. In all 5-AU treated root tips the frequency of labeled mitoses is greatly reduced. This is apparent even in treatment E, in which at the start of the 5-AU incubation period about 32 per cent of the dividing cells must have been labeled. This frequency declined to 19.2 per cent 1 hour later, indicating that few, if any, of the labeled cells entered mitosis after transfer to 5-AU. After another hour in 5-AU, less than 1 per cent of the dividing cells were labeled. The rapid decline in the mitotic index in this treatment precluded later observations. In the treatment involving a 2 hour interval (D), the appearance of labeled mitoses is delayed. However, the high labeling index at 10 hours is based on very few cells since the mitotic index at that time is very low (Table VIII).

The mitotic index of the above material is presented in Table VIII. The columns were shifted from the arrangement in Table VII to show the mitotic index after given lengths of time in 5-AU. The data for unlabeled controls (treatment F) correspond to those found by Duncan and Woods (1) in Allium cepa. Because of the rather slow decline in the mitotic index under such conditions, we had at first discounted the possibility that 5-AU affects the G2 stage. With respect to the mitotic index, there appeared to be an interaction between the effects of 5-AU and the time of TDR-H3 labeling. The mitotic index of the treatments labeled with TDR-H3 0.05 and 1 hour before the 5-AU incubation period (treatments B and C) was more variable than that of the control series. The 2-hour interval between TDR-H3 and 5-AU showed a decline in the rate of cell division similar to the one found in the control, where no TDR-H3 was used, whereas the 4-hour interval resulted in a decline which was faster than in the control.

### TABLE VII

<table>
<thead>
<tr>
<th>Interval between TDR-H3 labeling and start of 5-AU incubation</th>
<th>Labeled mitoses, hours after cessation of labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>A Control: no 5-AU</td>
<td>0</td>
</tr>
<tr>
<td>B 1/2 hour§</td>
<td>—</td>
</tr>
<tr>
<td>C 1 hour§</td>
<td>—</td>
</tr>
<tr>
<td>D 2 hours</td>
<td>0</td>
</tr>
<tr>
<td>E 4 hours</td>
<td>0</td>
</tr>
</tbody>
</table>

* Secondary roots labeled with 3 μc/ml of TDR-H3 for 20 minutes, rinsed, and returned to 1/6 Hoagland's. After stated interval, treatments B-E were incubated in 5-AU, 8 root tips fixed at each period.
† Low mitotic indices in treatments C, D, and E.
§ Labeled mitoses had few grains, especially in treatment B.
|| Same as control.
¶ Few mitoses found, all unlabeled, except in one out of 8 root tips fixed at 9 hours, where 31 out of 100 mitoses were labeled.

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TABLE VIII

Mitotic Index of Root Meristems Incubated in 5-Aminouracil after Labeling with TDR-H³*

<table>
<thead>
<tr>
<th>Interval between TDR-H³ labeling and start of 5-AU incubation</th>
<th>Mitoses, hours in 5-AU†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A Control: no 5-AU</td>
<td>10.7</td>
</tr>
<tr>
<td>B ½ hour§</td>
<td>--</td>
</tr>
<tr>
<td>C 1 hour</td>
<td>--</td>
</tr>
<tr>
<td>D 2 hours</td>
<td>--</td>
</tr>
<tr>
<td>E 4 hours</td>
<td>6.7</td>
</tr>
<tr>
<td>F Control: no TDR-H³</td>
<td>--</td>
</tr>
</tbody>
</table>

* Treatments identical with those in Table VII, except for the addition of an unlabeled control series.
† Data for treatment A are for hours after TDR-H³ labeling.
§ Data for treatment B were taken ½ hour earlier than indicated in column heading.

The large differences in mitotic indices were not anticipated, and therefore the experiment, as it stands, is not suitable for elucidating the cause for the range of effects actually observed. It is possible that the mitotic index of treatments B and C was influenced by the small amount of thymidine available to the root tips shortly before being transferred to 5-AU. This, however, does not explain the rapid decline of the mitotic index in treatment E. The failure of large numbers of labeled cells to enter mitosis could imply a superimposition of a 5-AU induced radiosensitivity to the beta radiation from the incorporated tritium. The mitotic index of the controls, which were labeled but not grown in 5-AU (treatment A), does not appear to have been greatly affected by the TDR-H³ treatment. It appears, therefore, that the structure of the data is markedly affected by unresolved factors, though it is highly probable that 5-AU affects the rate of progress of cells through the G₂ period.

Other Observations

It is clear that 5-AU, in depressing DNA synthesis, interferes somehow with thymidine metabolism in V. faba. We wanted to find out whether related 5-amino compounds had similar effects. Therefore, we tested the effect of a 24 hour incubation of V. faba roots in several concentrations of both 5-aminodeoxyuridine and 5-amino-orotic acid, but found no decrease in the mitotic index. With our techniques it has therefore been impossible to infer at what step in the biosynthesis of thymidilic acid 5-AU exerts its inhibitory effects on DNA synthesis.

DISCUSSION

In this study we were able to correlate the induced partial synchrony of cell division with a depression in DNA synthesis during the 5-AU incubation period, resulting in an accumulation of cells in the S phase. With a double labeling procedure involving an H³ label followed by a C⁴ label, it was possible to show that few, if any, cells leave the S phase. Since a maximum of about 80 per cent of the cells accumulates in the DNA synthetic phase during 24 hours of 5-AU treatment, one may be reasonably certain that the 5-AU treatment did not materially affect the rate at which cells left the G₁ phase and entered S. Howard and Pelc (5) obtained up to 75 per cent labeling under conditions of continuous labeling with P³, for 24 hours, in V. faba. Wimber (12), with repeated labeling during a 24 hour period with TDR-H³, found about 80 per cent of the cells labeled in Tradescantia root meristem cells. Consequently, this proportion apparently represents a value which is close to the maximum fraction of root meristem cells that it is possible to accumulate at any stage of the cycle.

A direct test of whether the rate of entry into DNA synthesis is affected by 5-AU could pre-
sumably be made with a C\textsuperscript{14} label followed by an H\textsuperscript{3} label (i.e., reversing the order of labeling used in the experiment in Table IV). We did not report the data from this experiment because the values obtained for the H\textsuperscript{3}:C\textsuperscript{14} ratios could not be interpreted in terms of the effect of 5-AU on the cell cycle. This is reasonable because of the large difference in specific activities between C\textsuperscript{14} and H\textsuperscript{3} labeled thymidine. At the specific activities available to us the concentration of thymidine is 128 times lower for comparable activity per milliliter when TDR-H\textsuperscript{3} is used than when it is substituted by TDR-C\textsuperscript{14}. However, since the rate of accumulation of cells in S shown in our experiments appears to be consistent with the data of Howard and Pelc, and of Wimber, the failure of the reverse double-labeling experiment is not a crucial point. Current work in our laboratory on the labeling kinetics of H\textsuperscript{3} after C\textsuperscript{14} labeling in untreated \textit{V. faba} bears this out; it appears that more attention will need to be paid to absolute concentrations of thymidine in double-labeling experiments, especially where the C\textsuperscript{14} label precedes or accompanies the H\textsuperscript{3} label.

Although we are reasonably certain that 5-AU does not affect the G\textsubscript{1} period, there is some evidence of a delaying effect in the G\textsubscript{2} period. Aside from the data in Tables VII and VIII, it is difficult to account for the incomplete labeling of the cells recorded in Table VI except by postulating some sort of effect in the G\textsubscript{2}. During and after the 5-AU treatment there was a much greater variability in TDR-H\textsuperscript{3} incorporation per cell in those cells which were scored as “labeled,” compared with controls. Part of the deficiency in labeled mitoses may therefore be due to less than detectable incorporation of TDR-H\textsuperscript{3} in some cells. However, G\textsubscript{2} cells are, by definition (5), those which do not incorporate detectable amounts of DNA precursor after the detectable DNA synthetic period has been completed. The data of Wimber and Davies (13) on the occurrence of FUDR-induced breaks in the G\textsubscript{2} phase, and observations on the apparent incorporation of TDR-H\textsuperscript{3} into chromosomes during pachynema of meiosis in male \textit{Triturus} (14) may be indicative of DNA synthesis in periods following the main DNA synthetic period. If that is the case, then the effect of 5-AU on the G\textsubscript{2} phase could occur by the same mechanism by which the S phase is affected, namely that of suppression of DNA synthesis by interference with thymidine metabolism. Nor would this be inconsistent with the data we obtained with CDR-H\textsuperscript{3} labeling, since the continuous labeling characteristic of this precursor could be responsible for the registration of cells as labeled when a pulse label would have placed them in the unlabeled category.

The authors wish to acknowledge the helpful discussions held in the course of this study with Doctors Donald E. Wimber and Philip S. Woods. They are also grateful to Mr. Keith H. Thompson for the statistical analysis and processing of the data. The photographs in this paper were taken by Mr. Robert F. Smith of the Photography Department, Brookhaven National Laboratory. The senior author held a United States Public Health Service Postdoctoral Fellowship (GM-12,602) during part of the time this research was being carried out. Work performed at Brookhaven National Laboratory was under the auspices of the United States Atomic Energy Commission.

Received for publication, March 20, 1964.

REFERENCES

8. PRENSKY, W., and SMITH, H. H., Incorporation of TDR-H\textsuperscript{3} in some cells. However, G\textsubscript{2} cells are, by definition (5), those which do not incorporate detectable amounts of DNA precursor after the detectable DNA synthetic period has been completed. The data of Wimber and Davies (13) on the occurrence of FUDR-induced breaks in the G\textsubscript{2} phase, and observations on the apparent incorporation of TDR-H\textsuperscript{3} into chromosomes during pachynema of meiosis in male \textit{Triturus} (14) may be indicative of DNA synthesis in periods following the main DNA synthetic period. If that is the case, then the effect of 5-AU on the G\textsubscript{2} phase could occur by the same mechanism by which the S phase is affected, namely that of suppression of DNA synthesis by interference with thymidine metabolism. Nor would this be inconsistent with the data we obtained with CDR-H\textsuperscript{3} labeling, since the continuous labeling characteristic of this precursor could be responsible for the registration of cells as labeled when a pulse label would have placed them in the unlabeled category.

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