Plant Cell Physiol. 31(1): 61–67 (1990)

Characteristics of the Inhibitory Effect of 5-Fluorodeoxyuridine on Cytodifferentiation into Tracheary Elements of Isolated Mesophyll Cells of *Zinnia elegans*

Munetaka Sugiyama, Hiroo Fukuda and Atsushi Komamine

Biological Institute, Faculty of Science, Tohoku University, Sendai, 980 Japan

The inhibitory effect of 5-fluorodeoxyuridine (FdU) on the differentiation into tracheary elements was characterized in isolated mesophyll cells of *Zinnia elegans*.

Both thymidine and uridine counteracted the inhibitory effect of FdU on the differentiation into tracheary elements, while only thymidine was effective in counteracting the effect of FdU on cell division. Higher concentrations of thymidine were needed for the restoration of the differentiation that was blocked by FdU than for the restoration of cell division. These results suggest that FdU prevents the differentiation via a mechanism that is different from the inhibition of thymidylate (dTMP) synthase by fluorodeoxyuridine monophosphate (FdUMP), derived from FdU, to which the blockage of cell division by FdU should be attributable.

The differentiation into tracheary elements was prevented when FdU was added earlier than the 36th hour of culture, and thymidine overcame the inhibitory effect of FdU only when added within the first 4 h of culture. Pretreatment with FdU before application of 6-benzyladenine (BA) and 1-naphthaleneacetic acid (NAA), which are essential for the formation of tracheary elements, also inhibited the differentiation. Thus, the aspect of the differentiation that is the target of inhibition by FdU appears to occur between the 4th hour and the 36th hour of culture and to begin even in the absence of exogenous plant growth regulators.

Key words: Cell division — Differentiation — Fluorodeoxyuridine — Tracheary element — *Zinnia elegans*.

5-Fluorodeoxyuridine (FdU), a structural analogue of deoxyuridine and thymidine, is very often used to inhibit DNA synthesis. Since fluorodeoxyuridine monophosphate (FdUMP) is a potent inhibitor of thymidylate (dTMP) synthase (Cohen et al. 1958), it is generally accepted that FdU, when administered to cells, inhibits dTMP synthase as FdUMP after it has been phosphorylated in vivo and, thereby, causes a deficiency of thymidine nucleotides, which results in cessation of DNA synthesis. It is also known that a small amount of FdU is incorporated into DNA in place of thymidine, possibly causing the interruption of the elongation of DNA chains (Dannenberg et al. 1981, Kufe et al. 1981, Schuetz and Diasio 1985). Some molecules of FdU may be decomposed to form fluorouracil (FUra). The incorporation of FUra into RNA has been reported in plant cells as well as in animal cells (Fraser 1975, Lerbs et al. 1980).

The effect of FdU on differentiation in plants has been examined in stem segments of *Coleus blumei* (Fosket 1968, 1970), in root cortical parenchyma of *Pisum sativum* (Shininger 1975), in pith explants of *Lactuca sativa* (Turgeon 1975), in tuber explants of *Helianthus tuberosus* (Phillips 1980), and in isolated mesophyll cells of *Zinnia elegans* (Fukuda and Komamine 1981a). FdU significantly inhibited the formation of tracheary elements in the various experimental systems examined, with one exception (Turgeon 1975). It has also been reported that exogenous thymidine can restore the differentiation that has been blocked by FdU, as is to be expected from the abovementioned mechanism of inhibition of DNA synthesis by FdU (Fosket 1968, 1970, Shininger 1975). Fukuda and Komamine (1981a) observed, furthermore, that FdU had no significant effect on the rate of protein synthesis, and

Abbreviations: BA, 6-benzyladenine; dT, thymidine (in Tables and Figures); dTMP, thymidine 5′-monophosphate; FdU, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FUra, 5-fluorouracil; NAA, 1-naphthaleneacetic acid; TE, tracheary elements (in Tables); U, uridine (in Tables); Ura, uracil (in Tables).

they concluded that the inhibition of the differentiation by FdU was not the result of a general impairment of gene expression. In their experimental system, it was apparent that neither cell division nor DNA replication in the S phase of the cell cycle is a prerequisite for the differentiation into tracheary elements (Fukuda and Komamine 1980a, 1981b). Thus, the action of FdU on the differentiation is not mediated by interruption of DNA replication in the S phase and the subsequent cessation of the progression of cell division cycle.

In the studies described in this paper, the effects of FdU on the differentiation into tracheary elements and on cell division were further investigated and characterized in *Zinnia* mesophyll cells. Based on the data obtained, a discussion is presented of a possible target for inhibition by FdU during the process of differentiation.

Materials and Methods

Isolation and culture of mesophyll cells—Zinnia elegans cv. Canary bird was grown at 25±1°C under a cycle of 14 h of light (approximately 8,000 lux) and 10 h of darkness. The first true leaves with lengths of 3 to 4 cm were harvested 14 days after sowing. The leaves were surface-sterilized in a solution of 0.15% NaOCl and then macerated with a homoblender (Type 500, Sakuma Seisakusho, Tokyo) in culture medium. Isolated cells were filtered through a nylon screen with a pore size of 50 μm, and the resultant suspension was subjected to centrifugation at 150×*g* for 1 min. The precipitated cells were washed with medium and resuspended at an initial concentration of approximately 10^5 cells/ml. Mesophyll cells prepared in this way were cultured in the dark at 27°C while being rotated at 10 rpm on a revolving drum.

The culture medium used, D medium, was a slightly modified version of that described by Fukuda and Komamine (1980b); it contained reduced concentrations of KH_2PO_4 and sucrose, and 0.1 mg/liter NAA and 0.2 mg/liter BA as plant growth regulators (Sugiyama et al. 1986). In one experiment, media that lacked both NAA and BA (C_O), only NAA (C_B), and only BA (C_N), were also used. Concentrations of growth regulators in media and the occurrence of differentiation and cell division when cells were cultured in each medium are shown in Table 1.

Determination of the number of tracheary elements and septa—The numbers of tracheary elements and septa were counted using a Tatai-type haemocytometer (Kayagaki Irikakogyo, Tokyo). The number of septa represents the frequency of cell division since initially, all of the cells in culture are single cells.

Results

Time course of the differentiation into tracheary elements and cell division—Localized thickening of secondary cell walls, characteristic of tracheary elements, was evident after about 48 h in culture (Fig. 1). Approximately 40% of cells differentiated into tracheary elements in the subsequent 24 h. Further formation of tracheary elements was hardly observed beyond the 72nd hour of culture. Cell division took place in two bursts, the first at around the 36th hour and the second later than the 72nd hour. The frequency of cell division was much lower than that in the original system (Fukuda and Komamine 1980b), probably as a result of the limitation of nutrients in the medium and some minor changes in the conditions used for culture of seedlings. Cell division occurred concurrently with but independently of the differentiation, as shown by Fukuda and Komamine (1981b).

Recovery from the inhibitory effect of FdU on the differentiation into tracheary elements as a result of simultaneous addition of thymidine and FdU—Cells were cultured for 96 h in the presence of various concentrations of FdU and thymidine. The numbers of tracheary elements and septa are plotted in Figure 2. Both the blockage of cytodifferentiation by FdU and that of cell division were overcome by addition of thymidine. As the concentration of FdU increased, the concentration of thymidine required to overcome the inhibition also increased. Cell division was restored by lower concentrations of thymidine than those required to restore the differentiation into tracheary elements.

Table 1 Concentrations of plant growth regulators in various media and occurrence of the differentiation into tracheary elements and cell division

Medium	BA (mg/liter)	NAA (mg/liter)	Differentiation	Cell division
C_O	0	0	−	−
C_B	0.2	0	−	−
C_N	0	0.1	−	+
D	0.2	0.1	+	+

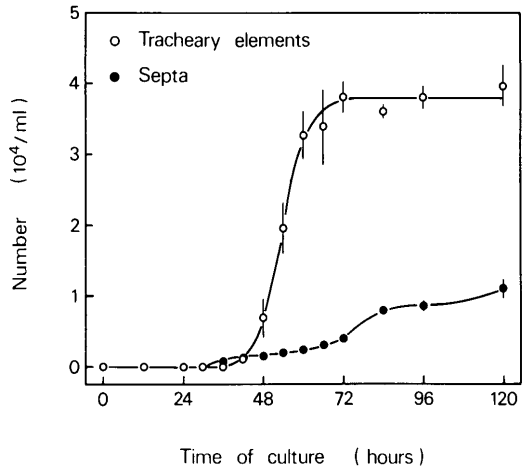

Fig. 1 Time course of the differentiation into tracheary elements and cell division. The initial concentration of cells was 8.6×10^4 cells/ml. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

Table 2 Effects of the simultaneous addition of thymidine, uridine, or uracil and FdU on the differentiation into tracheary elements and cell division

Addition	Number (10^4/ml)	
	TE	Septa
None	3.59±0.24	0.74±0.09
dT	3.51±0.12	0.78±0.03
U	3.64±0.14	0.80±0.14
Ura	3.22±0.11	0.53±0.06
FdU	0.05±0.02	0.01±0.00
FdU+dT	3.17±0.31	0.88±0.10
FdU+U	1.55±0.16	0.00±0.00
FdU+Ura	0.04±0.01	0.00±0.00

FdU was administered at a final concentration of 10^{-6} M, and the other chemicals were added at 10^{-3} M. The numbers of tracheary elements and septa were determined after 96 h in culture. The initial concentration of cells was 1.06×10^5 cells/ml. Values are means ± standard errors of results from 3 samples.

The effects of the simultaneous addition of uridine or uracil and FdU were also examined. As shown in Table 2, the differentiation into tracheary elements that was blocked by 10^{-6} M FdU was partially restored by 10^{-3} M uridine but not by 10^{-3} M uracil, and uridine was not as efficient as thymidine in overcoming the inhibition. Moreover, the blockage of cell division was not overcome by uridine.

Effects of FdU added at various times during culture on the differentiation into tracheary elements and cell division—FdU was administered to cell cultures at a final concentration of 10^{-6} M at various times, and the numbers of tracheary elements and septa were determined after a total of 96 h of culture. The results are shown in Figure 3.

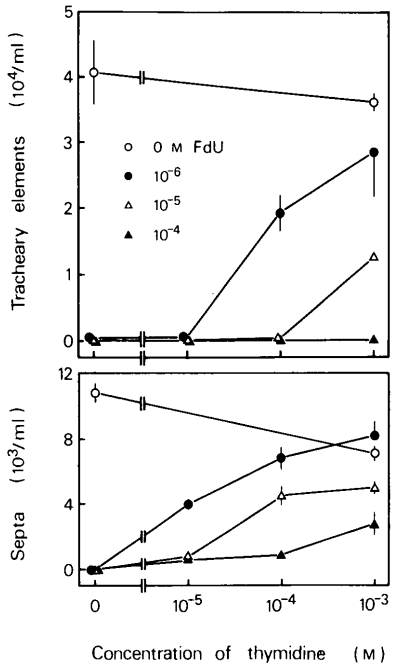

Fig. 2 Effects of simultaneous addition of various concentrations of FdU and thymidine on the differentiation into tracheary elements and cell division. The initial concentration of cells was 1.06×10^5 cells/ml. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

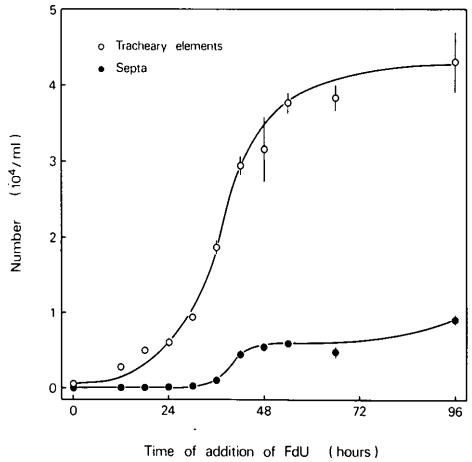

Fig. 3 Effects of FdU added at various times during culture on the differentiation into tracheary elements and cell division. The initial concentration of cells was 9.9×10^4 cells/ml. Fifty percent of the maximum number of tracheary elements formed in the absence of FdU was observed at 62 h. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

When FdU was added prior to the 24th hour of culture, the differentiation into tracheary elements was strongly inhibited. When FdU was added more than 48 h after initiation of cultures, the differentiation was hardly affected. Fifty percent inhibition was observed when FdU was added to the culture medium at the 36th hour. Cell division was almost completely blocked by addition of FdU prior to the 36th hour.

Effects of treatment with FdU followed by addition of thymidine on the differentiation into tracheary elements and cell division—Thymidine (10^{-3} M) was administered to cells cultured in the continuous presence of 10^{-6} M FdU, and the numbers of tracheary elements and septa were determined after 96 h of culture (Fig. 4). When thymidine was added later than the 12th hour of culture, few tracheary elements were formed but cell division still occurred. Addition of thymidine after 4 h of culture allowed 50% of the differentiation of that in control cultures treated with thymidine and FdU simultaneously.

In the next experiment, we examined the effects of the sequential addition of FdU and thymidine at different times during culture. Cells in cultures of various ages were treated with 10^{-6} M FdU, and then thymidine was added, at a final concentration of 10^{-3} M, after an interval of 6 h (Fig. 5, 6). This treatment was expected to realize a 6-h-pulse exposure of cells to FdU at different times, in view of the ability of thymidine to cancel out the effect of FdU. Exposure of cells to FdU within 48 h after the initiation of culture resulted in more than 50% inhibition of the differentiation into tracheary elements, and the strongest inhibition was achieved by treatment with FdU between the 12th and the 18th hours of culture (Fig. 5). Cell division was not completely blocked by a pulse exposure to FdU at any time during the course of the culture. In particular, treatment with FdU between the 54th and the 60th hour had the least effect on cell division. Such treatment is administered in a gap between two bursts of cell division. When FdU was applied at 10^{-7} M instead of 10^{-6} M, the result was almost the same as that shown in Figure 5, although it was somewhat ambiguous because of the incompleteness of the inhibition (data not shown). Figure 6 shows the time course of the formation of tracheary elements in the same cultures as those for which data are depicted in Figure 5. In the cultures treated with FdU in the first 24 h, the differentiation was not delayed but remained severely impeded for 4 days.

Effects of treatment with FdU in the absence of BA and/or NAA on the differentiation into tracheary elements and cell division—The formation of tracheary elements in the present system requires both exogenous auxin (NAA) and cytokinin (BA). However, these growth regulators may not necessarily induce all the events involved in the differentiation into tracheary elements. Thus, an experiment was designed to investigate whether or not some putative process that is part of the differentiation which is inhibited by FdU can take place without NAA and/or BA. Cultures were initiated in C_O (without BA and NAA), C_N (without BA), C_B (without NAA), and in D medium, all of which contained 10^{-6} M FdU. C_O, C_N and C_B media were converted to D medium at the 12th hour of culture by addition of BA and NAA together, BA alone, and NAA

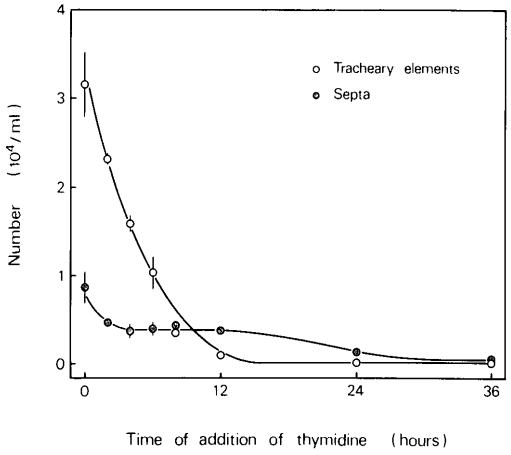

Fig. 4 Relationship between timing of addition of thymidine and recovery of the differentiation into tracheary elements and cell division after blockage by FdU. FdU was applied at the initiation of culture. The initial concentration of cells was 8.8×10^4 cells/ml. The numbers of tracheary elements and septa in cultures without FdU were $3.52 \pm 0.18 \times 10^4$/ml and $9.7 \pm 1.3 \times 10^3$/ml, respectively. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

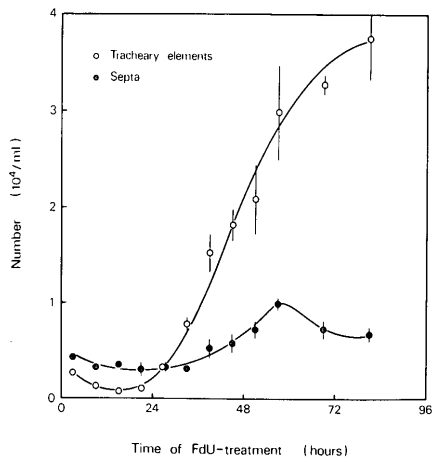

Fig. 5 Effects of exposure to FdU followed by addition of thymidine at various times, separated by intervals of 6 h, on the differentiation into tracheary elements and cell division. The numbers of tracheary elements and septa were determined after 96 h in culture. The initial concentration of cells was 9.6×10^4 cells/ml. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

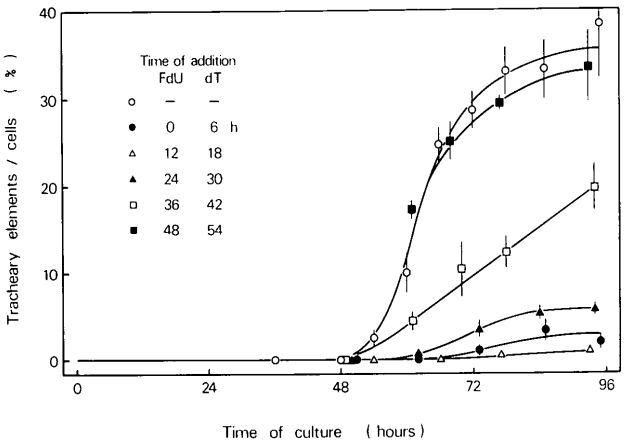

Fig. 6 Time course of the differentiation into tracheary elements, as influenced by sequential addition of FdU and thymidine at various times, separated by intervals of 6 h. Cultures were the same as those described in the legend to Figure 5. Each point represents the mean value of results from 3 samples and vertical lines show standard errors.

alone, respectively. Thymidine (10^{-3} M) was administered at the initiation, the 6th hour, or the 12th hour of culture, and the numbers of tracheary elements and septa were determined after a total of 96 h of culture. As shown in Table 3, in all of the culture media tested, the inhibition by FdU of the differentiation into tracheary elements was totally overcome, partly overcome, and barely overcome by thymidine added at the initiation, the 6th hour, and the 12th hour of culture, respectively. This result suggests that the process that is the target of inhibition by FdU during the differentiation can occur even in the absence of NAA and BA.

Discussion

It has been well established that the primary target of FdU in cell division is dTMP synthase. FdU is believed to act in the following manner. It inhibits dTMP synthase after being phosphorylated in vivo and causes a deficiency of thymine nucleotides. This deficiency interrupts DNA synthesis, with a resultant blockage of the progression of the cell-division cycle. In *Zinnia* mesophyll cells, cell division blocked by FdU was restored only by thymidine, which can compensate via the salvage pathway for the reduced supply of thymine nucleotides, but it was not restored by uridine (Table 2). This result supports the abovementioned mechanism of action of FdU on cell division.

The blockage by FdU of the differentiation into tracheary elements has also been attributed to the inhibition of dTMP synthase by FdUMP and the resultant interruption of cell division (Fosket 1968, 1970, Shininger 1975). However, the data obtained here make it necessary to reconsider the mechanism of action of FdU on the differentiation into

Table 3 Effects of treatment with FdU in the absence of BA and/or NAA on the differentiation into tracheary elements and cell division

Media	Time of addition (h)		Number (10^4/ml)	
	FdU	dT	TE	Septa
$C_O \rightarrow D$	0	0	2.92±0.14	0.63±0.03
$C_O \rightarrow D$	0	6	0.70±0.07	0.30±0.05
$C_O \rightarrow D$	0	12	0.01±0.00	0.36±0.06
$C_N \rightarrow D$	0	0	2.85±0.03	0.56±0.03
$C_N \rightarrow D$	0	6	0.98±0.02	0.20±0.02
$C_N \rightarrow D$	0	12	0.12±0.03	0.43±0.09
$C_B \rightarrow D$	0	0	3.36±0.19	0.62±0.05
$C_B \rightarrow D$	0	6	0.24±0.03	0.29±0.02
$C_B \rightarrow D$	0	12	0.03±0.00	0.33±0.09
D	0	0	3.26±0.38	0.61±0.06
D	0	6	1.24±0.05	0.34±0.06
D	0	12	0.21±0.10	0.25±0.02
D	—	—	3.79±0.41	0.68±0.17

Cultures were initiated in C_O, C_N, C_B, or D medium. At the 12th hour of culture, cultures in C_O, C_N, or C_B medium were supplemented with both NAA and BA ($C_O \rightarrow D$), BA alone ($C_N \rightarrow D$), and NAA alone ($C_B \rightarrow D$), respectively, to change each medium to D medium. FdU at a final concentration of 10^{-6} M and thymidine at 10^{-3} M were added at the indicated times. The numbers of tracheary elements and septa were determined after a total of 96 h of culture. The initial concentration of cells was 8.5×10^4 cells/ml. Values are means ± standard errors of results from 3 samples.

tracheary elements. In contrast to cell division, the inhibition of the differentiation into tracheary elements by FdU was overcome not only by thymidine but also by uridine, although only partially (Table 2). The mode of the inhibition of the differentiation into tracheary elements was distinguishable from that of cell division in two more respects, as follows. Much higher concentrations of thymidine were required for the restoration of the differentiation prevented by FdU than for the restoration of cell division (Fig. 2). The blockage of the differentiation by FdU was not overcome by thymidine added just a short time after the addition of FdU, while the blockage of cell division was more or less overcome by such treatment (Fig. 4, 5). These results suggest that the mechanism of prevention by FdU of the differentiation is different from the inhibition of dTMP synthase, to which the blockage of cell division by FdU should be attributable.

An alternative candidate for the primary site of action of FdU, other than the inhibition of dTMP synthase by FdUMP, is at the level of the synthesis of DNA or RNA after it has been incorporated into DNA or RNA (Wasternack and Hause 1987). The incorporation of FdU into DNA and RNA should be suppressed competitively by thymidine and by uridine, a hypothesis that is supported by the result that both thymidine and uridine counteracted the inhibitory effect of FdU on the differentiation into tracheary elements (Fig. 2). Thus, the inhibition of the differentiation by FdU may be mediated by the incorporation of FdU into nucleic acids (RNA and/or DNA) and the resultant interference of their synthesis. In particular, it is likely that the interference in DNA synthesis has a serious effect on the differentiation to tracheary elements, since all tested agents that affect DNA synthesis, such as arabinosyl cytosine, mitomycine C, and aphidicolin, prevent the differentiation (Fukuda and Komamine 1981a). The finding that thymidine was more effective in counteracting the inhibition of the differentiation by FdU than was uridine (Table 2) also suggests the greater importance of the interference in DNA synthesis than the interference in RNA synthesis in the mechanism of action of FdU on the differentiation. Such synthesis of DNA is not synonymous with the replication of total nuclear DNA in the S phase, because the differentiation is independent of the cell-division cycle (Fukuda and Komamine 1981b).

The timing of effective inhibition by FdU was also examined. The differentiation into tracheary elements was prevented when FdU was added earlier than the 36th hour of culture (Fig. 3), and thymidine overcame the inhibitory effect of FdU only when added within the first 4 h of culture (Fig. 4). It can be inferred from these results, in view of the ability of thymidine to cancel the effect of FdU on the differentiation, that the target of FdU that is part of the process of the differentiation, whatever it may be, involves events between the 4th hour and the 36th hour of culture. When cells were pulse-treated with FdU at various times during the course of culture by sequential addition of FdU and thymidine, the differentiation was most strongly inhibited by the treatment from the 12th to the 18th hour of culture (Fig. 5), a result that supports the abovementioned inference that the process that encompasses the target of FdU occurs between the 4th hour and the 36th hour. Furthermore, it also appears, from the effect of treatment with FdU prior to the application of NAA and BA (Table 3), that the targeted process can occur even in the absence of NAA and BA, although both growth regulators are required for the differentiation.

In conclusion, these studies revealed that FdU blocks the differentiation into tracheary elements by way of a different mechanism from that which results in the blockage of cell division by FdU. It is inferred that the targeted process occurs between the 4th and the 36th hours of culture and even in the absence of exogenous auxin and cytokinin. Furthermore, it is possible that the incorporation of FdU into DNA may be responsible for the prevention of the differentiation by FdU.

This work was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists from the Ministry of Education, Culture and Science of Japan.

References

Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R. and Lichtenstein, J. (1958) The mode of action of 5-fluorouracil and its derivatives. *Proc. Natl. Acad. Sci. USA* 44: 1004–1012.

Dannenberg, P. V., Heidelberger, C., Mulkins, M. A. and Peterson, A. R. (1981) The incorporation of 5-fluoro-2′-deoxyuridine into DNA of mammalian tumor cells. *Biochem. Biophys. Res. Commun.* 102: 654–658.

Fosket, D. E. (1968) Cell division and the differentiation of wound-vessel members in cultured stem segments of *Coleus*. *Proc. Natl. Acad. Sci. USA* 59: 1089–1096.

Fosket, D. E. (1970) The time course of xylem differentiation and its relation to deoxyribonucleic acid synthesis in cultured *Coleus* stem segments. *Plant Physiol.* 46: 64–68.

Fraser, R. S. S. (1975) Synchronous cell division in cultured explants of Jerusalem artichoke tubers: The effects of 5-fluorouracil on messenger RNA synthesis and the induction of cell division. *J. Exp. Bot.* 26: 555–568.

Fukuda, H. and Komamine, A. (1980a) Direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in a culture of single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol.* 65: 61–64.

Fukuda, H. and Komamine, A. (1980b) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol.* 65: 57–60.

Fukuda, H. and Komamine, A. (1981a) Relationship between tracheary element differentiation and DNA synthesis in single cells isolated from the mesophyll of *Zinnia elegans*.—Analysis by inhibitors of DNA synthesis. *Plant Cell Physiol.* 22: 41–49.

Fukuda, H. and Komamine, A. (1981b) Relationship between tracheary element differentiation and the cell cycle in single cells isolated from the mesophyll of *Zinnia elegans*. *Physiol. Plant.* 52: 423–430.

Kufe, D. W., Major, P. P., Egan, E. M. and Loh, E. (1981) 5-Fluoro-2′-deoxyuridine incorporation in L1210 DNA. *J. Biol. Chem.* 256: 8885–8888.

Lerbs, S., Wollgiehn, R. and Neumann, C. (1980) Effect of 5-fluorouracil on the synthesis of chloroplast and cytoplasmic ribosomal RNA in leaves of *Nicotiana rustica*. *Biochem. Physiol. Pflanzen* 175: 516–528.

Phillips, R. (1980) Cytodifferentiation. *Int. Rev. Cytol. Suppl.* 11A: 55–70.

Schuetz, J. D. and Diasio, R. B. (1985) The effect of 5-fluorouracil on DNA chain elongation in intact bone marrow cells. *Biochem. Biophys. Res. Commun.* 133: 361–367.

Shininger, T. L. (1975) Is DNA synthesis required for the induction of differentiation in quiescent root cortical parenchyma? *Dev. Biol.* 45: 137–150.

Sugiyama, M., Fukuda, H. and Komamine, A. (1986) Effects of nutrient limitation and γ-irradiation on tracheary element differentiation and cell division in single mesophyll cells of *Zinnia elegans*. *Plant Cell Physiol.* 27: 601–606.

Turgeon, R. (1975) Differentiation of wound vessel members without DNA synthesis, mitosis or cell division. *Nature* 257: 806–808.

Wasternack, C. and Hause, B. (1987) 30 years of 5-fluorouracil. *Pharmazie* 42: 73–79.

(Received April 3, 1989; Accepted October 27, 1989)