# THE EFFECT OF 5-FLUOROURACIL ON THE GROWTH AND NUCLEOLUS OF WHEAT COLEOPTILES

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#### Summary

Intact etiolated wheat coleoptiles grown from the beginning of imbibition in 5-fluorouracil (5-FU) show normal cell elongation, but division is inhibited. 5-FU-treated coleoptiles, 48 hr after imbibition, have enlarged nucleoli (165% increase in volume) in which the RNA is mostly confined to the periphery. Untreated and treated nucleoli were studied by light and electron microscopy. The 5-FU effects on the nucleolus, which occur at the time cell division usually occurs if 5-FU is not present, are of interest in relation to ribosome synthesis. Uracil or thymidine did not reverse the nucleolar effects, but uracil further inhibited growth, while thymidine partly reversed the cell division inhibition. Results with 5-FU and thymidine suggest that the coleoptile cells can divide at least once when they have abnormal nucleoli, but normal nucleolar metabolism is essential for the complete growth of the etiolated wheat coleoptile.

### I. INTRODUCTION

5-Fluorouracil (5-FU) has been widely used in studies on the growth of plant cells (Key 1969). 5-FU inhibits the synthesis of ribosomal RNA, as well as soluble RNA and DNA, but has little effect on an RNA fraction thought to be messenger RNA (Cherry and van Huystee 1965; Key 1966; Key and Ingle 1968).

Studies on a number of tissues have shown that 5-FU has no effect on cell expansion (Key and Ingle 1964; Masuda, Setterfield, and Bayley 1966; Lin and Key 1968; Nooden 1968). Key and Ingle (1964, 1968), using 5-FU have shown that auxin-induced growth is not dependent on the synthesis of ribosomal RNA. It has been concluded (Key 1969) that new ribosomes are not essential for cell elongation. However, under some conditions ribosomes limit expansion, as in fully expanded cells of tuber tissue after a period of dormancy. Thus, Setterfield (1963) found that 5-FU given during the "aging" period inhibited subsequent auxininduced elongation in Jerusalem artichoke tuber slices. Fowke and Setterfield (1968) have shown by electron microscope studies that ribosomes are synthesized in this aging period.

Fewer studies have been made on the effect of 5-FU on cell division. Masuda, Setterfield, and Bayley (1966) found that 5-FU completely inhibited cell division in oat seedling roots. Other authors indicated that 5-FU blocked division in soybean roots (Lin and Key 1968) and in artichoke tuber slices stimulated to divide, but did not present any data (Setterfield 1963).

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Masuda, Setterfield, and Bayley (1966) have studied the effect of 5-FU on both cell division and cell elongation in intact oat coleoptiles. If germinating oat seeds were transferred at 24 hr to 5-FU at 500 mg/l at 25°C, cell division and cell elongation were not affected. The embryonic oat coleoptile was shown to be rich in ribosomes and it was concluded that there were enough ribosomes in the mature embryo for all the cell division and elongation that occurred in the growth of etiolated oat coleoptiles.

We have examined the effect of 5-FU on both the growth and nucleolus of intact etiolated wheat coleoptiles. Cell division but not cell elongation was almost completely inhibited if wheat seeds were grown in 5-FU from the beginning of imbibition. The nucleolus, which is the site of synthesis of ribosomal RNA and probably of ribosome assembly (Birnstiel 1967; Maden 1968) was affected by 5-FU.

#### II. METHODS

The wheat (*Triticum aestivum* L.) cultivar Mendos was used in all experiments. Manipulations during the growth of the coleoptiles were done in dim green light (Withrow and Price 1957).

#### (a) Growth of Coleoptiles

Wheat seeds were germinated and grown in 9-cm Petri dishes as previously described by Rose and Adamson (1969). The seedlings were grown in the dark at  $23 \pm 1^{\circ}$ C. In most experiments test chemicals were added at the beginning of imbibition. However, in some cases the seeds were germinated in distilled water for 24 hr and then transferred to dishes containing 5-FU. In all cases there was 10 ml of the appropriate solution in each 9-cm Petri dish, and three dishes of 10 coleoptiles for each treatment.

#### (b) Cell Number and Cell Length Determinations

Ten coleoptiles were macerated for each determination, using a similar procedure to Wright (1961). Maceration was carried out in 5% acetic acid in 2n HCl. After a few minutes in a boiling water-bath, the tissue was left in a 40°C oven for 1–2 days before finally macerating the cells with a Pasteur pipette.

Final cell lengths of 100 cells were measured with an ocular micrometer in macerates from fully grown coleoptiles. Epidermal cells which do not divide (Avery and Burkholder 1936; Wright 1961) were not measured. Final cell numbers were determined when the coleoptiles had reached a length of at least 20 mm (if they grew this long), when cell division, but not cell elongation, had been completed (Rose and Adamson 1969). Haemocytometer counts were easier with the smaller cells. At least 1500 cells were counted for each treatment. The coleoptiles used for cell division determinations were growing parallel to the three lots of 10 coleoptiles used for height and final cell length measurements.

#### (c) Light Microscopy

The coleoptiles were fixed in 3:1 (v/v) absolute alcohol-glacial acetic acid, rinsed several times in 70% alcohol, and stored under refrigeration in 70% alcohol until required. Subsequently the tissue was dehydrated in a t-butanol series, embedded in paraffin, and sectioned at 10  $\mu$ m (Johansen 1940). The following staining procedures were used: azure B at pH 4.0, which stains DNA blue-green and RNA purple (Flax and Himes 1952; Swift 1955); naphthol yellow S for staining protein yellow, with or without Feulgen staining for DNA (Deitch 1955). Naphthol yellow S-stained material was viewed with a blue filter. After staining, all tissue was mounted in Euparal. In order to confirm that the material which stained purple with azure B was RNA, tissue sections were treated with ribonuclease at 0.04% (Swift 1955) for 2 hr at 37°C followed by 2% perchloric acid for 20 min at 4°C (Masuda, Setterfield, and Bayley 1966) prior to staining for RNA.

The diameter of selected circular nucleoli and nuclei were measured on azure B-stained  $10 \,\mu\text{m}$  paraffin sections. Measurements were made with a Meopta ocular micrometer. It was assumed that the circular nuclei and nucleoli were spherical and volumes were determined from these diameter values.

### (d) Electron Microscopy

Tissue from the central region along the length of the coleoptile was fixed for 2 hr in 2% osmium tetroxide, buffered to pH 7.4 with veronal acetate which contained both 1.5% sucrose (Caulfield 1957) and 0.01% calcium chloride (Porter and Machado 1960). The tissue was added to the fixative at 0°C and allowed to come to room temperature. Dehydration in an ethanol series and embedding in Araldite was according to Luft (1961). Sections were cut with glass knives on a LKB ultramicrotome, stained with lead citrate and uranyl acetate (Venable and Coggeshall 1965), and examined with an Hitachi HS-8 electron microscope.

#### (e) Chemicals

Ribonuclease was obtained from Worthington Biochemicals, Freehold, New Jersey, U.S.A., azure B and naphthol yellow S from Matheson, Coleman, and Bell, Norwood, Ohio, U.S.A., and 5-fluorouracil, uracil, and thymidine from Calbiochem, Los Angeles, U.S.A.

### III. RESULTS

5-FU at 500 mg/l markedly inhibited the growth of wheat coleoptiles if given at zero time (i.e. from the beginning of imbibition), whereas growth was inhibited much less if the germinating seeds were transferred to 5-FU at 24 hr (Fig. 1).



Fig. 1.—Effect of 5-FU on the growth of intact coleoptiles. Wheat seedlings were grown from the beginning of imbibition (0 hr) in distilled water ( $\Box$ ) or 5-FU ( $\bigcirc$ ), or were transferred to 5-FU at 24 hr ( $\bigcirc$ ). Each value represents the mean of three lots of 10 coleoptiles. 5-FU concentration was 500 mg/l.

Fig. 2.—Effect of 5-FU on the total nucleolar volume per nucleus of intact coleoptiles. 5-FU at 500 mg/l was added at the beginning of imbibition ( $\bigcirc$ ) or after 24 hr prior germination in distilled water ( $\bigcirc$ ). Values for the embryo, the water control ( $\square$ ) at 48 hr, and the 0 hr 5-FU addition at 48 hr were from nucleoli from 100 nuclei. All other nucleolar values were determined on 50 nuclei. In all cases an equal number of measurements were made on two different coleoptiles. Standard error of the mean indicated.

To determine what component of growth was affected, cell numbers and cell lengths were measured. 5-FU reduced the final number of cells but had very little effect on the final cell length (Table 1). Division was almost completely inhibited by 5-FU given at zero time.

Treatment from the beginning of imbibition is evidently more effective than the transfer to 5-FU at 24 hr probably because metabolic changes associated with the first divisions occur prior to 24 hr.

The inhibition of cell division by this compound, which is known to affect ribosome synthesis, prompted a cytological examination of the nucleolus. Nucleoli examined 2 hr after imbibition were used to represent the initial condition. Nucleoli were subsequently examined at 24, 48, and 93 hr after imbibition. These latter periods are just prior to the first wave of division, in the peak division period, and in the rapid elongation phase after division, respectively (Wright 1961; Rose and Adamson 1969).

#### TABLE 1

EFFECT OF 5-FU ON THE FINAL HEIGHT, CELL LENGTH, AND CELL NUMBER OF INTACT WHEAT COLEOPTILES

Wheat seedlings were grown from the beginning of imbibition (0 hr) in distilled water or 5-FU, or were transferred to 5-FU at 24 hr. 5-FU concentration was 500 mg/l. The initial coleoptile cell number present in the embryo is approximately 40,000. Standard errors of the mean are indicated

Character	Growth in Water from 0 hr	Growth in 5-FU	
		From 0 hr	From 24 hr
Coleoptile height (mm)	$60 \cdot 5 \pm 0 \cdot 6$	$14 \cdot 5 \pm 0 \cdot 9$	$44 \cdot 3 \pm 1 \cdot 0$
Cell length ( $\mu$ m)	$327\pm9$	$299 \pm 12$	$306\pm10$
Cell number	151,000	55,000	125,000

5-FU had cytological effects on the appearance and volume of the nucleolus. Figure 2 shows the effect of 5-FU on nucleolar volume. 5-FU added at 0 or 24 hr had caused large increases in nucleolar volume by 48 hr, the latter but not the former treatment causing a decline in volume by 93 hr. The reasons for this effect is not known, but may be related to the greater amount of division occurring in the coleoptiles transferred to 5-FU at 24 hr (Table 1). Further studies were confined to the effect of adding 5-FU at the beginning of imbibition.

Fig. 3.—Light micrograph showing nucleolus from a 48-hr-old coleoptile grown from the beginning of imbibition in 5-FU at 500 mg/l. Some "granular" material is present in the inner zone of the "doughnut" nucleolus. Lighter-staining nucleus surrounds nucleolus. Small vacuoles are visible within the densely staining nucleolar periphery. Azure B stained.  $\times 1900$ .

Fig. 4.—Nucleolus from coleoptile treated from the beginning of imbibition in 5-FU 500 mg/l. Inner zone (I) has many free granules (G). Outer zone (O) has a more dense fibrillar structure, with fibrils (F), granules (G), and vacuoles (V).  $\times 16,000$ .

Fig. 5.—Light micrograph showing nucleolus from 48-hr-old coleoptile grown from the beginning of imbibition in distilled water. Lighter staining nucleus surrounds densely staining nucleolus. A small central vacuole is visible in the nucleolus. Azure B stained.  $\times 1900$ .

Fig. 6.—Nucleolus from 48-hr-old coleoptile grown from beginning of imbibition in distilled water. Fibrillar areas (F), granular areas (G), and vacuoles (V) indicated. Nucleoplasm (Nu) surrounds the nucleolus.  $\times 16,000$ .



The cytological characteristics of the 5-FU-treated nucleolus are shown in Figure 3. The typical nucleolus resulting from such treatment had a "doughnut" appearance, with a peripheral band of varying width which stained for RNA with azure B. There was a very large central area which did not stain with azure B, except for some "granular" material (Fig. 3). Untreated nucleoli stained more uniformly with azure B, except for some small vacuolar areas which did not stain (Fig. 5) and which did not appear to contain large amounts of azure B-positive granular material. All of this material which stained with azure B in control and 5-FU-treated nucleoli was removed by ribonuclease, confirming the presence of RNA.

With the protein stain, naphthol yellow S, the 5-FU-treated nucleoli also showed an outer band, but the difference in the staining intensity of outer and inner zones was less marked than with azure B. The uniform staining of the inner zone by naphthol yellow S suggests a uniform distribution of protein in this zone, contrasting with the scattered "granular" material which stained for RNA.



Fig. 7.—Effect of 5-FU on the size class frequency distribution of the total nucleolar volume per nucleus.  $\Box$  Nucleoli from embryonic coleoptiles imbibed in water for 2 hr.  $\bigcirc$  Nucleoli from 48-hr-old coleoptiles grown in water.  $\bullet$  Nucleoli from 48-hr-old coleoptiles grown in 5-FU at 500 mg/l. Area shaded with horizontal bars represents those nuclei in the 5-FU treatment with part of the total nucleolar volume made up of at least one "doughnut" nucleolus. There was only one control nucleus with a nucleolus having a very large central vacuole giving the appearance of a 5-FU-treated nucleolus. Each distribution represents the total nucleolar volume from 100 nuclei from two different coleoptiles.

Fig. 8.—Effect of 5-FU on the size class frequency distribution of nuclear volume. □ Nuclei from embryonic coleoptiles imbibed in water for 2 hr. ○ Nuclei from 48-hr-old coleoptiles grown in water. ● Nuclei from 48-hr-old coleoptiles grown in 5-FU, at 500 mg/l. Each distribution represents the volume of 100 nuclei from two different coleoptiles.

In Figure 7, the size class frequency distributions are shown for nucleolar volumes in the embryo, and after growth for 48 hr in the presence or absence of 5-FU. Figure 7 also shows the number of nuclei with at least one doughnut nucleolus. In the presence of 5-FU there is a much greater volume containing RNA in the nucleolus at 48 hr,

566

than is present in the embryo. This can be concluded after subtracting the central clear zone volume from the total volume in 5-FU-treated nucleoli. Also, the volume containing RNA present in the outer band of 5-FU-treated nucleoli at 48 hr exceeds the total volume containing RNA which is present in untreated nucleoli at 48 hr.

While the frequency distributions of nucleolar volumes clearly show the effects of 5-FU on the nucleolus, they contrast with the frequency distributions of 5-FU on nuclear volumes (Fig. 8). 5-FU-treated and untreated nuclei, 48 hr after imbibition, show similar distributions and both show much larger volumes than nuclei in the embryo.

The nucleolar or nuclear volumes of epidermal cells were not included in Figures 7 and 8. They are different from other coleoptile cells and do not divide after germination (Avery and Burkholder 1936; Wright 1961).

#### TABLE 2

EFFECT OF URACIL AND THYMIDINE ON THE COLEOPTILE GROWTH INHIBITION AND NUCLEOLAR ENLARGEMENT CAUSED BY  $5 \cdot FU$ 

All compounds were added from the beginning of imbibition at the following concentrations: thymidine  $10^{-2}M$ , uracil  $10^{-2}M$ , 5-FU 500 mg/l ( $4 \times 10^{-3}M$ ). The height, cell length, and cell number values given are the final values at the end of coleoptile growth. Nucleolar volumes were determined on 50 nuclei from two different 48-hr-old coleoptiles. Standard errors of the mean are indicated

${ m Treatment}$	Coleoptile Height (mm)	$egin{array}{c} { m Cell} \ { m Length} \ (\mu{ m m}) \end{array}$	$10^{-3}  imes$ Cell Number	Nucleolar Volume (µm <sup>3</sup> )
Water	$61 \cdot 0 \pm 1 \cdot 2$	$347\pm11$	151	$61\pm 4$
Thymidine	$66 \cdot 1 \pm 0 \cdot 8$	$346\pm10$	140	$58\pm$ 4
Uracil	$55 \cdot 9 \pm 1 \cdot 0$	$319\pm12$	139	$56\pm~3$
Thymidine + uracil	$63 \cdot 3 \pm 1 \cdot 1$	$344\pm12$	141	$59\pm~4$
5-FU	$14 \cdot 6 \pm 0 \cdot 5$	$316\pm14$	51	$163 \pm 13$
5 - FU + thymidine	$29 \cdot 0 \pm 0 \cdot 8$	$239\pm12$	99	$126 \pm 11$
5-FU+uracil	$7 \cdot 0 + 0 \cdot 1$	153 + 4	39	134 + 8
$5  ext{-FU} +  ext{thymidine} +  ext{uracil}$	$9 \cdot 8 \pm 0 \cdot 2$	$185\pm 5$	41	$131 \pm 10$

In order to confirm the light microscope studies, the 5-FU-treated and untreated nucleoli were examined with the electron microscope. Electron micrographs show the outer band of 5-FU-treated nucleoli to consist of densely packed granules and fibrils, while the inner zone is electron-lucent and contains fibrils and many loosely scattered granules (Fig. 4). The control nucleoli have electron-lucent vacuoles and separate granular and fibrillar areas (Fig. 6). The granules occur around the periphery of the nucleolus and around the vacuoles. The size of the individual granules and fibrils are similar in 5-FU-treated and untreated nucleoli.

5-FU can be converted to 5-fluorodeoxyuridine (FUDR), which subsequently causes a deficiency of thymidylic acid (Cohen *et al.* 1958; Harbers, Domagk, and Muller 1968), leading to an inhibition of DNA synthesis. In order to see if the growth or nucleolar effects were related to effects on DNA, rather than RNA, synthesis, reversal studies were carried out using thymidine and uracil in combination with 5-FU.

Table 2 shows that uracil does not reverse the 5-FU growth effects but causes some additional inhibition. Thymidine causes partial reversal of the growth effects, which can be accounted for by an increase in cell number, even though the mean cell length is less than the water controls. The effects of 5-FU on growth cannot be completely accounted for by inhibition of DNA synthesis, as our experiments have shown that inhibition of coleoptile growth by FUDR can be completely reversed by thymidine. Rather surprisingly, uracil plus thymidine plus 5-FU causes greater growth inhibition than 5-FU alone, even though uracil plus thymidine alone are without effect on coleoptile growth (Table 2).

Thymidine, uracil, or uracil plus thymidine had no substantial effect on the nucleolar volume increase (Table 2) or the cytological abnormalities cause by 5-FU. However, in the presence of uracil the 5-FU-affected nucleoli appeared to contain more RNA, as judged by the smaller volume of the central area of the "doughnut" nucleoli. Uridine also failed to reverse the growth or nucleolar effects of 5-FU.

## IV. DISCUSSION

Wheat coleoptile cells usually divide two or three times after germination, followed by a period of cell elongation only (Wright 1961). Germination of wheat seeds in 5-FU prevents cell division in the coleoptile, but allows the existing embryonic cells to reach a similar length to the cells of untreated coleoptiles. The absence of a 5-FU effect on cell elongation is consistent with previous studies with plant tissue (Key 1969).

The addition of 5-FU at 24 hr after imbibition rather than at the beginning of imbibition probably accounted for the absence of an effect of 5-FU on oat coleoptile growth found by Masuda, Setterfield, and Bayley (1966) in their experiments. The same concentration of 5-FU (500 mg/l) given to wheat coleoptiles from the beginning of imbibition almost completely inhibited cell division and reduced growth. In wheat coleoptiles there was some inhibition of growth if 5-FU was given at 24 hr. 5-FU given from imbibition may also facilitate uptake by the coleoptile. In the wheat embryos, increases in DNA and some types of RNA are evident at 24 hr after germination (Vold and Sypherd 1968), and imbibition triggers off changes in nucleic acid metabolism (Marcus and Feeley 1964). DNA synthesis, in preparation for the first divisions, occurs during the first 24 hr (Setterfield 1961) in oat coleoptiles.

5-FU added at either zero time or 24 hr after imbibition caused enlargement and cytological abnormalities of the nucleolus. The effects of 5-FU on the nucleolus occurred in the 24–48-hr period, at the same time as the large increases in the volume of control nucleoli that are preparing for division. Wright (1961) has shown large increases in total RNA in the 24–48-hr period of growth of the etiolated wheat coleoptile.

The nucleolar abnormalities cannot completely explain the growth inhibition, as coleoptile cells divided at least once in the presence of 5-FU and thymidine, even though the nucleolar abnormalities still occurred. This is consistent with some conversion of 5-FU to FUDR, causing a thymidine deficiency and subsequently inhibition of DNA synthesis and cell division. Therefore, in wheat coleoptiles, cells can apparently divide and elongate without concomitant ribosomal synthesis. However, the much smaller final coleoptile heights in the presence of 5-FU plus thymidine, relative to the water controls, suggests that for the normal growth of the etiolated wheat coleoptile normal nucleolar metabolism is essential. The reason for 5-FU plus uracil causing greater growth inhibition than 5-FU alone is not known.

Embryonic cells of wheat coleoptiles are rich in ribosomes (Marcus and Feeley 1964) and the results in this paper suggest that at least one cell division can occur in the absence of normal nucleolar metabolism. Whether normal nucleolar metabolism is essential for division in permanent apical meristems is not known. McLeish (1954) has shown that micronuclei (induced by maleic hydrazide) in *Vicia faba* root tips can undergo mitosis only if a nucleolus is present. Studies with developing animal embryos suggest that a nucleolus is not necessary for cell division if the cells are rich in ribosomes (Hay 1968).

The effects of 5-FU on the appearance of the nucleolus must be due to large changes in nucleolar metabolism. The marked enlargement of the nucleoli is most likely due to the retention, or slow loss from the nucleolus, of ribosomal precursor material. 5-FU can be incorporated into the RNA of plant cells (Key 1966; Galun and Torrey 1969), so 5-FU-treated nucleoli may contain abnormal RNA (because of the incorporation of 5-FU) which prevents synthesis of normal ribosomes.

5-FU causes nucleolar but not nuclear enlargement in rat liver cells (Stenram 1966), together with cytological effects on the nucleolus, and the enlargement of the nucleoli appears to be related to an accumulation of ribosomal RNA precursors (Willen and Stenram 1967). The 5-FU-treated rat liver nucleoli do not show the doughnut appearance or as much enlargement as the nucleoli of 5-FU-treated wheat coleoptiles.

The nature of both the RNA and protein present in 5-FU-treated wheat coleoptile nucleoli warrants further investigation, as a means of obtaining additional information on ribosome synthesis in plant cells.

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