A Cytological Analysis of the Antimetabolite Activity of 5-Hydroxyuracil in *Vicia faba* Roots*

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ABSTRACT

The effects of 5-hydroxyuracil (5-HU) (isobarbituric acid) upon cell elongation, mitosis, and DNA synthesis were studied in *Vicia faba* roots.

5-HU had no consistent effect upon root elongation. It blocked DNA synthesis (analyzed by photometric measurements of Feulgen dye in nuclei) during the first 6 hours of treatment; the block spontaneously disappeared by the 12th hour of treatment.

Uracil and thymine had no effect upon this block of synthesis. Both thymidine and uridine reversed the block in 6 and 9 hours respectively.

In all cases blockage of DNA synthesis was followed by inhibition of mitosis (determined by changes in the percentage of cells in mitosis) and resumption of DNA synthesis was followed by resumption of mitosis. Inhibition indices calculated from the mitotic data indicated a competitive relationship between 5-HU and thymidine and 5-HU and uridine.

5-HU is considered to block DNA synthesis by competing with thymidine for sites on enzymes involved in the synthesis. It is suggested that uridine reverses the block in synthesis by undergoing a conversion to thymidine.

INTRODUCTION

The pyrimidine analog 5-hydroxyuracil (5-HU) (isobarbituric acid) and its nucleoside 5-hydroxyuridine have been reported to interfere with the growth of several lower organisms.

Kidder and Dewey (9) found that in a uracil-requiring strain of *Tetrahymena geleii* 5-HU competitively inhibited the growth-promoting effects of uracil. Hitchings *et al.* (6–8) reported that in *Lactobacillus casei* 5-HU caused inhibition of growth in the presence of thymine or folic acid and that this inhibition was reversed competitively by uracil. Puleston *et al.* (12), however, found that 5-HU at concentrations of 0.001 to 100 λ/ml. had no inhibitory effect upon the growth of *Streptococcus fecalis*.

In related studies Visser and his coworkers tested the nucleoside of 5-hydroxyuracil, 5-hydroxyuridine, on wild and mutant strains of *Neurospora*, on bacteria, on viruses, and on animal tumor tissue (for summary see Visser, 24). This compound inhibited the growth of the wild type *Neurospora*, but it was ineffective in the presence of each of four nucleosides: adenosine, guanosine, 5-bromouridine, and 5-chlorouridine. It inhibited the growth of the pyrimidine-requiring *Neurospora* 1298, an organism which was also inhibited by the four nucleosides (adenosine, guanosine, 5-bromouridine, and 5-chlorouridine). Reversal of the 5-hydroxyuridine inhibition in *Neurospora* 1298 was accomplished with uracil, uridine, and cytidine. In purine-requiring mutants of *E. coli*, 5-hydroxyuridine competitively inhibited the growth stimulation produced by adenine and hypoxanthine; these inhibitions were completely reversed by uridine or cytidine. 5-Hydroxyuridine effectively limited propagation of Theiler's G. D. VII virus in day-old mouse brain minces, and Sugiura and Stock (unpublished data quoted by Visser) obtained inhibition of the growth of sev-

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eral tumors in experimental mice with the same compound.

5-HU has thus been used in only a very limited number of experiments. It inhibited the growth of two of the three organisms to which it was supplied and in both cases its inhibition could be reversed only by uracil. Its nucleoside, 5-hydroxyuridine, is also a potent inhibitor; because of the complexity of its behavior, however, 5-hydroxyuridine was not used in the present study.

The experiments reported in this paper extend the investigation of 5-HU's activity to the angiosperms. The experiments test in the roots of *Vicia faba* (the broad bean) the effects of the compound upon the cytological aspects of three processes: root elongation, mitosis, and deoxyribonucleic acid (DNA) synthesis.

Materials and Methods

The *Vicia faba* seeds used in this investigation were obtained in several lots from the L. L. Olds Seed Company, Madison, Wisconsin.

Seeds selected for uniform size and normal appearance were soaked in tap water for approximately 36 hours with water changes at about every 12 hours. The seed coats were then removed and the naked embryos were planted in standard 10 inch pots approximately 1½ inches deep in soaked and tamped vermiculite which had been previously sifted free of dust. The potted seeds were placed in a dark constant-temperature room (21 ± 0.5°C.) and watered daily. In 4 to 5 days the roots were of sufficient length (3 to 5 inches) for experimental use.

Healthy seedlings were then replicated on the basis of root length and thickness, the number of roots in each replication corresponding to the number of treatment groups in the experiment. Since four roots (unless otherwise noted) were fixed from each treatment at each time period, four complete replications were required for each time period investigated. Four roots were fixed at the beginning of each experment to give the initial (0 hour) mitotic frequency. Plumules were removed from all seedlings at the beginning of treatment.

Treatment was carried out in 800 ml. beakers filled to within ½ inch of the lip with aqueous solutions of the chemicals being tested. The seedlings were placed on plastic plates which rested on the beaker tops and their roots were introduced into the solutions through holes in the plates. Short plastic tubes extending below each hole helped to keep the roots vertical in the solutions. Through a larger central hole in the plate a gas diffusion tube connected to an air line was passed to the bottom of each beaker, and the small stream of air from the tube agitated and aerated the treatment solution. The beakers with their seeds were kept in a dark constant-temperature room (21.0 ± 0.5°C.) for the duration of the experiment.

Five chemicals were used in this study: 5-hydroxyuracil (isobarbituric acid) from Dougherty Chemicals, Richmond Hill, New York; thymine and uracil from Schwarz Laboratories, New York; thymidine from California Foundation for Biochemical Research, Los Angeles; and uridine from Nutritional Biochemical Corporation, Cleveland. Treatment solutions were prepared by dissolving appropriate amounts of these chemicals in distilled water which had been "demineralized" (less than 1 P.P.M. ion concentration) in an ion-exchange column (Deaminizer Company). Heat (up to 60°C.) was required to dissolve 5-HU; all other chemicals dissolved at room temperature. All treatment solutions were adjusted to pH 6.0 ± 0.1 with dilute HCl or NaOH; none varied by more than half a pH unit during the course of the treatment.

Root elongation was determined by measuring before and after treatment the distance between the root tip and an India ink mark placed arbitrarily beyond the region of elongation (about 6 or 7 cm. from the root tip). Measurements are presented as arithmetic means of values from four roots taken at one time period of treatment.

The mitotic frequency was determined by the method described by Setterfield *et al.* (19). The first two millimeters of a Feulgen-stained meristem are macerated by treatment with pectinase to a suspension of free cells and small groups of cells. The separated cells are concentrated by centrifuging, the pectinase solution is decanted, and the cells are resuspended in a small amount of a Karo syrup–phosphate buffer mixture and mounted on slides. By examining the first 1000 cells encountered on such slides, random samples of cells throughout the meristem can be rapidly scored as to mitotic stage. All mitotic values given are derived from the arithmetic means of four roots macerated and scored separately and thus represent a total of 4000 cells examined for every time period reported.

The criteria used to separate mitotic stages for scoring purposes were those described by Patau and Swift (11). The exact extent of any stage is difficult to define; in experiments such as those described here, consistency of definition rather than precision of definition is the basic requirement. Particular care, however, was taken to detect cells in very early prophase in order not to miss the inception of a sudden wave of mitoses caused by the simultaneous overcoming of a mitotic block by many interphase cells.

Microphotometric measurements of deoxyribonucleic acid (DNA) were made on Feulgen-stained interphase nuclei prepared by a modification of the pectinase procedure. Eight roots taken at a single time period from each treatment were fixed 10 minutes in 3:1 alcohol-acetic acid, *in vacuo*, washed 10 minutes in water, hydrolyzed 8 minutes in 1 N HCl, stained 1 hour in leucobasic fuchsin, and washed in three 10 minute

changes of SO_2 water. After removal of the root cap under the dissection microscope, the first millimeter of the tip was cut from each of the eight roots and all eight 1 mm. pieces were placed together in 1 ml. of a 5 per cent aqueous solution of pectinase for 5 hours. The pectinase solution was then decanted and the root segments were given two washes in water (3 minutes each) and were finally shaken vigorously in about 1 ml. of water to give a suspension of free cells. Several drops of this aqueous cell-suspension were then placed on a slide and the cells were dried down to the slide for 12 hours at room temperature in a dust-free area. Each slide was passed through two changes (5 minutes each) of absolute alcohol, one of 1:1 absolute alcohol and xylene, and two of xylene. The dehydrated cells were flooded with HSR mounting medium dissolved in xylene and the mount was completed with a coverslip.

All roots taken from all treatments at the same time period were passed together through fixative, wash, HCl, Feulgen stain, and SO_2 water. Separation of the roots into individual treatment groups was then possible since roots from different treatments had originally been cut at different lengths for recognition purposes.

The microphotometric procedure used was that described by Ris and Mirsky (16) and Swift (22). Actual measurements were made with the instrument described by Srinivasachar (21) and Woodard (25), using a wave length of 4930 A. Random transects were made across the slides, and measurements were made of all reasonably homogeneous and approximately spherical nuclei encountered.

Values for the relative amount of DNA (Feulgen dye) per nucleus were obtained by measuring the extinction of a central core of the nucleus and multiplying this by the product of two right-angle diameters of the nucleus. Woodard (25) has calculated that this procedure gives values directly proportional to the amount of absorbing substance in spheres and spheroids.

RESULTS

Root Elongation Studies:

The elongation response of roots to 5-HU proved to be extremely variable. Elongation of roots treated with 2.0×10^{-4} M 5-HU, for example, at both 12 and 24 hours, was significantly stimulated in one experiment, significantly inhibited in a second, and unaffected in a third. A similar range of deviation was encountered at other concentrations of 5-HU. No consistent pattern of root-growth inhibition or stimulation appeared in the experiments reported below in which the pattern of mitotic inhibition was clearly evident and wholly reproducible. Since 5-HU appeared to affect root growth, if at all, in an essentially random manner, root elongation data, gathered routinely to detect possible root death, will not be further presented in this study.

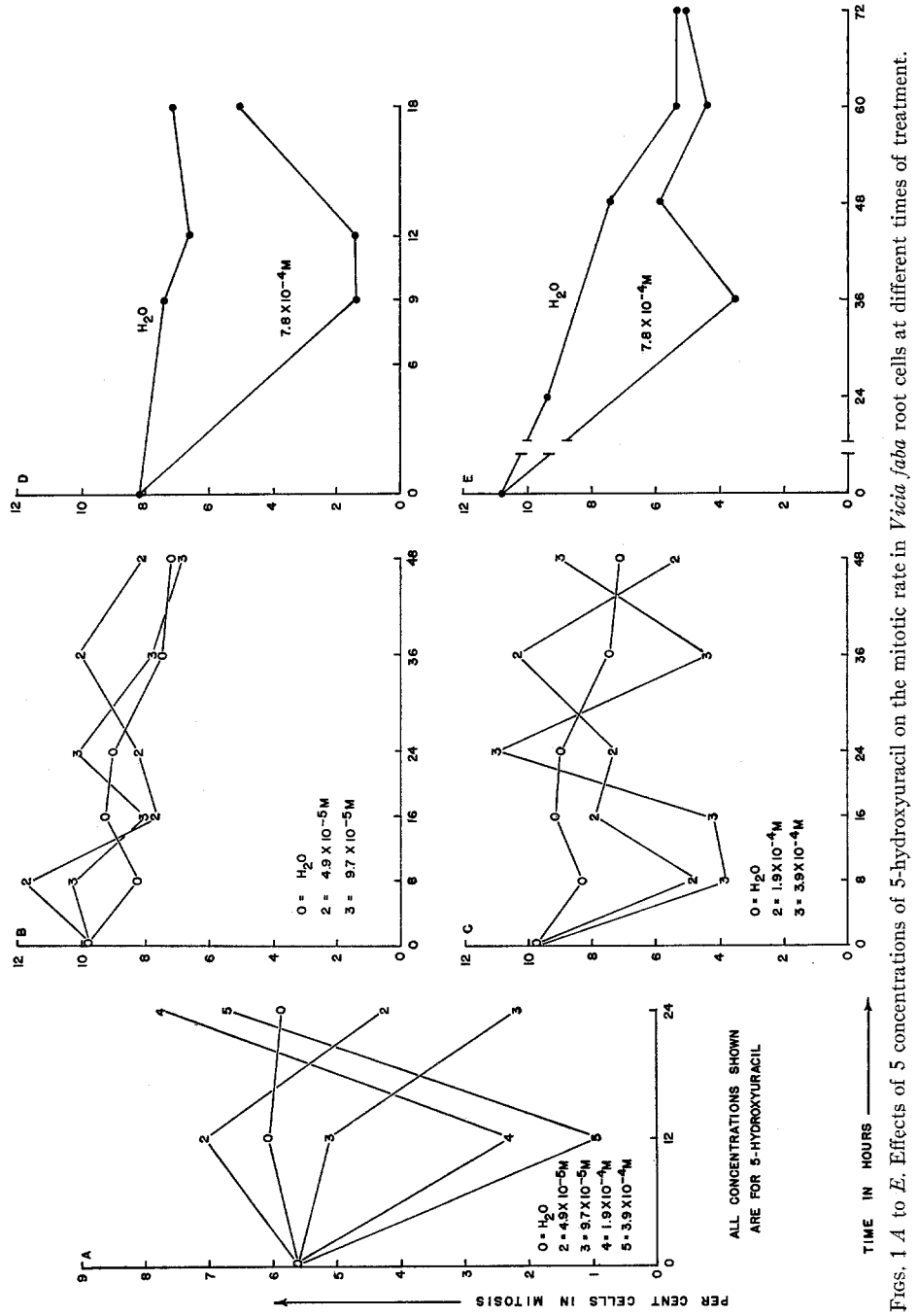
Mitotic Frequency Studies:

Each of four concentrations of 5-HU was tested at two time periods in an effort to establish the minimum time-concentration level at which inhibition[1] could be consistently produced. Fig. 1 *A* shows the results of the first experiment. At 12 hours 5-HU significantly inhibited mitosis at concentrations of 1.9×10^{-4} M (25 P.P.M.) and 3.9×10^{-4} M (50 P.P.M.), but had no significant effect at concentrations of 4.9×10^{-5} M (6.3 P.P.M.) and 9.7×10^{-5} M (12.5 P.P.M.). At 24 hours mitosis was occurring at the control rate in roots treated with 5-HU at concentrations of 4.9×10^{-5} M, 2.0×10^{-4} M, and 3.9×10^{-4} M, while a significant mitotic inhibition had appeared in roots treated with 9.8×10^{-5} M 5-HU.

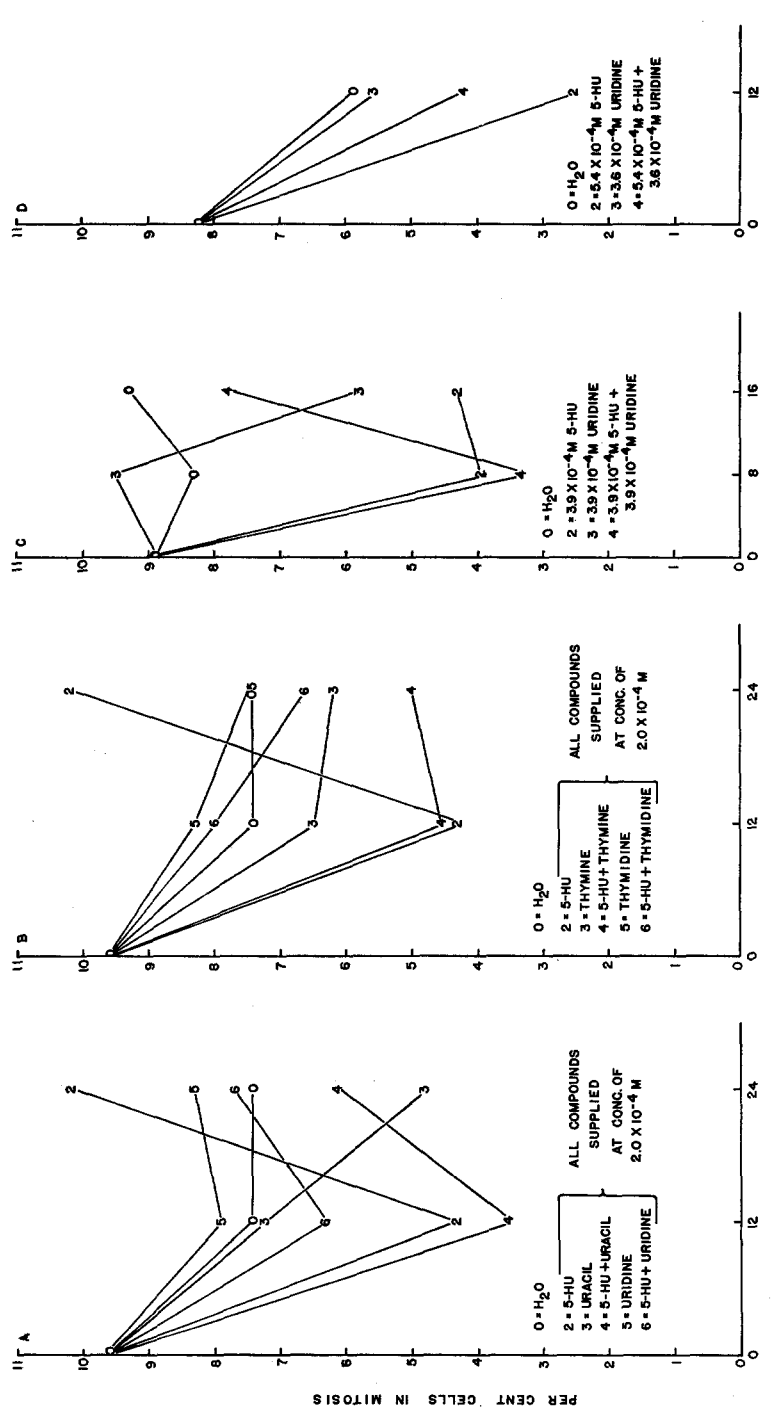
The next experiment (Figs. 1 *B* and 1 *C*) was an attempt to establish the reality of the spontaneous reversal of mitotic inhibition which was indicated in the first experiment. 5-HU at the same four concentrations was tested for its effect on the mitotic rate at five time periods: 8, 16, 24, 36, and 48 hours. 4.9×10^{-5} M 5-HU (Fig. 1 *B*) stimulated mitosis significantly at 8 hours but had no effect at any other time period. 9.7×10^{-5} M 5-HU (Fig. 1 *B*) had no effect on the mitotic rate at any time period. 1.9×10^{-4} M 5-HU (Fig. 1 *C*) significantly inhibited mitosis at 8 hours and then had no effect on the mitotic rate at the remaining time periods. 3.9×10^{-4} M 5-HU significantly inhibited mitosis at 8, 16, and 36 hours and had no effect on the mitotic rate at 28 and 48 hours.

Thus the mitotic activity of roots treated for 48 hours with 3.9×10^{-4} M 5-HU appeared to follow a regular pattern: inhibition at 12 hours, recovery at 24 hours, inhibition at 36 hours, and recovery at 48 hours. A similar pattern appeared during the first 48 hours in roots treated with 7.8×10^{-4} M (100 P.P.M.) 5-HU (Figs. 1 *D* and 1 *E*); the data presented in Fig. 1 *E* also indicate that the rate of mitosis, which had returned to

[1] It should be understood that the terms "inhibition" and "block" are used here synonymously to indicate the complete stopping of a process in a limited number of cells. "Reversal" and "recovery" are used to mean that the stopped process has resumed in the affected cells. The validity of these usages will be dealt with in the Discussion section.



FIGS. 1 *A* to *E*. Effects of 5 concentrations of 5-hydroxyuracil on the mitotic rate in *Vicia faba* root cells at different times of treatment.



FIGS. 2 *A* to *D*. Effects on the mitotic rate in *V. faba* root cells of treatment with 5-hydroxyuracil, uracil, uridine, thymine, and thymidine (each supplied separately) and with uracil, uridine, thymine, and thymidine (each supplied in combination with 5-hydroxyuracil) at different times of treatment.

the control level at 48 hours, remained at the control level at 60 and 72 hours.

Reversal of the inhibitory action of 5-HU on mitosis was then attempted. Four chemicals—uracil, uridine, thymine and thymidine—were tested alone and in combination with 5-HU (Figs. 2 *A* and *B*); all compounds were supplied at a concentration of 2.0×10^{-4} M. The mitotic rate in roots treated with 5-HU alone was significantly lower at 12 hours and significantly higher at 24 hours than the control rate. The free base uracil alone had no effect on the mitotic rate at 12 hours and significantly inhibited mitosis at 24 hours; in combination with 5-HU it had no effect at 12 hours on the inhibition of mitosis produced by 5-HU alone, whereas at 24 hours it cut in half the usual spontaneous 5-HU reversal. By itself uridine, the nucleoside of uracil, had no effect on the mitotic rate; when supplied in combination with 5-HU it produced at 12 hours approximately two-thirds reversal of the 5-HU inhibition whereas at 24 hours it kept the spontaneous 5-HU reversal to the control level.

Neither thymine nor thymidine when used alone had any effect on the mitotic rate. When used in combination with 5-HU, thymine at 12 hours also had no effect on the mitotic rate, but at 24 hours prevented the spontaneous reversal of the 5-HU inhibition. In combination with 5-HU, thymidine at 12 hours completely reversed the 5-HU inhibition and at 24 hours kept the reversal to the control level.

Thus at 12 hours both uridine and thymidine reversed the inhibition of mitosis caused by 5-HU while uracil and thymine had no effect on the inhibition. At 24 hours each of these four compounds when used in combination with 5-HU interfered with the usual spontaneous reversal of 5-HU inhibition.

Results of two additional experiments with uridine as the reversing agent are presented in Figs. 2 *C* and 2 *D*. In the first experiment (Fig. 2 *C*) 3.9×10^{-4} M 5-HU significantly inhibited mitosis at both 8 and 16 hours while 3.9×10^{-4} M uridine alone had no effect on the mitotic rate at 8 hours and was significantly inhibitory at 16 hours. Uridine supplied with 5-HU had no reversing activity at 8 hours but produced a 70 per cent reversal at 16 hours. In the second experiment (Fig. 2 *D*) the significant mitotic inhibition produced at 12 hours by 5.4×10^{-4} M 5-HU was 50 per cent reversed by 3.6×10^{-4} M uridine which had no effect on the mitotic rate when used alone. The uridine reversals in both these experiments were highly significant.

TABLE I

The Mitotic Rates in Vicia faba Roots Treated with 5-Hydroxyuracil and Uridine Supplied Separately and in Combination

5-Hydroxyuracil concentration	Uridine concentration	$\frac{\text{5-Hydroxyuracil concentration}}{\text{Uridine concentration}}$	Per cent of the control mitotic rate	Mitotic reversal	Time
				per cent	*hrs.*
—	—	—	100.0	—	12
1.95×10^{-4} M	—	—	58.5	—	12
3.90×10^{-4} M	—	—	46.2	—	16
5.46×10^{-4} M	—	—	44.3	—	12
—	1.95×10^{-4} M	—	107.5	—	12
—	3.64×10^{-4} M	—	105.9	—	12
—	3.90×10^{-4} M	—	62.4	—	16
1.95×10^{-4} M	1.95×10^{-4} M	1.00	85.0	63.9	12
3.90×10^{-4} M	3.90×10^{-4} M	1.00	83.9	70.0	16
5.46×10^{-4} M	3.64×10^{-4} M	1.50	71.0	50.0	12

Table I summarizes the uridine reversal data presented in Figs. 2 *A*, *C*, and *D*. Of interest is the fact that, although slightly different time periods were involved (12 and 16 hours), in the two mixtures of 5-HU and uridine which had the same molar ratio (mols 5-HU/mols uridine = 1.00) but different absolute concentrations, the per cent reversal of mitotic inhibition was approximately the same (63.9 per cent and 70.0 per cent). It should be noted that a molar ratio of 5-HU to uridine of 1.5 resulted in a 50 per cent reversal of mitotic inhibition; the inhibition index (mols of antimetabolite per mol of metabolite giving 50 per cent reversal) was therefore 1.5.

The reversing activity of thymidine was investigated further (Table II). Each of two concentrations of 5-HU (3.9×10^{-4} M and 7.8×10^{-4} M) was tested alone and in combination with three different concentrations of thymidine. The three molar concentrations of thymidine were equivalent to $\frac{1}{20}$th, $\frac{1}{10}$th, and $\frac{1}{5}$th of the molar concentrations of 5-HU. The results of this experiment (Table II) are highly significant for all reversals.

Of particular interest is the finding that for each pair of 5-HU-and-thymidine combinations

TABLE II

The Mitotic Rate in Vicia faba Roots Treated for 12 Hours with 5-Hydroxyuracil and Thymidine Supplied Separately and in Combination

5-Hydroxyuracil concentration	Thymidine concentration	5-Hydroxyuracil concentration / Thymidine concentration	Per cent of the control mitotic rate	Mitotic reversal
				per cent
—	—	—	100.0	—
3.90×10^{-4} M	—	—	75.7	—
7.81×10^{-4} M	—	—	51.7	—
3.90×10^{-4} M	1.95×10^{-5} M	20	69.6	0
3.90×10^{-4} M	3.90×10^{-5} M	10	90.0	59.2
3.90×10^{-4} M	7.81×10^{-5} M	5	99.6	98.5
7.81×10^{-4} M	3.90×10^{-5} M	20	53.1	0
7.81×10^{-4} M	7.81×10^{-5} M	10	78.0	54.4
7.81×10^{-4} M	1.56×10^{-4} M	5	97.0	93.7

which had identical molar ratios but different absolute concentrations the degree of reversal was approximately the same. Correlations of this type suggest a competitive relationship between antimetabolite and metabolite, a relationship commonly called "competitive inhibition" (Wooley, 26).

As indicated in Table II, the molar ratio of 5-HU to thymidine at which 50 per cent reversal of mitotic inhibition occurred was 10; the inhibition index therefore was 10.

No chromosomal abnormalities were detected in the course of these inhibition and reversal studies, nor in any treatment was the relative proportion of cells in each of the mitotic stages (prophase, metaphase, anaphase, and telophase) found to differ significantly from the pattern in the control.

Microphotometric Studies:

Microphotometric analysis of Feulgen-stained interphase nuclei was used to investigate the pattern of DNA synthesis in roots treated with 5-HU (the inhibitor) alone and in combination with either uridine or thymidine (the reversing agents).

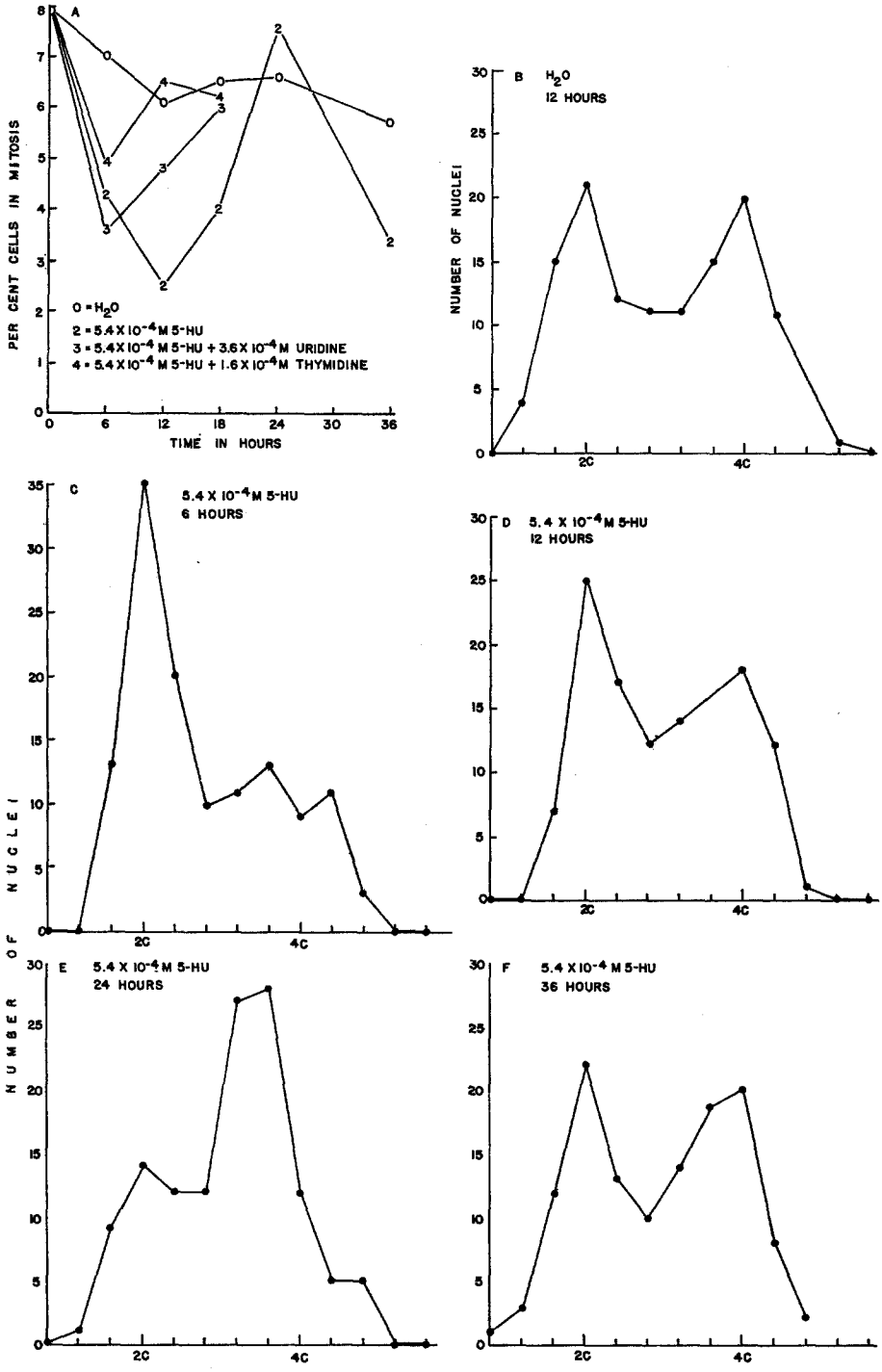
The graph in Fig. 3 *A* (repeated in Figs. 4 *A* and 5 *A*) shows the mitotic frequencies determined from the slides used for the photometric measurements. The 5-HU curve shows the expected inhibitions at 12 and 36 hours and the expected reversal at 24 hours. The 5-HU-and-uridine curve shows an inhibition at 6 hours, a 64 per cent recovery at 12 hours, and a recovery to the control mitotic frequency at 18 hours. The 5-HU-and-thymidine curve shows a previously undetected inhibition at 6 hours and a complete recovery at 12 hours, a recovery which is maintained at 18 hours.

The remaining graphs in Fig. 3 show the frequency distribution curves of the DNA content of individual interphase nuclei selected at random from control roots (3 *B*) and from roots treated with 5.5×10^{-4} M 5-HU (3 *C* to 3 *F*). Each graph represents measurements made on 125 nuclei taken at one time period of the treatment; measurements were made at 6, 12, 24, and 36 hours.

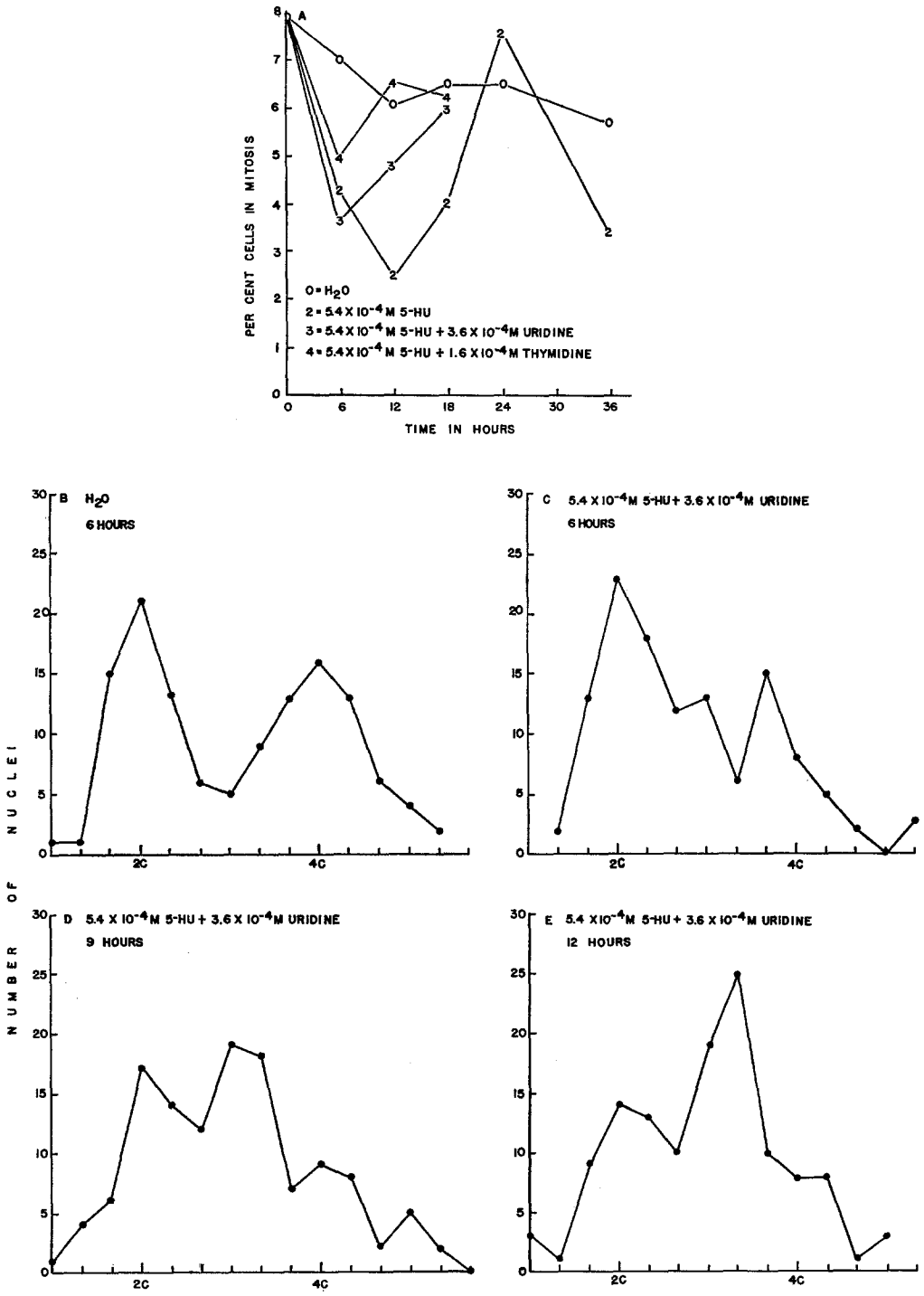
The bimodal curve obtained in the control roots (Fig. 3 *B*) is typical for untreated, randomly selected interphase nuclei: two groups of nuclei of approximately equal frequency, one of which contains twice as much DNA (designated as 4 *C*) as does the other (2 *C*). The pattern of a 2 *C*—4 *C* bimodal curve thus established in the control is the standard against which treatment effects are measured. Values intermediate between the 2 *C* and 4 *C* peaks represent nuclei undergoing DNA synthesis in preparation for the next cell division.

The effect on DNA synthesis of 6 hours of 5-HU treatment is shown in Fig. 3 *C*. The 2 *C* peak is larger and the 4 *C* peak is smaller than the corresponding control peaks. At 12 hours (Fig. 3 *D*) a 4 *C* peak is again apparent and the curve is bimodal. At 24 hours (Fig. 3 *E*) a single large peak is present and is skewed to the 2 *C* side of the control 4 *C* peak. At 36 hours (Fig. 3 *F*) the curve has the control pattern with relatively equal 2 *C* and 4 *C* peaks.

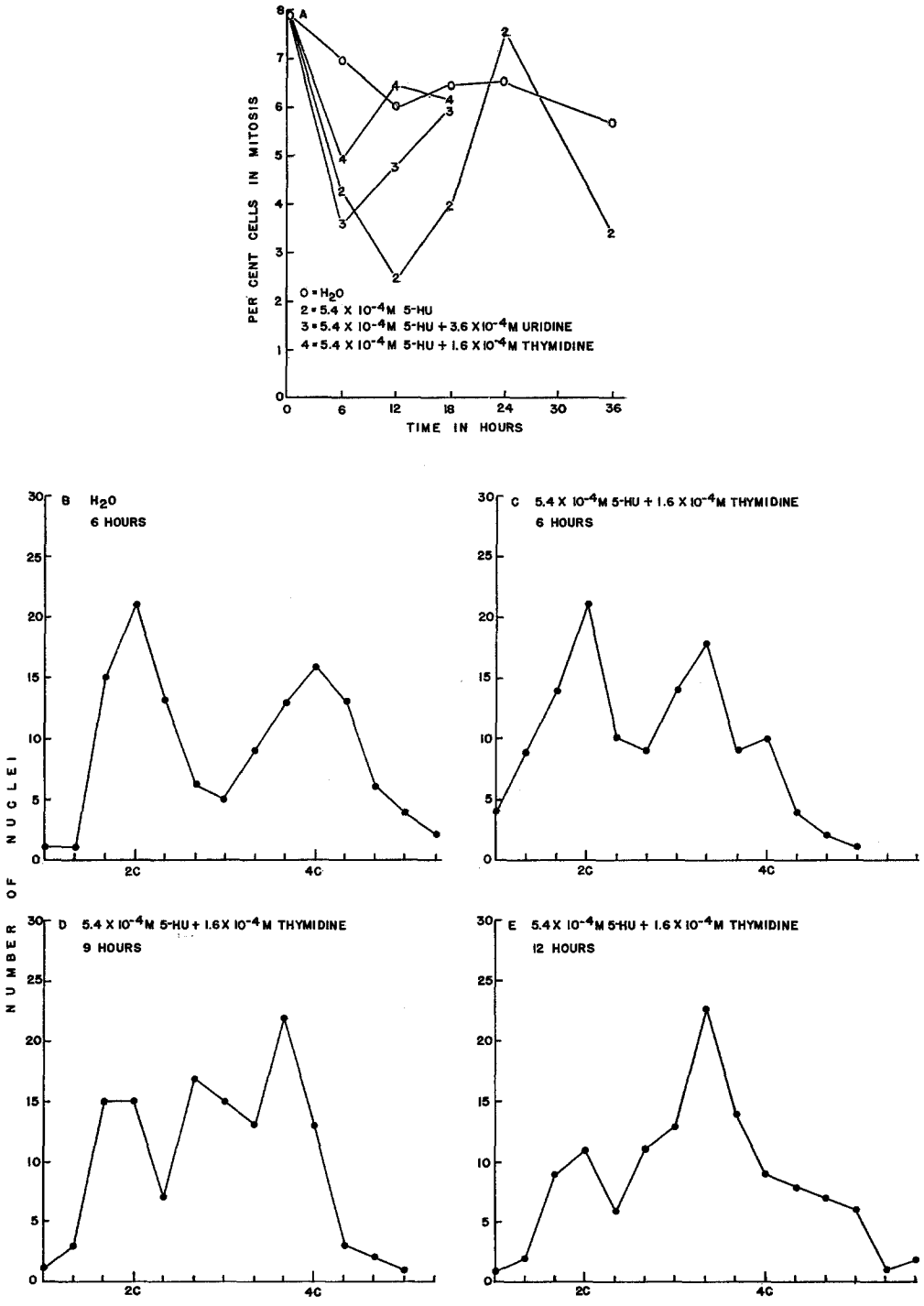
Fig. 4 shows the effect on DNA synthesis of 3.6×10^{-4} M uridine supplied in combination with 5.5×10^{-4} M 5-HU at 3 time periods. The mitotic frequency graph (Fig. 4 *A*) and the bimodal control curve (Fig. 4 *B*) are included for comparison. After 6 hours of treatment (Fig. 4 *C*) the 2 *C* peak is essentially similar to the control 2 *C* peak whereas most of the remaining nuclei are concentrated in the intermediate region of the curve. (Nuclei in this region contain more DNA than the 2 *C* nuclei and less than the 4 *C* nuclei.) At 9 hours (Fig. 4 *D*) the 2 *C* peak is smaller than the control 2 *C* peak and a definite peak is present in the intermediate region of the



FIGS. 3 *A* to *F*. Frequency distributions of randomly selected interphase nuclei in *V. faba* root cells according to their content of Feulgen dye (DNA) at 12 hours of treatment with distilled water (control, Fig. 3 *B*), and at four time periods during 36 hours of treatment with 5-hydroxyuracil (Figs. 3 *C* to *F*). Mitotic frequencies for these treatments are summarized in Fig. 3 *A*.



FIGS. 4 *A* to *E*. Frequency distributions of randomly selected interphase nuclei in *V. faba* root cells according to their content of Feulgen dye (DNA) at 6 hours of treatment with distilled water (control, Fig. 4 *B*), and at three time periods during 12 hours of treatment with 5-hydroxyuracil in combination with uridine (Figs. 4 *C* to *E*). Mitotic frequencies for these treatments are summarized in Fig. 4 *A*.



FIGS. 5 A to *E*. Frequency distributions of randomly selected interphase nuclei in *V. faba* root cells according to their content of Feulgen dye (DNA) at 6 hours of treatment with distilled water (control, Fig. 5 *B*) and at three time periods during 12 hours of treatment with 5-hydroxyuracil in combination with thymidine (Figs. 5 *C* to *E*). Mitotic frequencies for these treatments are summarized in Fig. 5 *A*.

curve. At 12 hours (Fig. 4 *E*) the 2 *C* peak has the same general shape as the 9-hour 2 *C* peak, but the peak in the intermediate region of the curve is more pronounced and is skewed toward the 4 *C* region.

Fig. 5 shows the effect on DNA synthesis of 1.6×10^{-4} M thymidine used in combination with 5.5×10^{-4} M 5-HU at 3 time periods. The mitotic frequency graph (Fig. 5 *A*) and the bimodal control curve (Fig. 5 *B*) are again included for comparison. After 6 hours of treatment (Fig. 5 *C*) the 2 *C* peak is essentially similar to the control 2 *C* peak and a definite peak is present in the intermediate region of the curve. At 9 hours (Fig. 5 *D*) the 2 *C* peak is slightly smaller than the 6-hour 2 *C* peak whereas the peak in the intermediate region is broader, overlapping the 4 *C* region. At 12 hours (Fig. 5 *E*) the 2 *C* peak is minor and the peak in the intermediate region of the curve is well established and skewed toward the 4 *C* region.

The 12-hour peaks established in the intermediate regions of the 5-HU-and-thymidine (Fig. 5 *E*) and the 5-HU-and-uridine (Fig. 4 *E*) curves are essentially similar and resemble the 24-hour peak of the 5-HU curve (Fig. 3 *E*).

DISCUSSION

The effect of 5-HU on bacteria and fungi has been described as a "growth inhibition"; results presented here permit a more precise understanding of how 5-HU interferes with cell processes involved in growth.

One process, cell elongation, is apparently largely unaffected by 5-HU. Gray and Scholes (4) have shown that in *Vicia faba* roots an elongating cell increases its length tenfold in 20 hours whereas a meristematic cell, undergoing a complete mitotic cycle in 24 hours, only doubles its size. An inhibition of cell elongation would therefore result in an inhibition of root elongation whereas an inhibition of mitosis in the meristem would have little, if any, measurable effect on root elongation during short-term experiments. The lack of consistent root-elongation response to 5-HU at the concentrations used in this investigation would appear to indicate that the chemical's major effect is not upon cell elongation. Since cell elongation is an energy-requiring process, it is therefore unlikely that 5-HU interferes with compounds (such as uridine diphosphoglucose) which are involved in energy production in the cell.

A second cellular process, mitosis, is inhibited by 5-HU treatment. The inhibition response to 5-HU concentration is linear: the higher the 5-HU concentration, the greater the mitotic inhibition. It, nevertheless, appears that the inhibition of mitosis is a secondary effect of the chemical's activity; there is evidence that mitosis is not directly affected by 5-HU.

One piece of evidence is the absence of detectable chromosomal abnormalities in treated cells, a finding which indicates that 5-HU has no apparent effect upon the structure or movement of chromosomes during mitosis. Further evidence is provided by data which show that in treated roots the proportions of cells in prophase, metaphase, anaphase, and telophase are nowhere significantly different from the proportions found in control roots. As pointed out by Setterfield and Duncan (18), the percentage of mitotic cells in a specific mitotic stage is in general equivalent to the percentage of total mitotic time taken by that particular stage. The finding in both control and treated roots of similar percentages of cells in corresponding stages indicates therefore that 5-HU has no effect upon the duration of any single mitotic stage.

Finally, there is evidence that 5-HU does not block the entrance of cells into prophase. Graphs of the microphotometric data from treated roots show that the 4 *C* peak, the peak which in the control represents nuclei possessing the doubled amount of DNA necessary for mitosis, is absent at 6 hours of treatment. If 5-HU were to block the entrance of cells into mitosis, the resulting pileup of 4 *C* cells would be reflected in a 4 *C* peak higher than that in the control. The absence of the 4 *C* peak suggests therefore that 5-HU has no direct effect upon the entrance of cells into mitosis.

It is upon a third cellular process, the biosynthesis of DNA, that 5-HU appears to have a direct effect. At 6 hours of treatment the 4C peak is absent and the 2C peak is higher than the control 2 *C* peak. This heightening of the 2 *C* peak is evidence for a blocking of DNA synthesis (Duncan and Woods, 2). Nuclei which at the beginning of treatment possessed the doubled (4 *C*) amount of DNA proceed normally through mitosis; the resulting 2 *C* sister nuclei added to the 2 *C* nuclei already present cause the pileup reflected in the heightened 2 *C* peak. The continuing presence of nuclei with the intermediate and 4 *C* amounts of DNA indicates that synthesis is not completely blocked by 5-HU at the concentrations used in

these experiments: complete blockage would result in the accumulation of all nuclei in a single 2 *C* peak. 5-HU thus appears to block DNA synthesis in a certain number of nuclei and, by preventing these nuclei from reaching the 4 *C* level, to reduce the number of cells in mitosis.

The inhibitory activity of 5-HU is reversed under these three conditions: (1) when 5-HU is supplied alone, (2) when 5-HU is supplied in combination with thymidine, and (3) when 5-HU is supplied in combination with uridine.

When 5-HU is supplied alone, it produces maximum inhibition of mitosis at 12 hours; then in all experiments the inhibition has disappeared at 24 hours. DNA synthesis is blocked at the end of 6 hours of treatment and is proceeding actively at 12 hours of treatment. This behavior suggests that 5-HU, shortly after being administered, begins to block DNA synthesis and that such a block results in an increasing inhibition of mitosis which becomes maximal in about 12 hours. The resumption of synthesis at some time before 12 hours permits the resumption of mitosis, and by 24 hours the number of cells in mitosis is at the control level. It thus appears that the inhibitory effect of 5-HU on DNA synthesis is exerted for somewhat more than the first 6 hours of treatment and that reversal of this inhibition is accomplished in the remainder of the first 12 hours of treatment.

When 5-HU is supplied in combination with thymidine, the inhibition of mitosis reaches its maximum at 6 hours and is completely reversed by 12 hours. DNA synthesis at 6 hours is proceeding at a normal rate as indicated by the normal 2 *C* peak; the presence, however of a large number of intermediate nuclei and a small number of 4 *C* nuclei suggests that DNA synthesis is recovering from a previous block. These findings indicate that thymidine produces in less than half the time the same reversal pattern which characterizes the spontaneous recovery obtained when 5-HU is used alone.

When 5-HU is supplied in combination with uridine, the inhibition of mitosis reaches its maximum at 6 hours, is two-thirds reversed at 12 hours, and is completely reversed at 18 hours. DNA synthesis is blocked at 6 hours but has resumed at 9 hours. These findings suggest that uridine and thymidine both act to produce more rapidly the same recovery pattern obtained spontaneously when 5-HU is supplied alone, but that uridine produces this recovery more slowly than does thymidine.

A competitive relationship between 5-HU and thymidine is indicated by the data presented in Table II: the same molar ratio of 5-HU to thymidine at different absolute concentrations produces approximately the same degree of mitotic reversal. This competitive relationship can be defined quantitatively in terms of an inhibition index which, antimetabolite theory holds, is an expression of the relative ability of antimetabolite and metabolite to compete successfully for the same receptor groups on an enzyme. The inhibition index for the pair of compounds, 5-HU (the antimetabolite) and thymidine (the metabolite), is 10. Thus when the concentration of thymidine molecules reaches one-tenth of the concentration of 5-HU molecules, the mitotic inhibition produced by 5-HU alone is 50 per cent reversed. Since 5-HU blocks DNA synthesis and since thymidine is after phosphorylation a component of DNA, it is reasonable to assume that 5-HU and thymidine compete for receptor groups on an intranuclear enzyme involved in DNA synthesis. Interference with thymidine incorporation would result in blockage of DNA synthesis; the extent of this blockage would define the extent of the subsequent inhibition of mitosis.

The finding that thymidine when administered with 5-HU produces an early unblocking of DNA synthesis is consistent with the hypothesis that these compounds are competitive. An increased thymidine level in the nucleus would be expected to unblock DNA synthesis by shifting the competitive advantage to the more numerous thymidine molecules. It is therefore logical to assume that in nuclei treated with 5-HU alone a high thymidine-to-5-HU ratio may also be responsible for the spontaneous unblocking of DNA synthesis. Such a ratio could become established either through increased synthesis of thymidine or through degradation of 5-HU by enzymes already present or adaptively formed in the nuclei. Either of these processes would eventually result in unblocking of DNA synthesis, although the unblocking would occur more slowly than if the thymidine were supplied exogenously.

The activity of uridine closely parallels the activity of thymidine in reversing the inhibition of mitosis produced by 5-HU. The probable competitive relationship between 5-HU and thymidine has been pointed out; a similar relationship between 5-HU and uridine can also be demonstrated. It is clear from the data in Table I that the same molar ratio of 5-HU to uridine at different abso-

lute concentrations produces approximately the same degree of mitotic reversal. The inhibition index calculated from these data is 1.5. A comparison of this index with the 5-HU-thymidine index—10— shows that approximately seven times as many uridine molecules as thymidine molecules are required to produce the same degree of mitotic reversal. Uridine is thus much weaker than thymidine as a reversing agent.

The inhibition and subsequent reversal of mitosis is considered to result from a prior blocking and unblocking of DNA synthesis. Uridine unblocks the 5-HU–induced block in synthesis in 9 hours, a time which lies midway between the 6 hours required for thymidine and the 12 hours required for the spontaneous recovery in 5-HU treated nuclei. It is obvious, however, that uridine, which is not a component of DNA, unblocks blocked DNA synthesis by some mechanism other than direct competition with 5-HU for sites on enzymes involved in DNA synthesis. The mechanism by which reversal is accomplished must, nevertheless, account for a competitive relationship between 5-HU and uridine.

The most logical mechanism would seem to be the conversion within the nucleus of uridine to thymidine. If such a conversion does occur, then the uridine reversal data can be explained as resulting from the activity of thymidine, the product of this conversion. This thymidine could then compete directly with 5-HU for enzyme sites. Moreover, the extra time required by uridine, as compared with exogenous thymidine, to unblock DNA synthesis would then be a measure of the time (approximately 3 hours) taken by the conversion of uridine to thymidine. Finally, the calculation derived from the comparison of inhibition indices that uridine is only one-seventh as effective as thymidine in unblocking synthesis would indicate that only one-seventh of the uridine molecules entering the plant are converted to thymidine. It is thus evident that the hypothesis evolved to explain the reversing ability of thymidine can account equally well for the reversing ability of uridine if uridine is being converted to thymidine.

Uridine to thymidine conversion would require the reduction of ribose and the methylation of uridine. Evidence for the existence of such a conversion pathway is accumulating. The reduction of ribose to deoxyribose, first suggested by Hammarsten *et al.* (5), has been demonstrated by Rose and Schweigert (17) and Roll *et al.* (15). Evidence exists for the conversion of uracil (Shive, 20; Rege and Sreenivasan, 13), uridine (Hammarsten *et al.*, 5), and uridylic acid (Brown *et al.*, 1) to the corresponding level of thymine. The findings of Friedkin and Roberts (3) and Reichard (14) that deoxyuridine-5′-phosphate is a good precursor of thymidine-5′-phosphate have led Kornberg (10) to suggest the following pathway of thymidine synthesis:

$$\text{Uridine-5′-P} \xrightarrow{+\ 2H} \text{deoxyuridine-5′-P} \longrightarrow \text{thymidine-5′-P}$$

The conversion of uridine to thymidine proposed for *V. faba* would, if proved, suggest that ribonucleosides can function as DNA precursors in higher plants. The fate of labelled uridine in *V. faba* roots treated with 5-HU is under investigation by the senior author; results of these experiments will be reported in a later paper.

Whatever the mechanisms involved, recovery from the effects of 5-HU occurs in definite patterns which can be summarized as follows: DNA synthesis resumes before 6 hours in cells treated with 5-HU and thymidine, by 9 hours in cells treated with 5-HU and uridine, and by 12 hours in cells treated with 5-HU alone. Resumption of synthesis results, as would be expected, in subsequent resumption of mitosis: the mitotic rate is at the control level by 12 hours in cells treated with 5-HU and thymidine, by 18 hours in cells treated with 5-HU and uridine, and by 24 hours in cells treated with 5-HU alone.

When DNA synthesis is examined in treated cells which have recovered from 5-HU activity and are at the control mitotic level, the pattern of synthesis differs widely from the control pattern. A striking similarity is evident in the microphotometric data secured from cells treated for 24 hours with 5-HU alone and from cells treated for 12 hours with either 5-HU and thymidine or 5-HU and uridine. In all three cases the 2 *C* peak is smaller than the control peak, the 4 *C* peak is absent, and a large peak, skewed toward the 4 *C* region, is present in the intermediate zone of the curve.

The absence of high 2 *C* and 4 *C* peaks indicates that there is no blocking of entrance into synthesis and entrance into mitosis. The heavy concentration of nuclei in the intermediate region of the curve suggests on the other hand that DNA synthesis cannot go to completion in these nuclei. Such an effect, if it is real, is at odds with Swift's proposal (23) that DNA synthesis is an all or nothing reaction. Support for the reality of the

effect is provided by the finding of Setterfield and Duncan (18) that DNA content is significantly below normal in prophase cells of *V. faba* roots treated with diaminopurine (which blocks DNA synthesis when used alone) in combination with adenine (which prevents the effects of diaminopurine).

If DNA synthesis is blocked short of completion, then a subsequent drop in the rate of mitosis would be expected since mitosis normally is initiated only in 4 *C* cells. An inhibition of mitosis does in fact appear 12 hours later (at 36 hours) in cells treated with 5-HU alone (the only treatment followed for more than 18 hours).

The concentration of nuclei into a relatively narrow area of the intermediate region of the curve suggests that DNA synthesis proceeds to approximately the same point in all these nuclei and is then blocked. One possible explanation of the blockage is that it results from the exhaustion of a part or of all of the DNA-precursor pool because of synchronous synthesis in an abnormally large number of nuclei. Such a mass synthesis could occur if the 5-HU–induced block in synthesis were removed simultaneously in all blocked nuclei.

Whatever the cause of the large number of nuclei with the intermediate amount of DNA, the effect is transitory: synthesis returns to normal by 36 hours and the rate of mitosis returns to the control level by 48 hours and remains there for the duration of the study.

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