5-AMINOURACIL
SYNCHRONIZATION OF THE CELL CYCLE OF *VICIA FABA* ROOT TIP MERISTEMS

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ABSTRACT

This study was undertaken to try and find how the thymine analogue 5-aminouracil induces cell synchrony in the cell cycle of plant root meristems. It has previously been used as a synchronizing agent without knowing its mode of action.

The experiments confirmed the synchronization effect and that the removal of plants from 5AU stimulated the cells to divide. Results indicated that the late S and early G_2 phases of the cell cycle were the most affected, with DNA synthesis continuing in the presence of 5AU at a reduced rate. The inhibition of division caused by 5AU could be reversed by other bases and mixtures.

The G_1 phase was found not to be affected by 5AU but it was postulated that cells in early G_2 were slowed down or halted by the chemical. DNA density measurements were taken of nuclei treated continuously for varied times with 5AU, and these results confirmed a buildup of cells in the latter third of the S phase found by other workers. The presence of Feulgen-negative regions in chromatids of the 5AU treated tissue was noted and linked with possible interference in heterochromatin synthesis. The possibility of some enzyme function important in the final joining together of DNA units being interfered with by 5AU is also discussed. Suggestions are made for further possible avenues of work into DNA synthesis.

The significance of cell cycle studies and their experimental design has recently been reconsidered and is mentioned in view of this work and other cell population studies.

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Finally to my husband Mike must go thanks for his continued encouragement, and especially for assistance with photography and diagrams.

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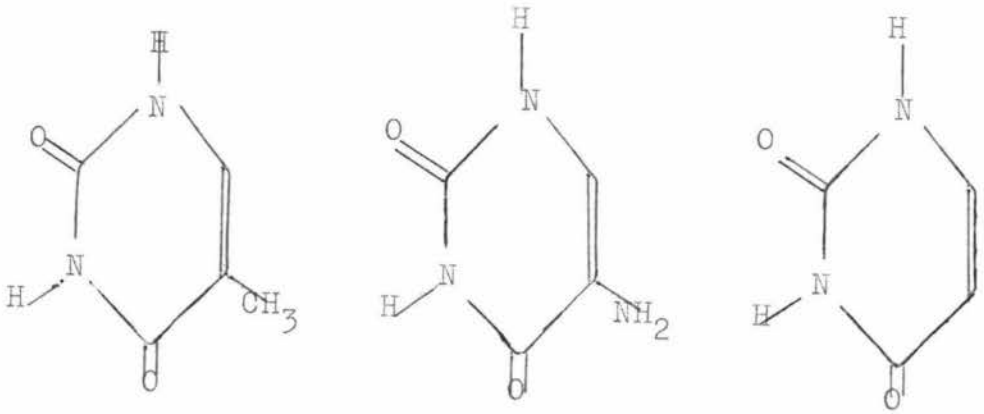
ABBREVIATIONS USED IN TEXT

5AU	5-aminouracil
BAO	2,5-bis[4'-aminophenyl-(1')]-1,3,4-oxadizole
CdR	deoxycytidine
H^3-CdR	tritiated deoxycytidine
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
FUdR	5-fluorouracil-2-deoxyriboside
H^3	tritium
LI	Labeling Index
MI	Mitotic Index
ppm	parts per million
RNA	Ribonucleic acid
RNase	Ribonuclease
TdR	thymidine
H^3-TdR	tritiated thymidine

INTRODUCTION

5-aminouracil is a structural analogue of thymine



thymine (5-methyluracil) | 5-aminouracil | uracil

and uracil. Smith, Fussell, and Kugelman (1963), (working with plant root material), published the first report of a partially synchronized population of root cells after a treatment with 5AU.

Previous to this publication, 5AU had been used as an inhibitor in growth studies of bacteria (Puleston et al, 1950; Hitchings et al, 1950; Shive and Skinner, 1958.). The distinct inhibition of growth in Streptococcus faecalis R caused by 5AU could be reversed by uracil or thymine (Puleston et al, 1950) while folic acid or thymine could similarly reverse the action of 5AU in Lactobaccillus casei (Hitchings et al, 1950). These authors and others (Rogers and Shive, 1948; Skipper et al, 1950) suggested that 5AU inhibits the synthesis or utilization of the pyrimidine bases of the nucleic acids, and thus DNA synthesis in the cells since the inhibitory action could be reversed by the addition of such pyrimidines. Wacker et al (1950)

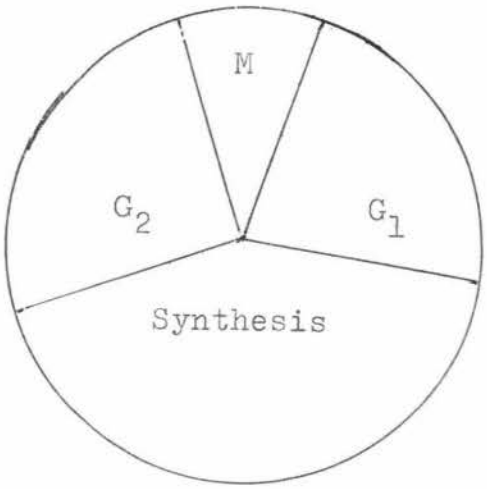
studying the metabolism of labeled $[^{2}\text{-}C^{14}]$-5AU, found that it was directly incorporated in bacterial DNA and used in a very small amount for DNA synthesis.

5AU is now used as a synchronizing agent for both plant and animal studies concerning cell division, cell metabolism, effect of mutagenic chemicals and effects of radiation and resulting chromosome aberrations (Chu, 1965; Regan and Chu, 1966; Eriksson, 1966).

However, the mode of action of this pyrimidine analogue in higher organisms has still not been elucidated. The problem has been studied by several scientists and approached from different aspects. Reversal studies (Jakob and Trosko, 1965; Prensky and Smith, 1965; Scheuermann and Klaffe-Lobsien, 1973) have produced contradictory results, but the latest data suggest no thymine/5AU or TdR/5AU competition. There have been consistant reports of a partial synchronization occurring after a certain time, when roots are kept continuously in 5AU (Jakob and Trosko, 1965; Mattingly, unpublished data 1965; Wagenaar, 1966). Thus it appears that the knowledge gained from the studies of 5AU action on bacteria cannot be extrapolated to higher organisms, which clearly have a much more complicated metabolism.

Howard and Pelc (1951) found that the cell cycle could be divided into several stages, each having different functions which all contribute to the final result - division of the cell. The greater part of the cell cycle is interphase which is further sub-

divided into G_1 a pre DNA synthetic phase, S equated



with DNA synthesis, and G_2 a post DNA synthetic phase. 5AU could affect one of these stages, or possibly a combination of them. It is generally accepted that mitosis itself is not interfered with in any way. Labeling studies of the G_1 phase (Mattingly, unpub. data, 1965) showed no difference between 5AU treated roots and controls.

In order to try and answer the question of how 5AU causes cell synchrony, attention has now been focused on the DNA synthesis phase, and the G_2 phase which seems to be prolonged (Socher and Davidson, 1971; Wolff and Luipold, unpublished data, 1964, in Mattingly 1966a). However there are anomalies in the published results concerning occurrence of peaks of division after 5AU treatment, (Smith, Fussell and Kugelman, 1963; Jakob and Trosko, 1965), and concerning actual DNA synthesis in the S phase (Woods and Duncan, 1952; Jakob and Trosko, 1965; Scheuermann and Klaffe-Lobsien, 1973).

The purpose of this study is to try and elucidate some of the anomalies as mentioned above, in the work already done in this field, and to also proceed further in answering the question of how 5AU causes cell synchrony.

Synchronization of division only occurs naturally in specialized stages of higher plants eg. gametogenesis, which has been used extensively to describe biochemical events by Hotta and Stern (1963a, 1963b, 1965). The advantages of using artificially synchronized cell populations has already been noted by Regan and Chu (1966) for assaying DNA synthesis in cultures of human tissue, and by Prensky and Smith (1964) for studying chromosomal proteins using tritiated arginine in plant tissue.

A knowledge of how the synchronizing agent functions is consequently very important if 5AU is to continue being used in this field of research, to ensure that it is in no way going to interfere with what is being studied.

Synchronization studies involving DNA synthesis require pulse labeling of tissue as a method of marking when DNA synthesis is occurring in cells. H^3-TdR and H^3-CdR are the most commonly used as it is now known that they are only incorporated into DNA (Wimber, 1960; Davidson, 1968). Autoradiography is required to pinpoint incorporation and most commonly, the tissue is prepared for viewing using squash techniques. Mattingly (1966b) used serial sections to reveal the pattern of mitotic divisions in the root tip

during and after 5AU treatment, which could explain reports of subpopulations of cells (Socher and Davidson, 1971).

This work is repeated and correlated with squash preparations from various experiments using 5AU to help determine whether the data obtained from squashes is still significant. Caffeine was used to try and determine between possible subpopulations of cells obtained with 5AU treatments, as well as to morphologically label cells for a short study of the cell cycle.

All the repeated synchronization experiments agree with the literature. DNA synthesis does occur in the presence of 5AU but at a slower rate resulting in a buildup of cells in the latter third of the S phase. This has led to the suggestion that the synthesis of the heterochromatin regions is being affected in some way. Previous chromosomal aberration studies using 5AU examining Feulgen-negative 'gaps' have also pointed towards interference with heterochromatin synthesis (Kihlman, 1966; Martinez-Pico and Duncan, 1955). However only one recent paper examining the problem of action of 5AU has suggested this (Scheuermann and Klaffe-Lobsien, 1973). The build up of cells in the latter third of the S phase, together with the prevention of labeled early G_2 cells entering mitosis in the presence of 5AU could suggest possible interference at the enzyme level of the final stages of

DNA synthesis. This could be overcome by using an alternative repair pathway but only after a critical time period of approximately 40 hours has passed.

Further work at the enzyme level could provide more interesting information on the problem of mode of action of 5AU.

MATERIALS AND METHODS

2-01 Growth of Seedlings

Vicia faba (var Exhibition Longpod) seeds 2 cm long were rinsed in 1% sodium hypochlorite solution for five minutes, then soaked for 24 hours in running tap water at $15^{\circ} \pm 1^{\circ}C$. The seed coats were removed before the seeds were planted, radicle downwards, nine per 16 cm^2 pot in vermiculite (exploded mica). They were left in the dark at $25^{\circ} \pm 1^{\circ}C$ to germinate and watered when necessary with tap water. After six days, all those plants six to eight cm high with at least 20 lateral roots were transferred to continuously aerated 1/4 strength Hoagland's mineral nutrient solution, (Table 1) also in the dark at $20^{\circ} \pm 1^{\circ}C$. This was renewed every 24 hours. Because lateral roots were required, the tip of the primary root was removed on transferring the plants to nutrient solution. After a day in nutrient solution the lateral roots were two to three cm long and ready for use in experiments. The temperature of the growth room was thermostatically controlled as this was critical to results during both treatment and recovery times. (Figure 1).

Germination and growth of seedlings as well as all experiments were carried out in the dark, as this avoided any possibility of diurnal rhythms masking 5AU induced synchronization patterns, and resulting MI. Some workers (Gray and Scholes, 1951) remove the plumule to avoid this possibility, but because all the hormones involved in lateral root initiation are

Table 1: Hoagland's Mineral Nutrient Solution 2.

(modified by Johnson et al 1957)

Macronutrients

Compound	Mol wgt	Concn Stock Soln M	Concn Stock g/l	Vol Stock per 1 final soln ml	Element
KNO_3	101.10	1.00	101.10	6.0	N, K
$Ca(NO_3)_2.4H_2O$	236.16	1.00	236.16	4.0	Ca
$NH_4H_2PO_4$	115.08	1.00	115.08	2.0	P
$MgSO_4.7H_2O$	246.49	1.00	246.49	1.0	Mg, S

Micronutrients

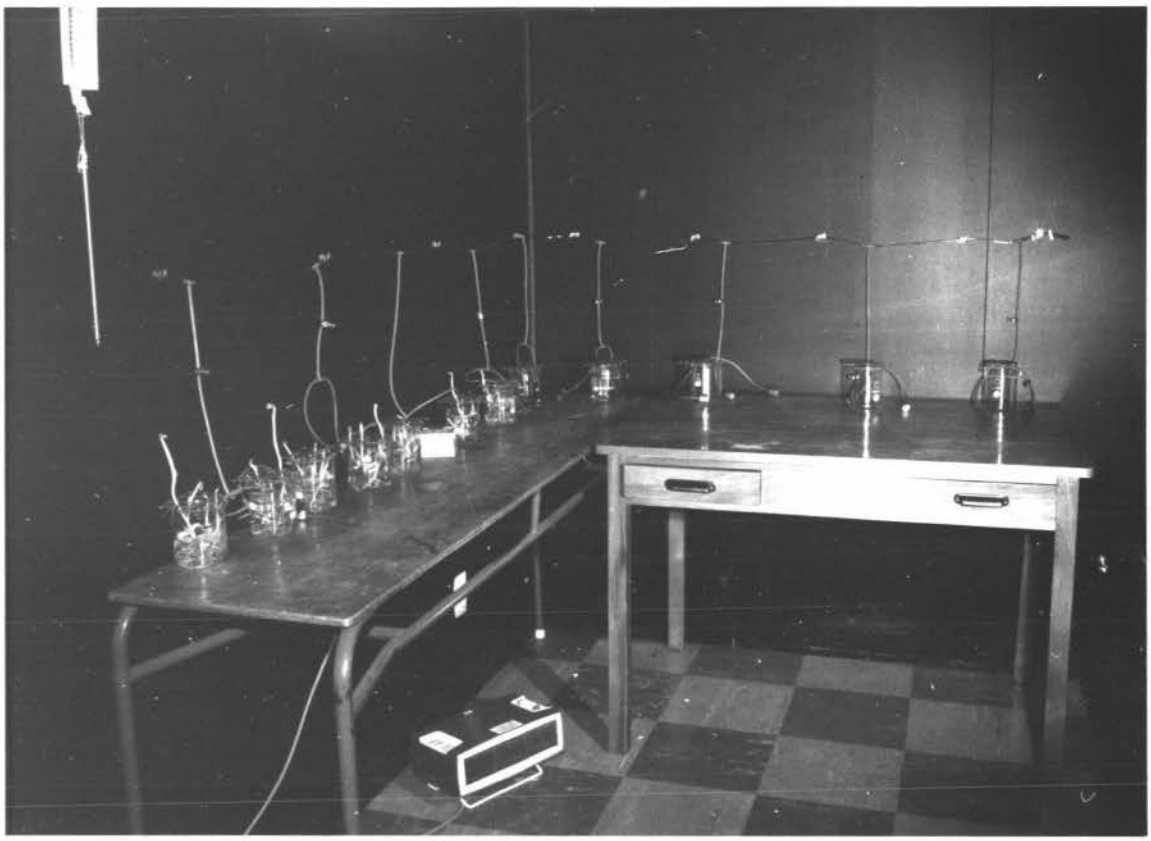
		mM			
KCl	74.55	50	3.728		Cl
H_3BO_3	61.88	25	1.546		B
$MnSO_4.4H_2O$	223.06	2.0	0.4461	1.0	Mn
$ZnSO_4.7H_2O$	287.55	2.0	0.575		Zn
$CuSO_4.5H_2O$	249.71	0.5	0.125		Cu
H_2MoO_4 (85% MO_3)	161.97	0.5	0.081		Mo
Na-Fe-EDTA				1.0	

Na-Fe-EDTA: Dissolve 5g of NaOH in 800 ml of distilled water, add 33.2g EDTA (tetrasodium salt), stir until dissolved, add 24.9g $FeSO_4$ and stir. Make up to 1 litre and aerate the solution over night using an aquarium air pump.

Adjust pH of final nutrient solution to 6.5.

From Epstein - Mineral Nutrition of Plants.

FIGURE 1 : *Vicia faba* plants continually being supplied with air, growing in a thermostatically controlled darkroom.



still not completely understood, it seemed best to avoid any possible interference with normal growth.

When it was necessary to examine the growing plants and take samples, a green safety light was used which was known to exclude both blue and red wavelengths of light.

2-02 5-aminouracil Treatment

5AU (Sigma) was dissolved in nutrient solution at a concentration of 3.93×10^{-3}M (500ppm), for all experiments, so that comparisons could be made between individual experiments and with the published literature. If the duration of an experiment exceeded 24 hours, the nutrient or 5AU solution was renewed at the end of 24 hours.

It was observed that over 24 hours the 5AU solution often deepened in colour. To check whether 5AU was being metabolized, samples of the solution were taken at 0, 12 and 24 hours and checked for absorption on an Hitachi recording spectraphotometer using UV wavelengths (210 - 360nm). The resulting spectrum was similar for all three solutions recording a peak at 290nm. From this data it was concluded that the colour change was not an indication of metabolism of 5AU and it could be assumed that 5AU was functional for the complete duration of an experiment. It was suggested that phenolic excretions from the roots combined with aeration could have caused this effect.

2-03 Cytological Procedures

2-031 SAMPLING:

When sampling, lateral roots were put directly into Carnoy's fixative (3:1 ethanol:glacial acetic acid) and left overnight. Next day they were transferred to 70% ethanol and refrigerated at 4°C.

2-032 STAINING:

A staining procedure had to be chosen that made the interphase nucleus and chromosomes easy to see for mitotic counting of squash preparations. Several stains were tried.

i) Acetocarmine - (for method see Appendix 1) Reasonable results were obtained but the handling of individual root tips made it impractical for the volume of tissue that needed to be processed.

ii) Gomori's Haematoxylin - (for method see Appendix 1) Excellent results with nuclei and chromosomes clearly stained; cytoplasm also stained lightly. Good squashes were only obtained if the roots had been left in fixative and 45% acetic acid for the maximum time. Often large clumps of cells, not thoroughly squashed were too dark to distinguish nuclei for counting. The first few synchronization experiments were examined using this stain but it became clear that the results were not completely satisfactory.

iii) Feulgen - (for method see Appendix 1) This seemed the most obvious replacement to Haematoxylin as it was also required in the autoradiography

experiments. However the first few attempts using this stain resulted in poor squashes with the nuclei difficult to see clearly. It was thought that the basic fuchsin could have been too old. Several batches of basic fuchsin were tried before a supply was found (BDH) that produced a clear solution and stained the nuclei and chromosomes clearly, leaving the cytoplasm almost invisible. Consequently tissue from all repeat experiments was stained with Feulgen and it was also used for all the squash preparations required in the isotope incorporation experiments.

The Feulgen stain was unsuitable for examining binucleate cells obtained in the experiment using caffeine, as the cell walls could not be distinguished to aid in counting the marked cells. Nomarski interference microscopy was also found to be unsuitable. Consequently the tissue had to be embedded and sectioned so that a double staining technique could be used. The safranin-fast green method (see Appendix 1) as described by Jensen (1962) was chosen as the most suitable, staining nuclei and chromosomes red, and the cell walls a greenish-blue.

2-033 PREPARATION OF SLIDES:

Microscope slides used for squash preparations were soaked for at least one hour in acidified 95% ethanol (100:1 ethanol:conc HCl). When required they were air dried and wiped, and squashes were made directly onto these.

Microscope slides used for mounting sectioned tissue were soaked in Decon[i] (diluted 20:1 with distilled water) for at least one hour. When required the slides were air dried and wiped, then a drop of Mayer's albumen (Gray 1954) was smeared across the slide then wiped off. The ribbons of tissue were then floated on with distilled water, positioned correctly, then allowed to dry at $30^{\circ} \pm 1^{\circ}C$ overnight.

2-034 USE OF COLCHICINE:

For the purposes of counting mitoses it is much easier to count an accumulated number of cells over a shorter time for sampling, rather than combine small groups of data obtained over a longer period. For this reason, colchicine (BDH) which destroys the mitotic spindle and accumulates metaphases, was used when sampling was carried out over peaks of division. 0.05% colchicine solution was used to avoid the abnormalities that higher concentrations cause, (Evans, Neary and Tonkinson, 1957; Evans and Savage, 1959; MacLeod and Davidson, 1968). Colchicine was used in both controls (nutrient only), and 5AU solutions for treatments up to six hours, with samples being taken during the latter five hours of this time.

2-035 USE OF CAFFEINE:

This chemical produces a synchronous, morphologically labeled population of cells by virtue of preventing cytokinesis in cells in telophase, and thus

(i - registered trade mark)

resulting in binucleate cells,(Kihlman 1955). Caffeine has been further studied by Gimenez-Martin, Gonzalez-Fernandez, and Lopez-Saez (1965), who emphasize three aspects of this method.

'i) its specificity upon cytokinesis

ii) rapidity with which it acts and with which its effects are eliminated.

iii) low toxicity 0.1% in the course of the cell division cycle. Treatment for one hour is sufficient.'

Caffeine 0.1% (Sigma) was used to mark the small population of cells in telophase which could then be labeled with H^3-CdR (short pulses of 15 mins 4uc/ml or 2uc/ml) to catch the beginning and end of the S phase in order to determine the lengths of the different stages of the cell cycle. This was only done as a preliminary study to determine whether it is a feasable alternative to the more conventional methods of using only pulse labeling with H^3 or double labeling with H^3 and C^{14},(Quastler and Sherman, 1959; Evans and Scott, 1964; Wimber and Quastler, 1963).

2-036 ENZYME TREATMENT:

In order to confirm the incorporation of H^3-TdR solely into DNA a short experiment was done using enzymes. 3:1 ethanol:glacial acetic acid roots were thoroughly rinsed in water, then treated for two hours in a 5% pectinase solution (ex Aspergillus niger, Koch Light Laboratories Ltd.). The root tips were then squashed onto cleaned slides and the coverslips were

removed with dry ice and the slides rinsed in distilled water to remove the pectinase before storing the slides in 70% ethanol until ready for the enzyme treatment. Slides were treated with RNase or DNase in order to ascertain the position of the H^3 silver grains after autoradiography. RNase treated slides were incubated at room temperature for three hours, whereas DNase treated slides were incubated at 37°C for six hours. RNase (Sigma, ex bovine pancreas) was used at a concentration of 1 mg/ml in phosphate buffer (pH 7), and DNase (Koch Light Laboratories Ltd, ex bovine pancreas) was used at a concentration of 0.2 mg/ml in phosphate buffer (pH 7) with 0.005M $MgSO_4$,(Jakob and Trosko 1965). After incubation the slides were again rinsed in glass distilled water and prepared for Feulgen staining. DNase treated slides remained colourless due to the removal of all nuclear material that normally stains with basic fuchsin. Consequently after autoradiographic processing the slides were examined using Nomarski Interference microscopy so that cell outlines could be seen. On RNase treated slides the nuclei stained normally with Feulgen and silver grains could be seen over the nuclei (using oil emersion), after autoradiographic processing.

2-037 EXAMINATION OF SLIDES:

Once preparation of slides was complete, they were all mounted in Eukitt (O Kindler W. Germany) so they could be examined as time permitted. Preparations of

basic synchronization experiments were scanned randomly using a Visopan microscope (100x lens) to determine the mitotic index;

$$\frac{\text{no of mitotic cells}}{\text{total no of cells examined}} \times 100 \text{ (MI)}$$

counting 500 cells per slide and three slides per time sample. These were compared with slides of sectioned material to locate regions of observed divisions in relation to other tissues in the root tip. Slides from experiments involving label were scored as to presence or absence of silver grains, either in general or specifically over mitotic figures.

2-04 Isotope Incorporation Studies

DNA synthesis can be closely studied in plant cells by supplying them with DNA precursors that are radioactively labeled. In this study H^3 was used because it is a low energy B particle emitter and has a high resolving power which is suitable at the cellular and subcellular level. The labeled compounds used were [methyl-3H]thymidine, which is exclusively incorporated into DNA (Reichard and Estborn 1951), and deoxy-[5-3H]cytidine which has often been suggested as an alternative (in discussion of paper by Howard and Dewey, 1960; Prensky and Smith, 1965). Chemicals were from the Radiochemical Centre, Amersham, UK. Specific activity 2.0 ci/mmol. The amounts of isotope used are mentioned later with discussion of individual experiments in the results.

The isotope was supplied to the plant roots in the nutrient or 5AU solutions and aerated as in all other experiments. Pulses ranged from 15 to 30 mins depending on the concentration of label used. Samples were taken and fixed in the usual way. When deciding to use 3:1 absolute alcohol:glacial acetic acid, it was noted that certain fixatives result in substantial losses of radioactivity,(Kapriwa and Leblond, 1962).

The tissue was stained with Feulgen, squashed on slides, passed through on alcohol series to water prior to autoradiography. Again some loss of radioactivity due to hydrolysis and rinsing of tissue cannot be avoided, (Baserga and Malamud, 1969).

Ilford L4 liquid nuclear emulsion in gel form (Ilford, UK) with a very fine grain size (0.14u) was used for autoradiography. The required amount of emulsion was removed under Ilford S902 safelight conditions and heated to 45°C in a waterbath to melt the gel. The stock was kept in a refrigerator at 4°C. The emulsion was diluted 50:50 with warmed distilled water and agitated slowly to avoid excess froth being formed. Dilution allows a thinner layer of emulsion to cover the tissue which is important when working with H^3.

The prepared slides were then dipped into the emulsion, withdrawn and lightly drained, at a rate of five seconds per slide. The coated slides were then put in black slide boxes with a small gauze satchet of self indicating silica-gel, then when dry, sealed with black electrical tape, wrapped in aluminium foil

and stored in the refrigerator at 4°C.

After exposure for varying lengths of time depending on the concentrations of radioactivity used, (three weeks for 4uc/ml and six weeks for 2uc/ml or less) the slides were developed in Kodak D19 for 3-4 minutes at 20°C. A 1% acetic acid stopbath (10 secs) was used, followed by five minutes in Amfix 1:3 (plus 1 part S type hardener to 40 parts diluted fix) (May and Baker), then five minutes in running tap water, followed by one minute in distilled water as recommended by Caro (1964). The slides were then air dried and made permanent by mounting in Eukitt (O Kindler, W. Germany).

The tissue on each slide was scanned systematically using an Olympus E binocular compound microscope with wide field 10x oculars, and where required a labeling index was determined using oil emersion as:

$$\frac{\text{no of labeled cells}}{\text{total no of cells examined}} \times 100 \quad \text{(LI)}$$

2-05 Reversal Experiments

In early studies of bacterial growth 5AU was used as an inhibitor. Reversal experiments showed that growth of bacteria would continue if uracil or thymine were added to the growth medium, (Puleston et al, 1950). From these results it was suggested that 5AU inhibited the synthesis or utilization of the pyrimidine oases of nucleic acids and thus DNA synthesis.

Thus it seemed of interest to add pyrimidine bases

in the presence of 5AU knowing that in higher plants DNA synthesis is not completely halted by this thymine analogue, (Jakob and Trosko, 1965; Mattingly 1966a). The information gained could possibly give further insight into observed action of 5AU. Equimolar quantities of a selection of bases were added to 5AU solutions and sampled at the end of the following times:

a) 12 hours treatment
b) 24 hours treatment
c) 12 hours treatment followed by 12 hours recovery in nutrient solution

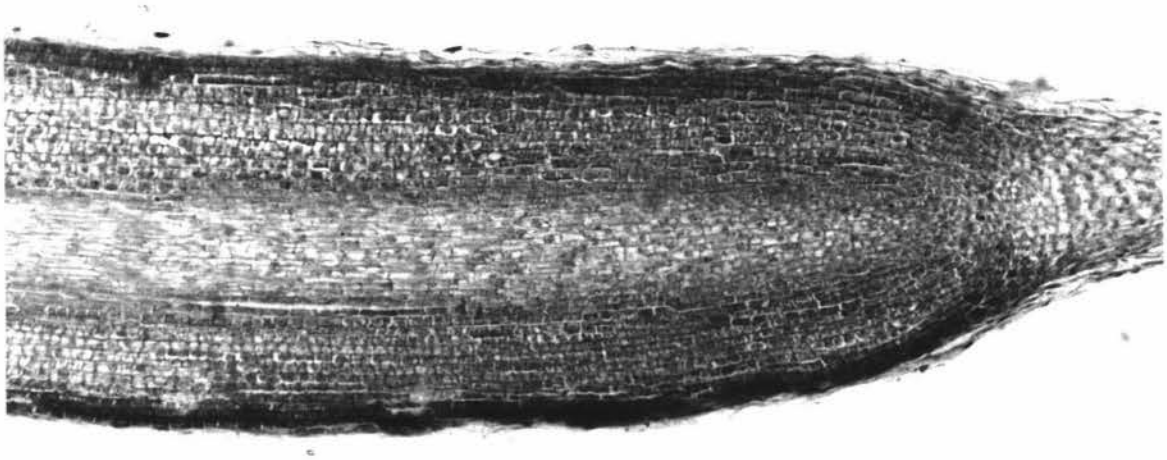
Mitotic figures were counted and compared with control experiments where no bases were added to the growth medium. The bases used were: uridine, thymine, uracil (Calbiochem), and thymidine, deoxyuridine, thymidylic acid (Nutritional Biochemicals), each at a concentration of 4mM, the same as the 5AU solution. The first five base solutions were also combined in equal quantities to give a 20mM mixture which was also used in the reversal experiments.

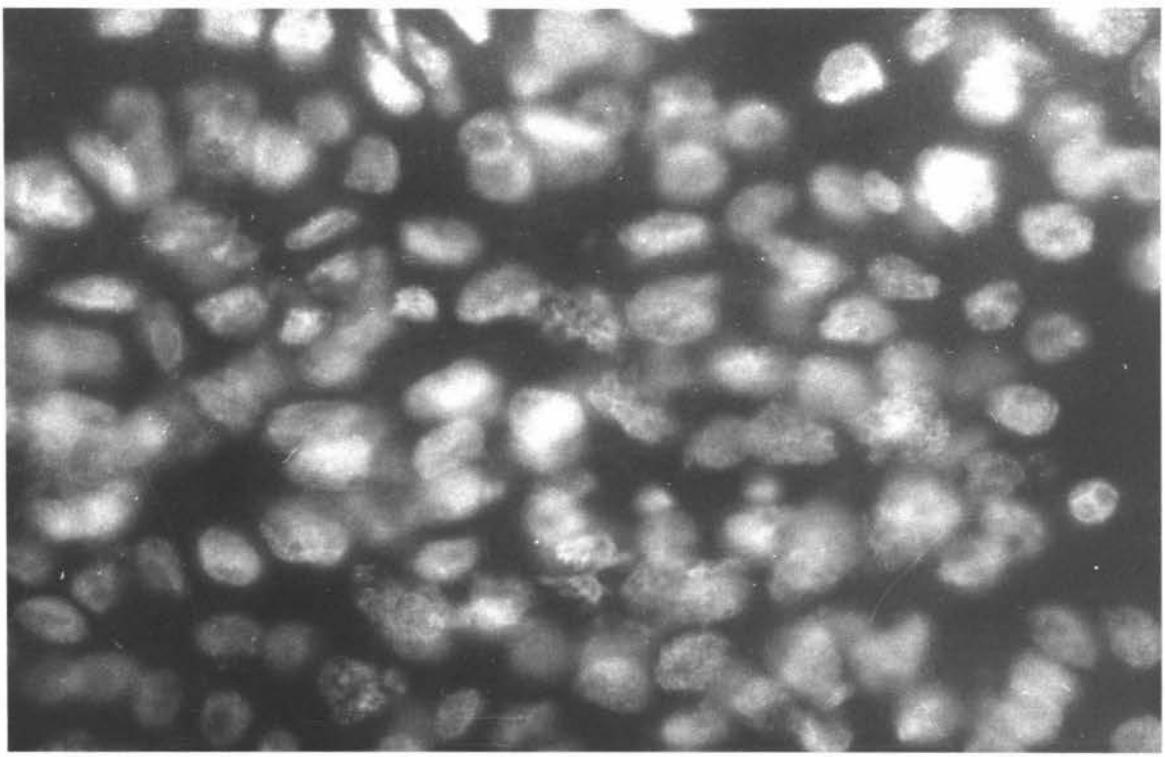
2-06 Sectioning of Material

To enable examination of longitudinal sections of the root tip, tissue was infiltrated and embedded in paraffin, then sectioned at 10μm for double staining in safranin-fast green, or 6μm for auto radiographic procedures. (For full details see Appendix 2). This enabled squashed preparations to be compared with sectioned material, in order to orientate the distribution of patches of dividing cells seen in squashes, with longitudinal sections, (Figure 2).

FIGURE 2 : Longitudinal median section through a root showing the different cell types: stele, cortex, root cap and meristem that are present in the squash preparations. (x 80)

FIGURE 3 : Fluorescing nuclear DNA of cells in squash preparations stained with BAO. (x 600)





2-07 Measurement of Relative DNA content per cell nucleus

2-071 To be able to accurately measure the relative DNA content of individual cell nuclei, tissue preparation had to be very uniform. An ammended procedure of Feulgen staining was followed: a 10 minute hydrolysis in 1N HCl at 60°C, followed by a 30 second rinse in distilled water then staining for one hour in Schiff's reagent. The tissue was squashed and mounted on cleaned slides as described in Appendix 1. For the best results, it was found by experience that the slides had to be permanently mounted in Eukitt rather than just viewed under oil.

The relative DNA content of the cell nucleus was measured with a Vickers M85 Integrating Microdensitometer at the Cytogenetics Unit, Christchurch Hospital, under the following conditions: ocular 10x; objective 100x (oil emersion) nA 1.25; size of measuring spot - 0.4um. A wavelength of 590nm was used for all measurements as recommended by Dr P Fitzgerald of the Cytogenetics Unit. (Maximum absorption for Feulgen - 575nm in Atkin, 1970, which cannot be used due to the high optical density of cell nuclei.). The subtractive method of measurement was used because the slides were permanently mounted. This involved taking measurements of several individual nuclei within a small area, and then taking a density measurement of the clear background close to each group of nuclei measured. This second reading was subtracted from each nucleus

reading to give the density of the nuclear DNA. Several nuclei were measured three times to determine a reproducibility of measurements. A typical example gave 716, 715, 710. In general, any mean measurement could be considered correct within 3 units but after subtraction eg. $(764 \pm 3) - (623 \pm 3) = 141 \leq 6$, ie there may be an error of 6 units in any individual measurement. But this averages out as seen in Table 6 where there is very good agreement between prophase metaphase and anaphase, and telophase values being half of these. The results are given in relative units.

2-072

Before the availability of the Vickers M85 Integrating Microdensitometer was known other methods were used in an attempt to measure DNA content. Root meristem tissue was stained with a fluorochrome BAO (Fluka AG),(Ruch 1966), (See Appendix 1 for details) suitable for the Feulgen reaction and relatively stable to UV irradiation. Using a Carl Zeiss (67896 W.Germ) fluorescent microscope, the fluorochrome was excited using a BG3 filter which peaked at 365 nm, and viewed using a BG12 barrier filter which cut out any wavelengths below 500 nm. Photographs were taken of the fluorescence using Kodak Tri-X film at 400 ASA, (Figure 3) Exposure times of 5 to 10 minutes were necessary and this increased with the length of time the tissue had been stained. Kodak Tri-X film was also pushed to 2400 ASA using Diafine[i] two bath film developer to try

(i - registered trade mark)

and have shorter exposure times, but this was unsuccessful. The developed negatives were scanned using an Automatic Recording Microdensitometer (Joyce/ Loebl). However the maximum width of the scanning beam was too small compared with the diameter of individual nuclei, and the machine only satisfactorily scanned whole frames measuring parts of several nuclei, rather than going backwards and forwards over one nucleus.

2-073

A second method again used the fluorochrome BAO, but utilized a photocell which directly measured the amount of fluorescence and recorded it directly onto an Electrometer 610C (Keithley Instruments). The system was suitably sensitive but only an average value of fluorescence from approximately 20 to 30 nuclei in the field of view could be obtained.

The possibility of using a computer programme designed for analysing data gained from X-ray Crystallography was also considered. Because the cells were randomly distributed in the squash preparations, rather than uniform, the programme was unable to integrate the values correctly. It would have taken several months to write and test a satisfactory programme, so consequently this idea was abandoned.

RESULTS

Each experiment was designed to provide information to help in answering specific questions which when considered together would provide further knowledge of how 5AU achieved synchronization of root meristem cells.

3-01 Does 5AU Induce Synchrony?

The first experiment using 5AU to partially synchronize a population of root cells was reported by Smith, Fussell and Kugelmann (1963). These authors gave a 24 hour treatment of 5AU (5.5×10^{-3}M) and obtained a peak of mitotic figures (42%) at 14 hours after removal from 5AU. This was not repeated through a second cell cycle, although their data did show a small rise another 12 hours later (26 hours after removal from 5AU), which they could not explain. Jakob and Trosko (1965) repeating the experiment had a peak of divisions 15 hours after removal from a 24 hour 5AU treatment.

I repeated the experiment using a 24 hour treatment (3.93×10^{-3}M), then sampled half hourly over the expected peak, and two hourly before and after. One seedling was used per sample, but it was found that the timing of divisions in individual seedlings varied considerably (as with Jakob and Trosko, 1965), so that the graph (Figure 4) gave sharp peaks and dips which were not characteristic of the general trend.

To check that these sharp peaks were due to different seedlings having different cell cycles, repeat experiments used two roots per seedling from three seedlings, for each sample. The results gained from the three seedlings were then averaged and are included in Figure 6A. All repeat experiments had the sampling period extended to 36 hours to investigate a sharp rise which appeared at the end of the first experiment, (Figure 4). In all cases the control samples, taken whenever 5AU treated plants were sampled, fluctuated about a mean of 10 divisions per 100 cells.

These results confirm that 5AU definitely causes the partial synchronisation of a population of root cells. The peak of divisions occurs between 14 and 15 hours after 5AU treatment, (Figure 7), with a maximum MI for one root of 45. The data plotted in Figure 6A is averaged from three duplicate experiments involving 3000 to 5000 cells per sample time.

The synchronization only lasts one cell cycle, and after the peak, the MI often varied considerably in a random fashion. There was always a small peak at 10 hours which may have been a fast dividing population of cells.

3-011

Most workers used a treatment of 5AU then removed the tissue to gain synchronization. Control tissue had always been in nutrient solution. Was the synchronizing effect caused by the period of treatment in 5AU, or the removal from the 5AU solution? I thought that a

treatment using continuous 5AU should be given as a different type of control. Tissue was sampled 3 hourly from 0 to 48 hours and showed a peak of divisions occurring between 37 and 41 hours, (Figure 6B), which indicated that the plants had been able to overcome the effect of 5AU. This peak occurred at the same total time as the peak obtained in the 24 hour treatment, (Figures 6A&B). Is then, the removal of tissue from 5AU important in obtaining the synchronous division of cells? How is 5AU causing this effect? The continuous 5AU experiments will be discussed in detail in section 3-05; but it was the result of this experiment that led to the design of a third synchrony experiment.

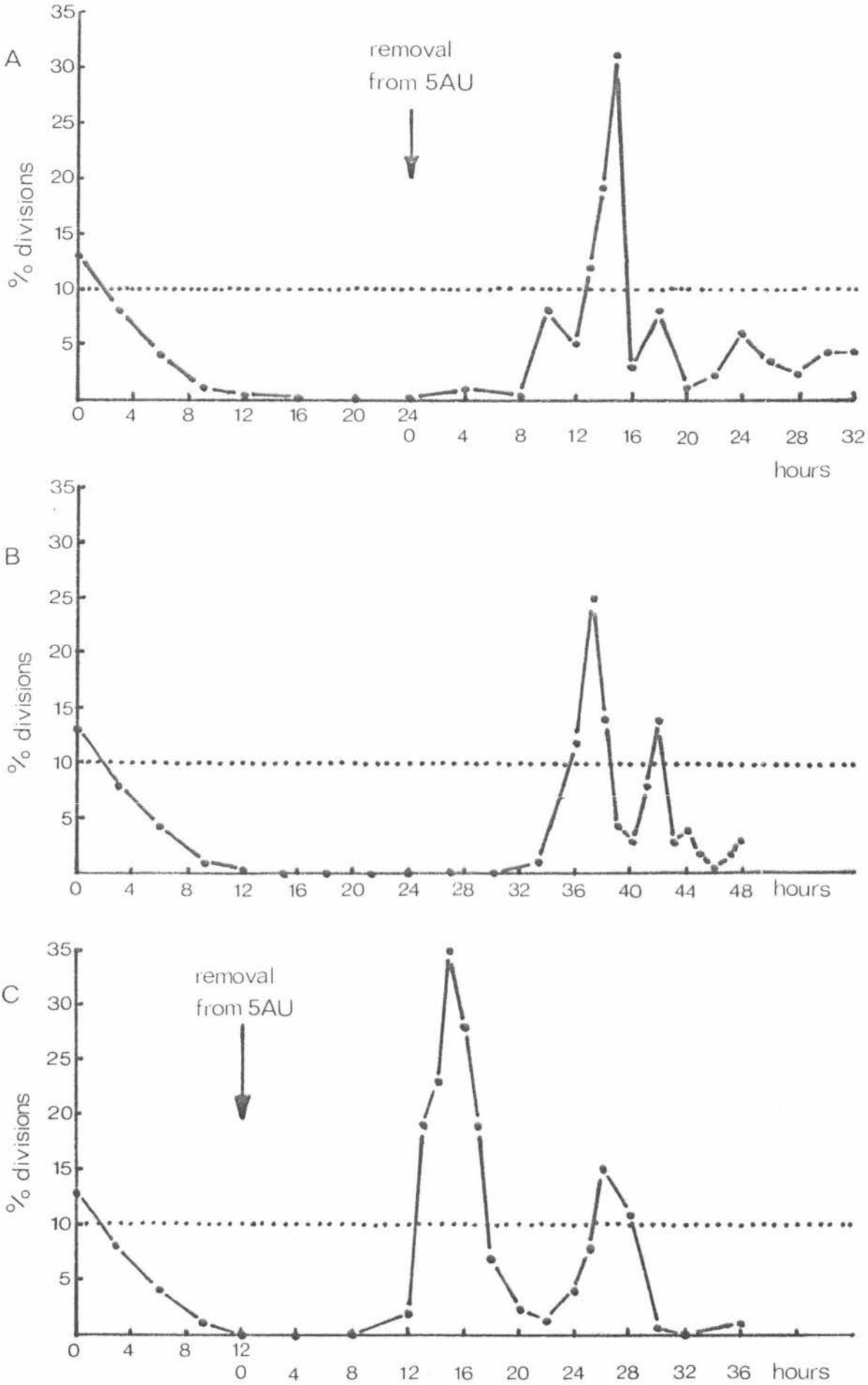
3-012

Would the observed peak of divisions still occur at the same total time if a 12 hour 5AU treatment was given instead of a 24 hour one, or would the peak of divisions appear 12 hours earlier because of the shorter 5AU treatment? This would indicate whether the observed peak of divisions is a response by the seedlings to being removed from 5AU, or whether it is just that the plants can overcome the effect of the chemical after a certain minimum treatment time anyway as observed in Figure 6B. Smith et al (1963) suggested that the removal of roots from 5AU to nutrient resulted in the synchronous division they observed, while Mattingly (unpublished data 1965)found that after recovery from mitotic inhibition, cells in roots exposed to 5AU for several days went through repeated divisions.

FIGURE 6A : Graph showing recovery after a 24 hour 5AU treatment (500 ppm) using averaged results from duplicate experiments. (Dotted line is average control)

FIGURE 6B : Graph of mitotic index during continuous 5AU treatments (500 ppm) using averaged results of duplicate experiments. (Dotted line is average control)

FIGURE 6C : Graph showing recovery after 12 hour 5AU treatments (500 ppm) using averaged results from duplicate experiments. (Dotted line is average control)

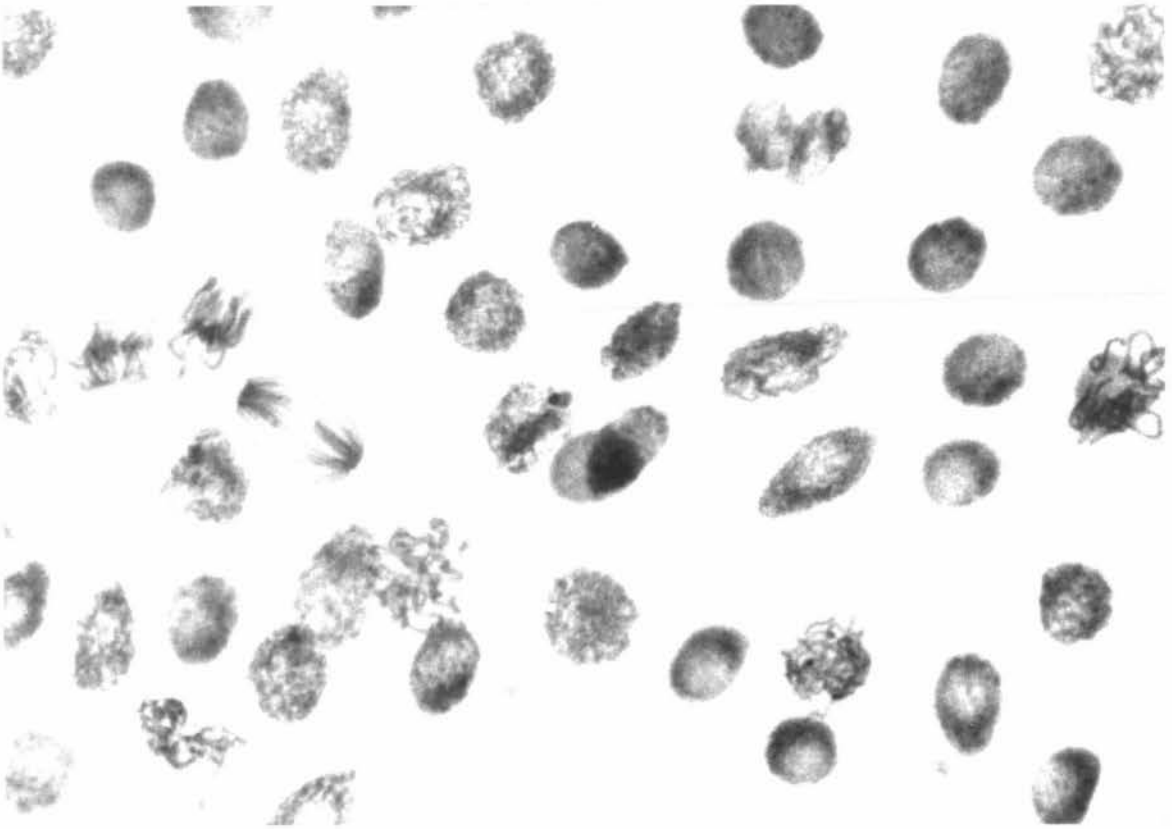

A
removal
from 5AU
% divisions
35
30
25
20
15
10
5
0 4 8 12 16 20 24
0 4 8 12 16 20 24 28 32
hours
B
% divisions
35
30
25
20
15
10
5
0 4 8 12 16 20 24 28 32 36 40 44 48 hours
C
removal
from 5AU
% divisions
35
30
25
20
15
10
5
0 4 8 12
0 4 8 12 16 20 24 28 32 36 hours

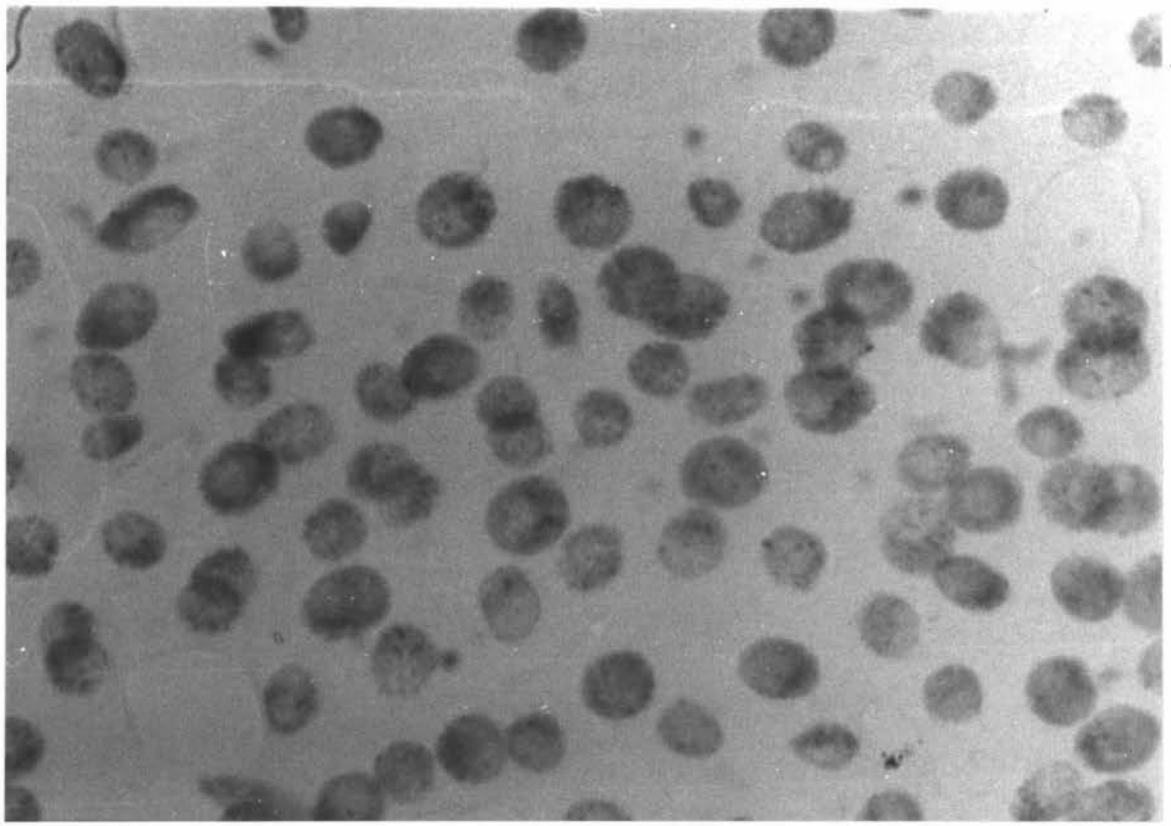
Plants were given a 12 hour 5AU treatment (3.93 x 10^{-3}M) then returned to nutrient and sampled hourly for the next 36 hours, (Figure 6C). Averaged results from duplicate experiments (at least 3000 cells examined per sample) showed a sudden rise 13 hours after removal from 5AU, peaking at 15 hours and dropping below control levels again by 18 hours. A second peak (although smaller) was evident at 26 hours after removal from 5AU but the MI had dropped to 0 again by 30 hours. This second peak is at the same time (38 hours after 5AU addition) as the peak of 15 hours in the 24 hour treatment, (Figures 6A & C). In both Figures 6A and 6C there is a peak approximately 15 hours after removal from 5AU, which shows that it is probably removal itself stimulating the cells to divide, with a delay of at least 13 hours before mitotic figures are seen.

These results suggest that removal from 5AU stimulates the cells to divide which would explain the appearance of the first peak at 15 hours after removal from 5AU, while the second peak, 26 hours after removal could be due to cells naturally recovering from the 5AU treatment. Would the cells that are seen dividing at the same total times in the different experiments all be found in the same regions of the root if the tissue was examined longitudinally? This was done with a view to determining the geographic distribution of dividing cells seen after 5AU treatment.

FIGURE 7 : Partially synchronized cell divisions 14 hours after a 24 hour 5AU treatment (500 ppm) Note the presence of prophase, metaphase and anaphase. (x 640)

FIGURE 8 : No cells dividing at the end of a 24 hour treatment with 500 ppm 5AU. (x 640)



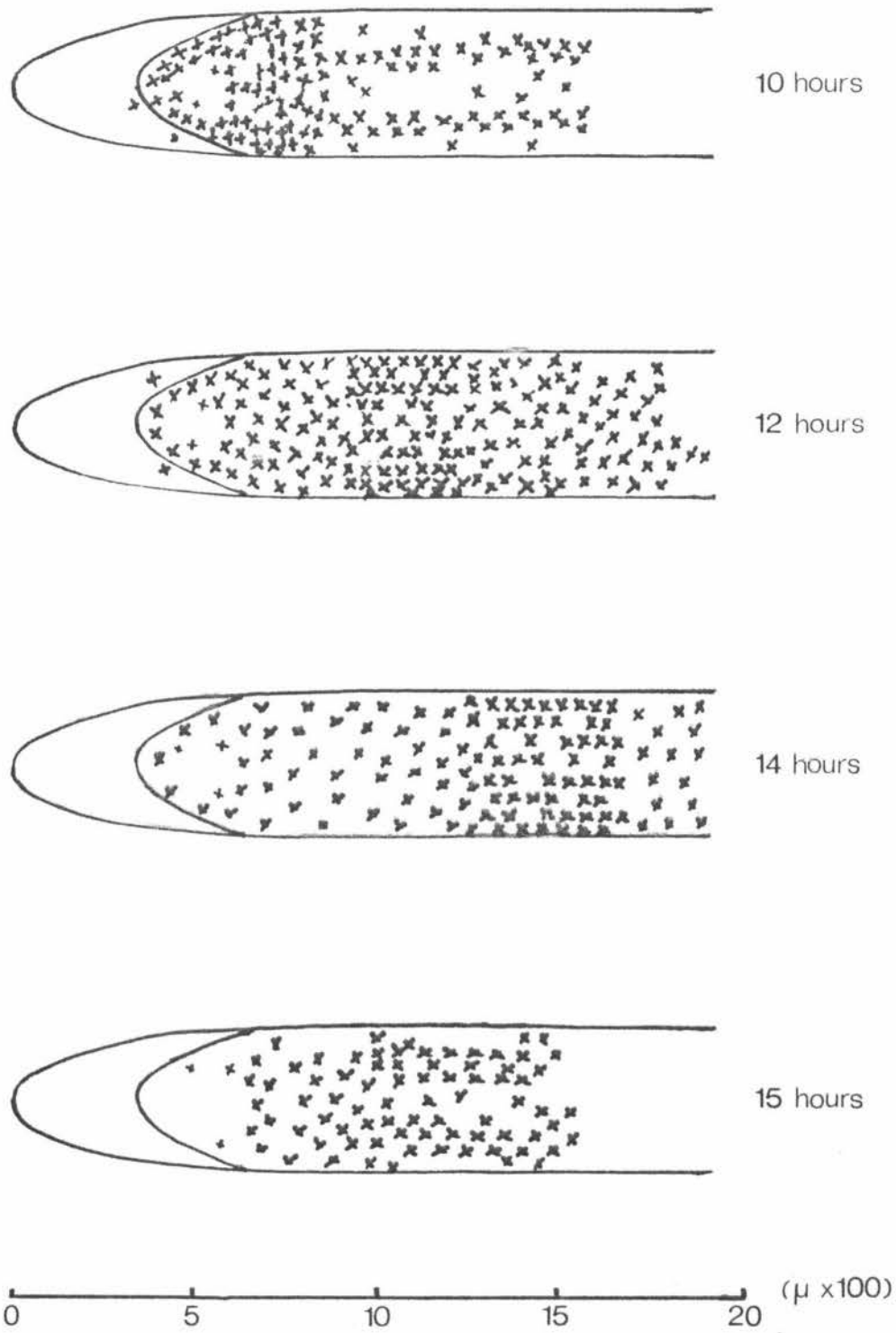


3-02

Longitudinal sections were prepared from tissue sampled over the peaks of division, in order to determine the distribution of the mitotic figures seen in squash preparations of the respective synchrony experiments, (Figure 6A, B & C). All mitotic figures were recorded diagrammatically from 6 to 8 sections passing through the centre of the root, which made the distribution of cell divisions very clear.

In the 24 hour 5AU treatment, tissue was prepared from samples taken between 10 and 15 hours after removal, (Figure 9). This clearly showed that the small rise at 10 hours (Figure 6A) was mainly due to cells dividing that were surrounding the quiescent centre. The distribution of mitotic figures over the main peak was more even, extending up into the cortex and stele for the 1½ to 2 mm that were examined. The densest area of divisions, (when present), progressed further back the root with time.

These observations at first seemed difficult to account for compared with those of Mattingly (1966b). This author sampled up to 10 hours and used colchicine to collect metaphases, as she was following the distribution of the few divisions that occur before the synchronous peak. My observations started when Mattingly's were completed. The lack of mitotic figures in the vascular cylinder at 10 hours (as compared with Mattingly's data) could have been due to my not using colchicine, which would have collected any cells that divided in this region a few hours before sampling.


10 hours
12 hours
14 hours
15 hours
0
5
10
15
20
(μ x100)

3-021

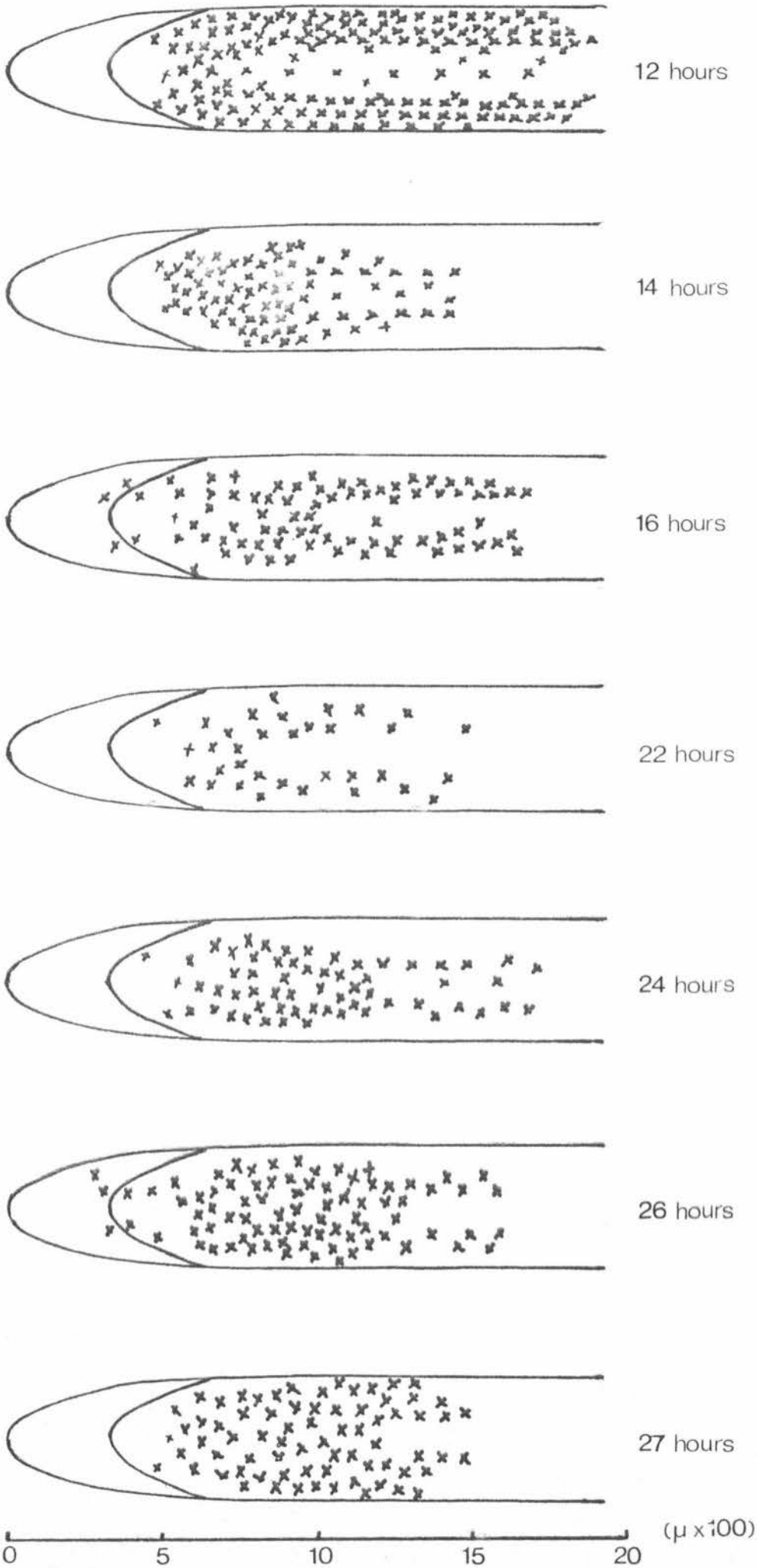
In the 12 hour 5AU treatment divisions occurring at 12, 13 and 14 hours after 5AU removal were mostly distributed in the first millimetre behind the root cap, but also extended up the cortex for the next 1½ mm in smaller numbers, (Figure 10). The majority of figures present over the 24 to 27 hours peak were also directly behind the root cap, but extended further back in smaller numbers as well.

These results suggest that there are not two geographically separate cell populations dividing at different times to give the two synchronous peaks after the 12 hours 5AU treatment. However there is still the possibility that a few cells close to the quiescent centre are dividing twice, while those further back into the cortex only divide once. This possibility is discussed later in section 3-11.

Preparations from the peak of the continuous 5AU treatment 37 to 44 hours showed similar results to the previously discussed longitudinal sections, with mitotic figures being evenly distributed but in smaller numbers in each section.

Now that the synchronous effects of 5AU have been confirmed, they must be studied to find out how these effects are achieved. This involves looking at each stage of the cell cycle separately using different experimental techniques of which pulse labeling of cells in the S phase , with radioactive DNA synthesis precursors, is the most universally used.

FIGURE 10 : Diagrams of longitudinal median sections of roots collected over peaks of divisions after a 12 hour 5AU treatment showing the geographical distribution of the dividing cells seen in the squash preparations.


12 hours
14 hours
16 hours
22 hours
24 hours
26 hours
27 hours
(μ x 100)
0
5
10
15
20

3-03 Effect of 5AU on G_1 and G_2 phases

Duncan and Woods in 1953 working with Allium cepa reported that 5AU affected the metabolism of nucleic acids. It was also about this time that Howard and Pelc (1951, 1953) published evidence for the cell cycle showing that interphase had three important stages: G_1, S, G_2; which had to be completed before cell division could occur. Duncan and Woods had shown that 5AU affected the S phase, but it was not until 1966 that the G_1 or G_2 phases were studied by Mattingly to determine whether they were also interfered with by 5AU.

3-031 G_1 phase

The experiment reported by Mattingly (1966a) was repeated and similar results were obtained. Seedlings were pulsed over seven hours with 2 uc/ml H^3-TdR in the presence or absence of 5AU solution (3.93×10^{-3}M). Samples were taken hourly from 2 to 7 hours, stained with Feulgen, autoradiographed, and were compared for label which indicated entry into the S phase from G_1. If cells were being held up in the G_1 phase and prevented from entering S, the initial LI of 20 should not have significantly increased. In each case results were similar (Table 2), especially at the later times indicating no significant effect on the G_1 phase. This conclusion is also confirmed by Prensky and Smith (1965).

Table 2: Labeling Index recorded in the presence or absence of 5AU over seven hours.

TIME	CONTROL	5AU
2 hrs	26	20
3 hrs	44	20
4 hrs	42	30
5 hrs	40	40
6 hrs	46	40
7 hrs	40	40

3-032 G_2 phase

Several authors including Mattingly (1966a), Wolff and Luipold (1964) have suggested the possible elongation of the G_2 phase as a direct result of 5AU treatment. An unpublished experiment of Wolff and Luipold (1964), reported by Mattingly (1966a), who independantly confirmed their results, was repeated to consider the effects again. Plants were pulsed for one hour with H^3-TdR (4 uc/ml) in nutrient solution, then some were transferred to fresh nutrient while others were put into 5AU solution. Samples were taken at 2, 4, 6, 8, 10 and 11 hours. Metaphases were collected with 0.05% colchicine. Slides were examined to determine the appearance of labeled mitotic figures. All cells in S and those that passed from S into G_2 during the time of pulsing were labeled. Therefore if labeled cells were not seen in mitotis, the G_2 cells must have been held up. This is what

was observed. Label was present over chromosomes in the control by 4 hours after pulsing, indicating an approximate G_2 duration of 4 hours (Table 3). There

Table 3: Labeling Index of mitotic figures observed over 11 hours 5AU treatment or nutrient control.

TIME	CONTROL	5AU
2 hrs	0	0.0
4 hrs	6	0.0
6 hrs	14	0.0
8 hrs	7	0.1
10 hrs	10	0.0
11 hrs	-	0.5

was no label over mitotic figures in 5AU treated samples after 11 hours. What was seen at 8 and 11 hours is dubious because of heavy background. These results are similar to those obtained by other authors who have interpreted them as the G_2 phase being interfered with by 5AU such that cells are prevented from continuing into mitosis. My suggestion is that only cells early in the G_2 phase are affected by 5AU. A longer period of labeling prior to 5AU treatment could possibly confirm this theory.

After examining the G_1 and G_2 phases of the cell cycle for their response to 5AU treatment, the S phase of cells dividing in the synchrony experiments was studied using pulse labeling.

3-04 DNA synthesis at the end of a 24 hour 5AU treatment

Pulse labeling in the last hour of a 24 hour 5AU treatment resulted in a LI of 3. Samples taken over the peak of divisions 14 and 15 hours later had a MI of 17 and 24 respectively but there was no label in these mitotic figures. From this it could be concluded that DNA synthesis for cells appearing in the peak of divisions at 14 to 15 hours after the end of a 24 hour 5AU treatment, does not occur in the last hour of 5AU treatment. Possibly these cells were held in early G_2 by the 5AU after having undergone DNA synthesis near the beginning of 5AU treatment.

3-041 DNA synthesis after a 12 hour 5AU treatment

The appearance of the second peak in the 12 hours 5AU treatment at 26 hours after removal from 5AU (Figure 6C) immediately posed further questions. Is this a second population of cells dividing, or is it the same population as the first peak having undergone a rapid cell cycle? Or could it be cells that had an extended G_2 phase but were still all the same population?

Pulsing with H^3-TdR would indicate whether DNA synthesis was occurring between the observed peaks. Two different pulse experiments were carried out. They used H^3-TdR (2 uc/ml) pulsing from

1) 2 to 6 hours
2) 16 to 20 hours

after removal from 12 hours 5AU treatment. The LI at

the end of the 2 to 6 hour pulse was 72. Samples collected over the two peaks were examined for labeled mitotic figures which would indicate that the dividing cells had undergone DNA synthesis during the time of the pulse. Over both peaks all mitotic figures seen were labeled; but the MI did not suggest the presence of a peak of divisions as observed in all previous replicate experiments. Consequently these results could not be considered as a completely representative sample. However they do support the idea that the two peaks observed in the original experiments could be all from the same group of cells with some having a longer cell cycle than others.

At the end of the second pulse (16 to 20 hours) a LI of 55 was recorded. A MI of 22 was noted at 20 hours, which immediately explained the lack of mitotic figures over the sampling of the first pulse - the peak of divisions had been delayed by five hours. Consequently, as was expected, the MI over the second peak was also low, and it was questionable whether the figures were labeled compared with the heavy background. This apparent lack of label again supports the conclusion that the cells seen dividing over the two peaks in the original experiments are not from two different groups of cells or parts of the root. This experiment could not be repeated due to the two months required for its completion, but further information can be gained from another labeling experiment designed to determine DNA synthesis recovery after a 12 hour 5AU treatment.

3-042

Half hour pulses of H^3-TdR (4 uc/ml) were given in nutrient solution at 3, 6 and 9½ hours after the 12 hour 5AU treatment. Some tissue was fixed immediately after the pulse, while the rest was allowed to continue growing so that further samples could be taken at 12, 13 and 14 hours, the beginning of the first expected peak of divisions (Figure 6C). The initial LI was recorded after each pulse, and then the MI and percentage of labeled mitotic figures noted for each pulse time over the peak of divisions.

Table 4: MI and percentage of labeled figures occurring over the peak of division after a 12 hour 5AU treatment.

Initial samples :	LI	
3 hours pulse	11	
6 hours pulse	18	
9½ hours pulse	16	
12 hours samples :	**MI**	**% labeled mitotic figures**
3 hours pulse	3	50%
6 hours pulse	3	80%
9½ hours pulse	5	10%
13 hours samples :		
3 hours pulse	5	0%
6 hours pulse	3	90%
9½ hours pulse	10	50%

Table 4 Continued

14 hours samples :		
3 hours pulse	16	85%
6 hours pulse	10	50%
9½ hours pulse	22	0%

In the 12 and 13 hour samples the LI for the 6 hour pulses was high, but the MI was notably low. These few cells could have been strays from the main population in which the MI had increased by 14 hours. This sample clearly showed that the majority of cells entering the peak of division between 13 an 16 hours underwent DNA synthesis between 3 and 6 hours after removal from 5AU, and is confirmed by Jakob and Trosko (1965).

However the question of whether the second peak at 26 hours after removal from 5AU, were different cells compared with the first peak has still not been clearly answered.

3-043

Another method used to help determine whether the cells in the second peak had undergone DNA synthesis after the first peak of divisions, and consequently a rapid cell cycle, was the use of caffeine to mark cells in telophase at the end of the first peak. Tissue collected over the second peak (25 to 28 hours) was then examined for the presence of binucleate cells in division. These preparations were longitudinally sectioned and double stained with safranin and fast-

green to clearly distinguish cell walls and chromosomes. No dividing binucleate cells were observed in any preparations.

The longitudinal sections discussed in section 3-021 (Figure 10), of preparations of tissue sampled over both peaks also suggested that there are not two distinct populations of cells dividing at different times.

All these experiments lead to the conclusion that DNA synthesis for cells appearing in both peaks occurred before 12 hours after removal of the tissue from 5AU. Therefore the second peak is not due to some of the cells from the first peak dividing a second time.

Further half hour pulsing experiments would be required to indicate the exact times and duration of the S phase for each of the groups of cells from the different peaks.

3-05 Effect of 5AU on DNA synthesis itself

This set of experiments was designed to see whether DNA synthesis and ultimately cell division will occur in the presence of 5AU. Samples were taken 3 hourly through a 36 hour treatment of 5AU (3.93×10^{-3}M). Because additional seedlings were available samples were also taken at 40, 42, 44 and 48 hours continuous 5AU. The resulting graph of MI showed a decline of divisions to almost zero by 12 hours and the MI stayed at this low level until 36 hours (Figure 8). However

some divisions were present in the 42 hour sample, (Figure 5). There could have been a peak between 36 and 40 hours which was not sampled so this time was included in a repeat experiment.

Samples were taken hourly from 36 to 48 hours in the repeat experiment. The MI reached a peak between 37 and 42 hours (Figure 6B), which was similar to data of Jakob and Trosko (1965). These authors found that root tip cells of some seedlings did not divide at all, resulting in large ranges of data. As temperature was kept constant at $20^{\circ} \pm 1^{\circ}C$ and could be excluded as a reason for this large variation, the fact that other authors have found wide variation between seedlings, could explain why the peak in my data ranged over five hours in duplicate experiments.

The results of this experiment indicate that 5AU does not completely block the cell cycle (and consequently DNA synthesis), as a synchronous division of cells can occur in its presence. These findings confirm those of Jakob and Trosko (1965).

3-051

If DNA synthesis is not completely halted in the presence of 5AU, when does it occur for those cells observed in division between 37 and 42 hours? Two times were chosen for pulse labeling before the appearance of mitotic figures. One was of 4 hours duration: 14 to 18 hours (pulse A), and the other was 6 hours: 24 to 30 hours (pulse B). Samples were collected at

the end of the pulse, and over the peak of divisions 34 to 42 hours. Duplicate experiments were done using H^3-TdR and H^3-CdR at 2 uc/ml, as well as a 'cold' thymidine (equimolar) control, to ensure that thymidine was not overcoming any effect of 5AU as Prensky and Smith (1965) suggested. The resulting preparations were examined for label in the mitotic figures, which would indicate when the DNA was synthesized for the cells dividing between 37 and 42 hours.

At the end of pulse A (14 to 18 hours), the labeling index was 30 for both H^3-TdR and H^3-CdR. The LI rose to between 70 and 80 for both labeled compounds between 34 and 42 hours indicating a buildup of cells in the S phase before cells are seen in division.

Table 5: LI and MI obtained after pulse A using 'cold' thymidine, H^3-TdR and H^3-CdR.

'cold' thymidine:	MI	H^3-TdR:	MI	LI
Initial	7/2000	Initial		26
34 hours	0	34 hours	0	75
36 hours	0	36 hours	1.4	80
37 hours	0	37 hours	0.5	70
38 hours	0	38 hours	0	75
41 hours	0	41 hours	0	80
42 hours	25/800	42 hours	0	85

H^3-CdR	MI	LI
Initial		40
34 hours	0	80
36 hours	0.1	80
37 hours	0	80
41 hours	3	70

The expected rise in the mitotic index did not occur. There were a few figures present in the 42 hour sample and a large number of preprophase cells which suggested that cell division was being slowed down by the presence of these precursors at a critical time. The control experiment using equimolar thymidine had the same effect (low MI with preprophase cells at 42 hours), but also resulted in misshapen nuclei as seen also in reversal experiments in section 3-08 (Figure 17). Those mitotic figures seen at 42 hours were labeled, and therefore those cells must have synthesized DNA during the time of the pulse between 14 and 18 hours which was 24 to 28 hours previous to their dividing. The S phase of these cells has definitely been prolonged.

Pulse B (24 to 30 hours) gave similar results to Pulse A. The equimolar thymidine control did not damage the nuclei to the same extent even though the treatment was longer, which suggests that 24 to 30 hours is a less critical time for DNA synthesis of cells appearing in the expected peak of divisions between 34 to 42 hours. The LI after pulsing was 25 for both precursors and this rose to 60 between 34 and 42 hours, again indicating a buildup of cells in the S phase. Mitotic figures started appearing by 42 hours, but it was difficult to determine whether they were labeled due to the heavy background.

It appears that pulses of several hours duration delay the observed peak of divisions that occur in

control experiments (5AU only), as this same phenomenon was observed in section 3-041 with pulsing after a 12 hour 5AU treatment. This delaying effect of thymidine will be referred to again in the discussion.

Because of the 24 to 28 hours between pulse time and appearance of labeled mitotic figures in pulse A, indications are a prolonged S phase or possibly an extended G_2 phase. The investigation of these possibilities led to a further pulse labeling experiment.

3-052

Thirty minute pulses of H^3-TdR (4 uc/ml) were given in the presence of 5AU to plants being treated continuously with the thymine analog. Two plants per sample were pulsed then transferred to aerated, distilled water for 10 minutes to permit depletion of the H^3-TdR pool before fixing. Samples were taken at selected times between 3 and 36 hours. Preparations were examined and a labeling index and mitotic index recorded (Table 6).

The LI reached a peak by 9½ hours and then gradually declined, while the MI had dropped to 0 by 9½ hours and only fluctuated a few hours before the expected increase in MI. These results show the same trends as those of Jakob and Trosko (1965), but the LI is lower (30 to 50 compared with 50 to 60) in the 5AU samples. This could be due to pulsing for only 30 minutes compared with 2 hours.

Table 6: LI and MI during continuous 5AU treatment.

TIME	LI	MI
Control	40	12
3½ hrs	24	12
9½ hrs	56	0
15½ hrs	49	0
21½ hrs	47.5	0
27½ hrs	44.5	8
30½ hrs	31.5	0
33½ hrs	40	3
36½ hrs	34.5	1

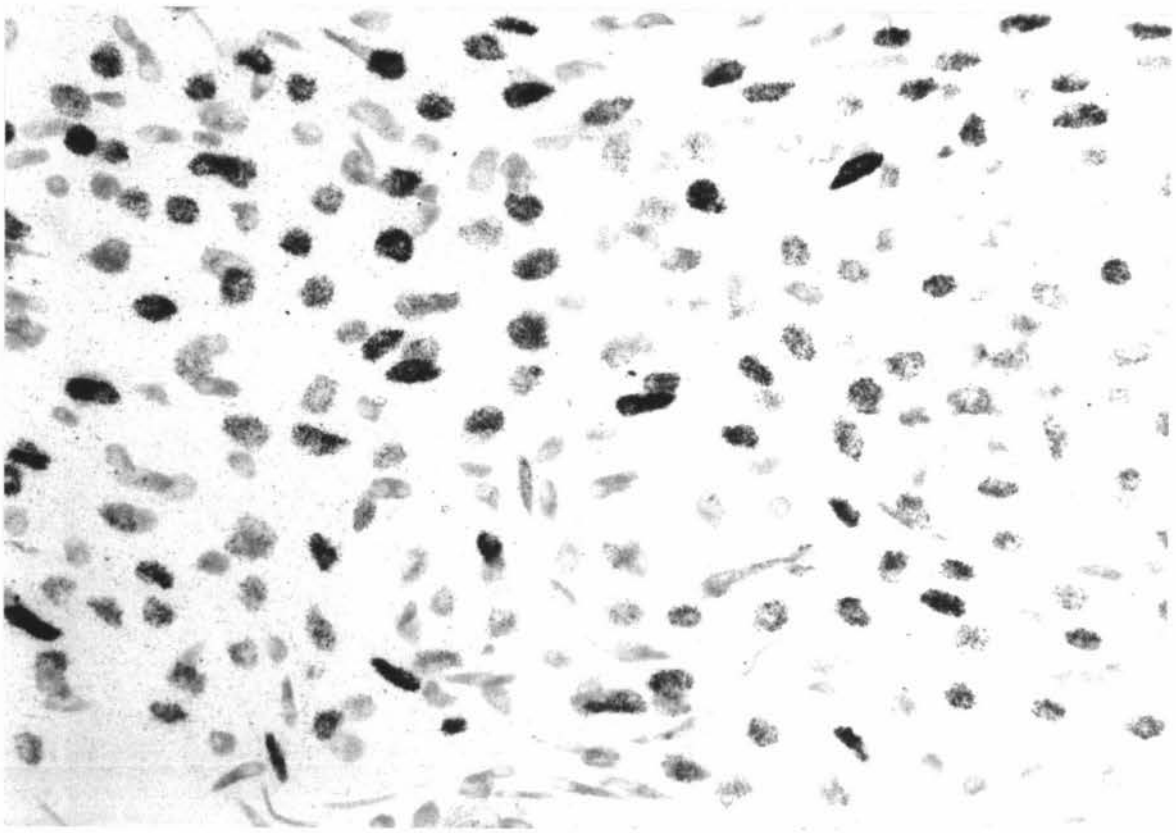
Cells continued to synthesize DNA in the presence of 5AU (Figure 11), with a peak in the LI at 9½ hours and this slowly declined closer to the expected peak of divisions, as the number of cells heldup in the S phase gradually started declining. If DNA synthesis is occurring as early as 3½ and 9½ hours into 5AU treatment, but mitotic figures are not seen for another 27 to 30 hours, then either the S phase is prolonged (ie DNA synthesis is occurring at a slower rate), or the cells are held up in the G_2 phase. The short 30 minute pulses of H^3-TdR did not seem to delay the progress of cells into mitosis as the longer pulses did.

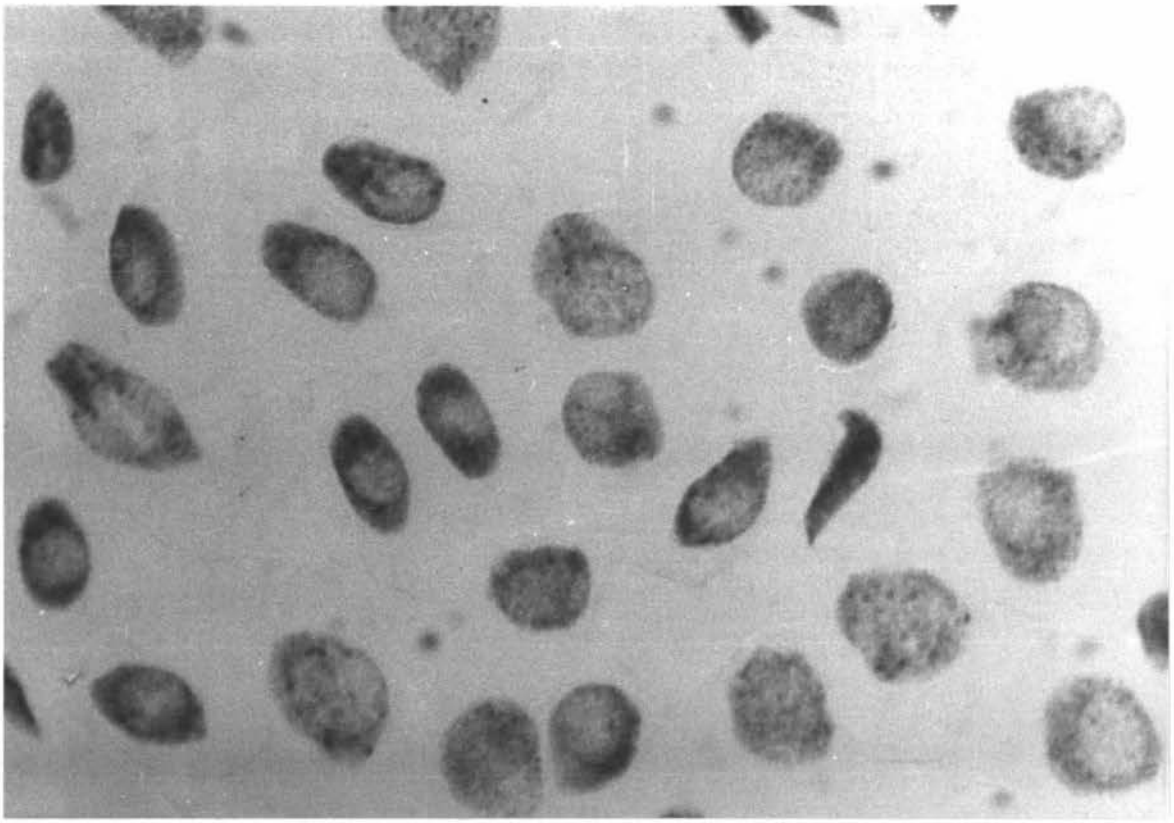
3-06 Longitudinal sections of pulse labeled tissue

Longitudinal sections of pulse labeled tissue from section 3-052 were also prepared to examine the distribution of label in the root. The 3½ hours 5AU sample

FIGURE 11 : 21½ hours continuous 5AU treatment showing incorporation of label immediately after a 30 minute pulse during the last half hour of treatment. (4 uc/ml concentration of radioactivity) (x 640)

FIGURE 12 : Large nuclei seen in samples of 42 hours continuous 5AU treatment. Diameter of 22 microns compared with 14 microns in controls. (x 640)



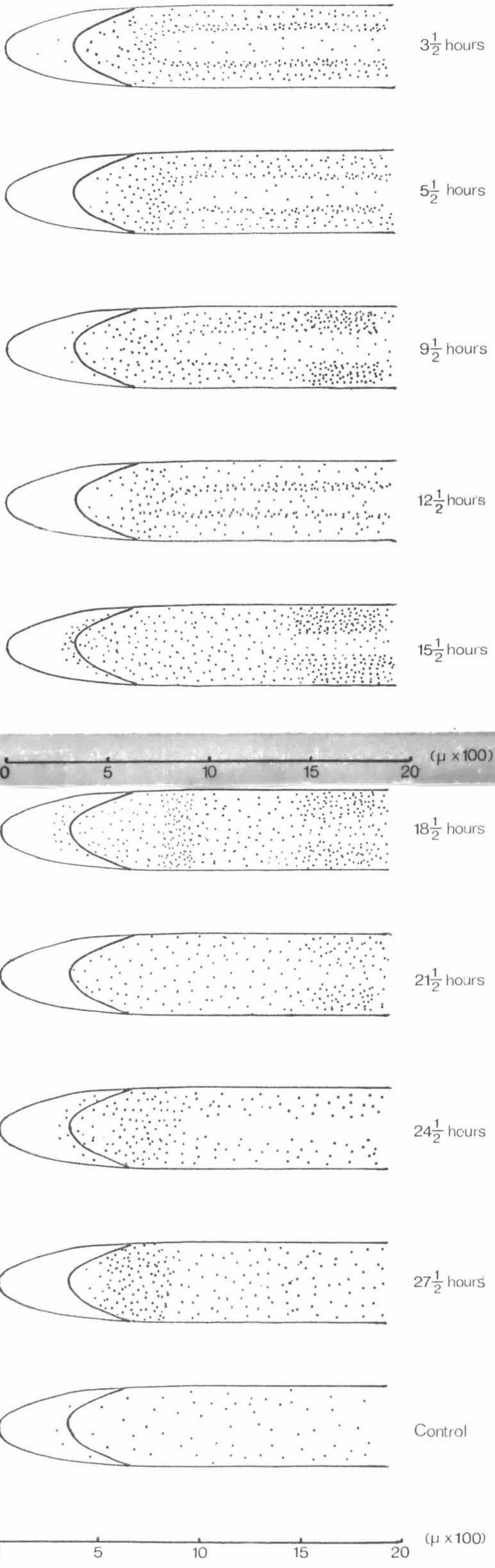


had label fairly evenly distributed up the meristem with the quiescent centre of about 20 cells, in any one 6u section clearly unlabeled (Figures 18 and 19). By 9½ hours the label was present in a greater proportion of cells, implying a delay in the S phase, with rows of heavily labeled cells surrounding the stele approximately 1½ to 2 mm from the root tip (Figure 13). 15½ hours was very similar. After this time, as with the squash preparations, the number of labeled cells declined, as did the number of silver grains per nucleus. These observations support the data gained from the squash preparations that the number of cells undergoing DNA synthesis increases up to 9½ hours, levels off at 15½ hours, then gradually declines as the DNA synthesis slows down (fewer grains per cell and fewer cells labeled), and some cells begin leaving S.

3-07 DNA density measurements

A method distinguishing between cells held up in a prolonged S phase or blocked in the G_2 phase is to measure the amount of DNA per nucleus for a large number of cells per sample, which would give an idea of the overall trend at each time of sampling. Several techniques were tried (see Materials and Methods) but the successful method was to use an Integrating Microdensitometer. Slides of 6, 12, 15, 24, 30, 36, 42and 48 hours in continuous 5AU were examined. Only 100 cells per slide could be measured due to the

FIGURE 13 : Diagrams of longitudinal median sections of pulse labeled tissue showing distribution of label in the root tip fixed immediately after a 30 minute pulse. (4 uc/ml H^3-TdR)


3½ hours
5½ hours
9½ hours
12½ hours
15½ hours
0
5
10
15
20
(μ x100)
18½ hours
21½ hours
24½ hours
27½ hours
Control
0
5
10
15
20
(μ x100)

limited time available. Control slides were also measured for density values of interphase and mitotic cells. As was mentioned in Materials and Methods, there was individual variation between readings with a reproducibility of measurement being ±6. Table 7 gives a selection of DNA densities from control slides showing the excellent correlation between 2C and 4C values, especially when considering the averages.

Table 7: A selection of DNA densities of mitotic figures from several control slides.

	Prophase	Metaphase	Anaphase	Telophase
	224	234	220	120
	244	239	243	134
	255	242	259	116
	257	232	244	123
	246	220		120
	258	245		106
	225	254		117
	249	255		123
	259	262		111
	252	261		118
	256	279		115
	235	268		126
	241	246		125
	244	249		124
	247	184		121
MEAN:	246	245	242	120

The data for the 5AU treated tissue are presented as histograms in Figure 14 with any mitotic figures

being blocked in. The results show that by 15 hours there is a buildup of cells in G_1, but because we know that the G_1 phase is not affected by 5AU (section 3-031) this must actually be due to a slowing down of the progress of cells through the S and possibly the G_2 phase. This point is verified by following the shift of the bulk of cells from G_1 at 15 hours through to G_2 by 36 hours. The cell nuclei at 42 hours were very large and diffuse compared with controls, (Figure 12). The density values for this slide were much higher than any of the others, with measurements on dividing cells being in the same range. This phenomenon has also been observed in human cells treated for long periods in 5AU (Regan 1966), up to 20 times normal size after 60 hours treatment. There were quite a few cells (36-not on histogram) that had DNA values up to 700 which could have been 6C (although this seems unlikely in the first 2 mm of root tip), but none were found in the 48 hour sample. If the Vicia cells were following the pattern of the human cells, then the 48 hour sample should also have had larger, diffuse nuclei. The area of individual nuclei was larger than control cells, but the density values were in the same range, and the nuclei did not look blotchy as was noticed in the 42 hour sample. When trying to explain the unusual results of the 42 hour samples, the unavoidable variation in intensity of the Feulgen reaction must be remembered and according to Richards (1966, quoted by Atkin 1970) '..... virtually precludes

the comparison of amounts of Feulgen colour in situations other than between cells in the same area of the slide.'

The similarity of data obtained for mitotic figures between slides (Table 7) and with other published results (Deeley et al 1957; M^{c}Leish and Sunderland, 1961) support the validity of my comparison of slides for general trends. The peaks occurring on the histograms showing G_1 and G_2 do vary one column either way between roots. This is directly due to the variation in Feulgen intensity between slides of different roots.

More recently, published work measuring DNA densities using a Barr and Stroud Integrating Microdensitometer gave data for 2000 cells per 5AU treatment (14, 24 and 48 hours); 1000 cells for G_1(telophase) and G_2(prophase); and 3000 cells for control interphase, (Scheuermann and Klaffe-Lobsien, 1973). These authors found a buildup in the latter third of the S phase by 48 hours. My results although based on smaller samples than the above authors, still show the same trend of a buildup of cells towards the 4C DNA level with prolonged 5AU treatment. This data also corresponds with pulse labeling experiments of this same tissue treated continuously with 5AU, discussed in section 3-052. The cells seem to be held late in the S phase or possibly very early in the G_2 phase.

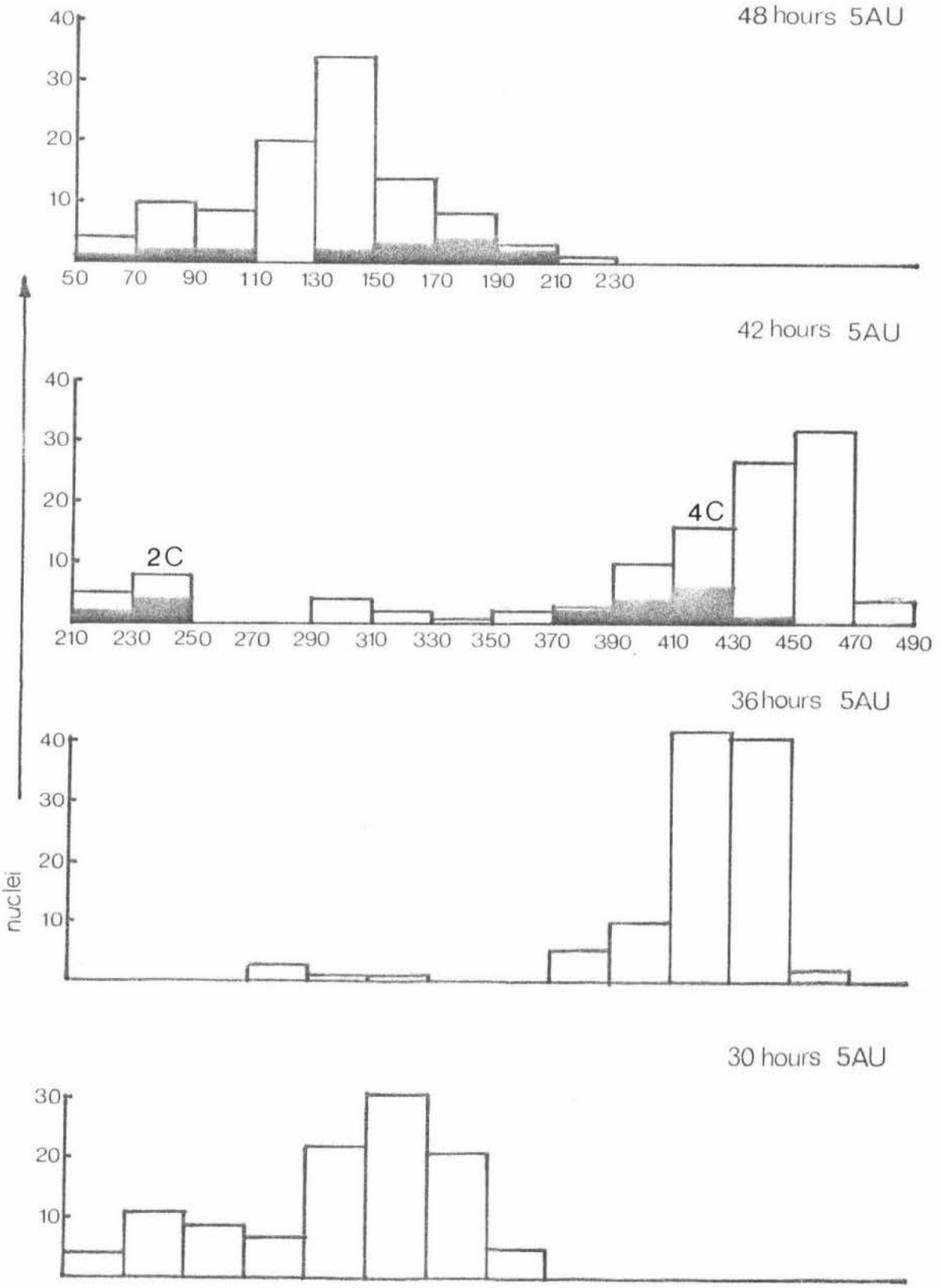
3-071

Further useful data can be obtained using the microdensitometer by measuring DNA density of preparations pulse labeled with H^3-TdR. All labeled cells must be in the S phase, therefore by measuring all unlabeled cells ie G_1 or G_2, the data will indicate if there is any cell arrest occurring.

Only small numbers of cells (approximately 50 per slide) could be measured due to the short time available. Even so, a pattern emerged showing that there is a buildup of cells with 4C DNA by 15½ hours continuous 5AU treatment, (Figure 15). From this time to 27½ hours, cells are congregating in G_1 which coincided with the observed MI of 8 at this time. These cells dividing 'prematurely' and those seen in the 27½ and 33½ hour samples were unexpected as they had not been observed in previous duplicate experiments, but they still fitted in with the general observations of a shift of cells from G_2 through mitosis to G_1 and back towards G_2 again by 33½ hours. It must be remembered that these samples are very small and therefore may be biased, but if 3½ hours is considered similar to a control situation, samples treated for longer times in 5AU definitely have the progress of cells through the cell cycle impeded. Alternatively, it could be said that cells are becoming synchronized to a small degree by 9½ hours and continue like this until a major peak of divisions occurs at approximately 37 to 42 hours.

In the previous section 3-052 the conclusions drawn

FIGURE 14 : Histograms showing a change in nuclear DNA content of the interphase cell population during continuous 5AU treatment (500 ppm). Shaded areas indicate mitotic figures.


48 hours 5AU
40
30
20
10
50 70 90 110 130 150 170 190 210 230
42 hours 5AU
40
30
20
10
2C
4C
210 230 250 270 290 310 330 350 370 390 410 430 450 470 490
36 hours 5AU
40
30
20
10
nuclei
30 hours 5AU
30
20
10

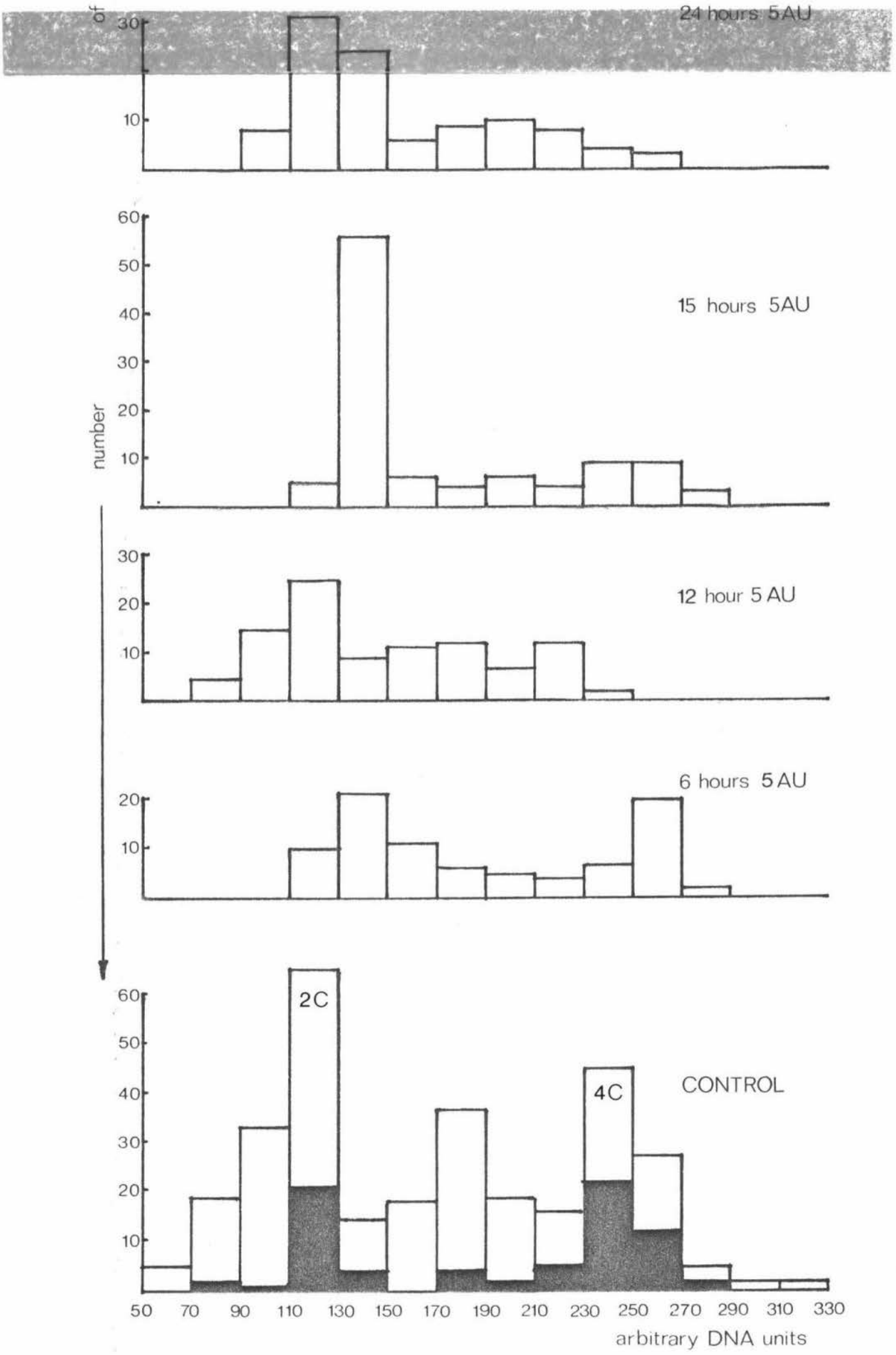
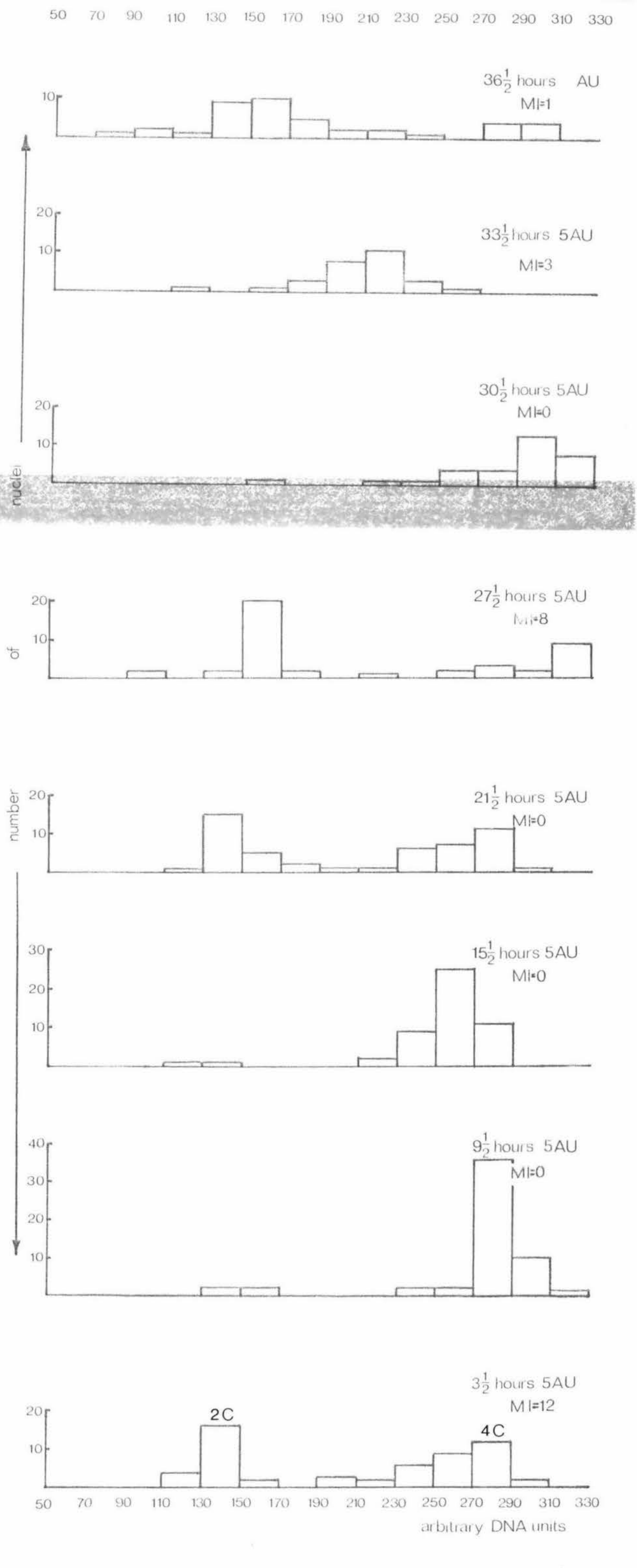

24 hours 5AU
15 hours 5AU
12 hour 5 AU
6 hours 5 AU
CONTROL
2C
4C
number of
arbitrary DNA units
50 70 90 110 130 150 170 190 210 230 250 270 290 310 330

FIGURE 15 : Histograms showing the range of DNA content in G_1 and G_2 interphase cells treated for varying lengths of time with continuous 5AU, 500 ppm.


50 70 90 110 130 150 170 190 210 230 250 270 290 310 330
$36\frac{1}{2}$ hours AU
MI=1
10
20
10
$33\frac{1}{2}$ hours 5AU
MI=3
$30\frac{1}{2}$ hours 5AU
MI=0
20
10
nuclei
$27\frac{1}{2}$ hours 5AU
MI=8
20
10
of
number
20
10
$21\frac{1}{2}$ hours 5AU
MI=0
30
20
10
$15\frac{1}{2}$ hours 5AU
MI=0
40
30
20
10
$9\frac{1}{2}$ hours 5AU
MI=0
$3\frac{1}{2}$ hours 5AU
MI=12
2C
4C
20
10
50 70 90 110 130 150 170 190 210 230 250 270 290 310 330
arbitrary DNA units

from the pulse labeling results were that the cells treated with 5AU are either held up late in the S phase, or possibly very early in the G_2 phase. If the latter postulation was correct, on first reflection one would have expected to see a buildup of G_2 cells in arrest as they would not have been labeled and would have been measured in this experiment. No cell arrest in G_2 was observed which tends to confirm the conclusion of Scheuermann and Klaffe-Lobsien (1973) that cells are held towards the end of the S phase.

However if cells very early in the G_2 phase that have just completed synthesis are being held up, they would in fact be labeled in these preparations and therefore would not have been measured which would still account for no G_2 arrest being found.

A rather complicated and time consuming procedure is required to measure the labeled S phase cells in order to determine their DNA content. Map type photographs must be taken so that individual cells can be relocated, then the silver grains are bleached from the preparations. The slides are re-examined and DNA density measured, using the map to determine which were the previously labeled cells. This could provide further information in a repeat experiment if at least 100 labeled and 100 unlabeled cells were measured.

3-08 Reversal Experiments

The addition of pyrimidine base precursors to 5AU solutions is a different approach in gaining further

understanding of the effects of 5AU on the lowering of the MI and synchronization of cell division. Reversal experiments have previously been carried out by Prensky and Smith (1965) and Jakob and Trosko (1965). The former authors studied the lowering of the MI using 1.6×10^{-3}M (200 ppm) 5AU and equimolar bases, sampling primary root meristems at the end of 24 hours treatment. Jakob and Trosko (1965) studied the synchronization effect by giving a 12 hour 5AU treatment (3.93×10^{-3}M) then sampling over the following 15 hour recovery period.

My experimental design incorporated both these aspects as well as sampling after 12 hours 5AU treatment as this time was common to both experiments. A mixture (20 mM) of five of the bases (excluding thymidylic acid), was also used to investigate if there was a synergistic effect that would otherwise not have been noticed.

Two slides were counted per treatment with at least 5000 cells examined per slide and the mean taken. The results are recorded as number of mitotic figures seen per 5000 cells and are compared with controls, Table 8.

After 12 hours treatment deoxyuridine and the mixture showed the only significant increases in the number of mitotic figures above the controls. The higher number of mitotic figures in the mixture could have been due to the deoxyuridine, but in combination with other bases its effect was decreased. The 24

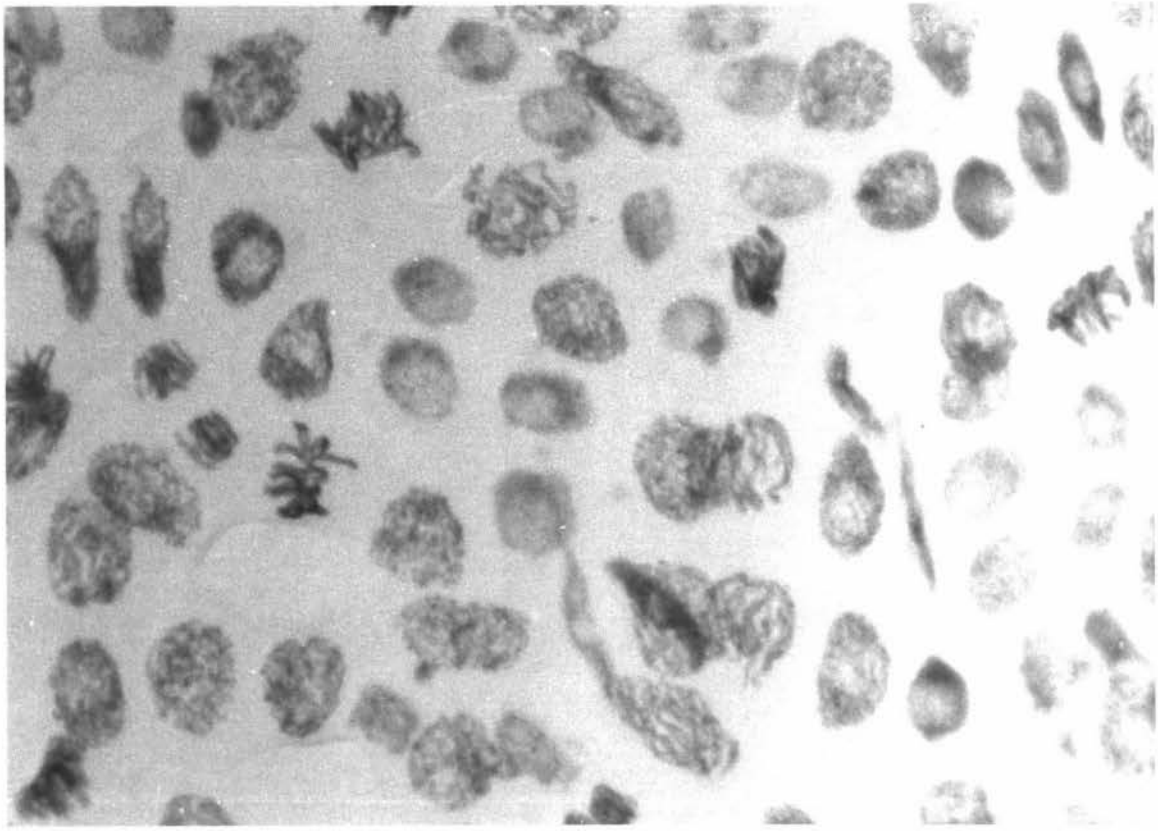
Table 8: Mitotic figures seen per 5000 cells in the reversal experiments.

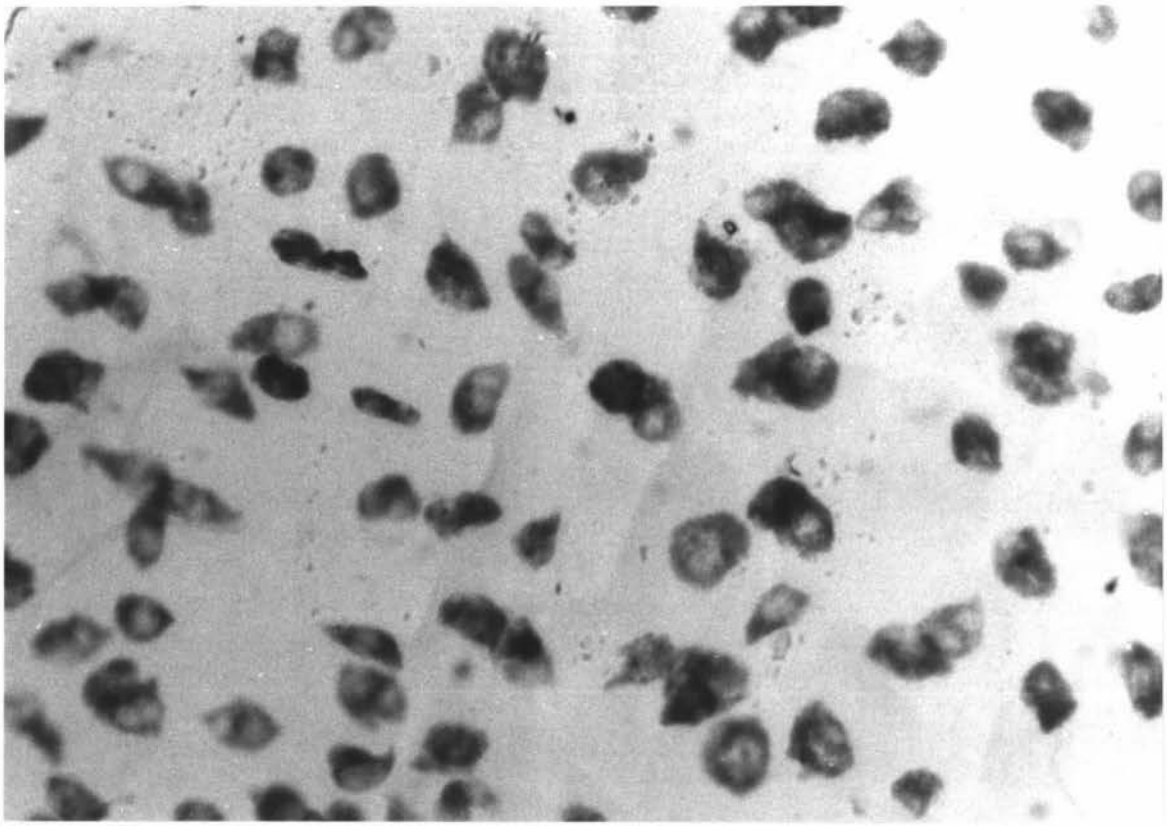
	24 hours	12 hours	12 hours treatment and 12 hours nutrient
deoxyuridine	55	151	257
uridine	32	17	748
uracil	133	17	83
thymine	2.1	7.5	240
thymidine	2.1	2.7	80
mixture	962	83	214
thymidylic acid	29		
Control (5AU only)	2.0	3.8	225

hour treatment produced different results. Thymine and thymidine were the same as the control. Deoxyuridine, uridine and thymidylic acid increased the number of mitotic figures 15 to 20 fold. The effect of deoxyuridine had diminished since the 12 hour sample. Uracil produced an increase in the number of mitotic figures 65 times the control which had increased 8 fold since the 12 hour sample. The mixture caused the most dramatic alteration increasing the number of mitotic figures seen 480 times, (Figure 16). Again it is clearly a combination of bases that can overcome the effect of 5AU, with uracil individually being the most successful. The results of Prensky and Smith (1965) did not agree with those mentioned above. They found that thymidine and thymidylic acid completely reversed

FIGURE 16 : High mitotic index seen in reversal experiment after 24 hours treatment with 500 ppm 5AU plus a mixture of pyrimidine bases. (x 640) (See text for full details)

FIGURE 17 : 'Damaged' nuclei seen after prolonged treatment with thymidine in recovery after 12 hour 5AU treatments and reversal experiments. (x 640)





the effects of 5AU, while uracil did not. They commented that their observations were only general and there is the possibility that primary meristems react differently to lateral roots at different 5AU concentrations.

The 12 hour treatment followed by 12 hours recovery in nutrient solution produced yet different results again. This time deoxyuridine, thymine and the mixture did not affect the expected peak of divisions. Uracil and thymidine showed inhibitory effects on the synchronized cell division by more than halving the number of mitotic figures seen in control slides. Uridine more than trebled this value. The peaks of division in Jakob and Troskós(1965) experiments were the same as if 5AU had been given alone. Uridine was only used in combination with thymidine which could possibly have been the reason no change in frequentcy of mitotic figures was observed compared with my results where it was used alone and markedly increased control values.

Examining the results generally, deoxyuridine and uracil cause the most change to the observed number of mitotic figures after 12 and 24 hour treatments in combination with 5AU. Possibly these are the bases causing increased values in the mixture too. These bases could be having a specific effect acting at a site possibly related to nucleic acid synthesis enabling cells to complete DNA synthesis and continue through to division. The high concentration (20 mM) of the

mixture cannot be excluded as a possible reason for the increased number of mitotic figures observed in these treatments. The increase in the number of cells dividing caused by uridine after the cells had had 12 hours to recover from 5AU and uridine treatment is also very interesting. If the experiment of Jakob and Trosko (1965) showing that DNA synthesis is slow to recover after a 12 hour 5AU treatment, is considered at this point, it could be argued that the presence of uridine prior to the removal of 5AU, enhances the DNA synthesis after the removal of the thymine analog. It would be interesting to follow cells into division after a 24 hour treatment with 5AU and uridine. Thymine was the only base not to cause any alterations in the observed numbers of cells in division as compared with controls.

These observations could possibly be explained by considering the delicate balance of purines to pyrimidines required for DNA synthesis. The enzyme aspartate transcarbamylase controls pyrimidine synthesis and 5AU as a pyrimidine analogue in excess, would be expected to alter the regulation of the enzyme and reduce synthesis. Purine synthesis would continue unaffected. In the absence of exogenous bases, excess purines promote pyrimidine synthesis (Mahler and Cordes 1971), which could explain the eventual recovery of cells in the presence of 5AU. The addition of other bases, singularly or as a mixture, could further alter the balance of purines to pyrimidines resulting in continued DNA synthesis.

These results are very encouraging and this new approach is well worth further investigation which could provide further clues into how 5AU achieves a lowering of the MI and the synchronization effect.

In the 12 and 24 hour thymidine and 5AU treatments, samples had nuclei that appeared 'damaged' compared with controls (Figure 17). This phenomenon was also apparent after four hour treatments with labeled and 'cold' thymidine used in experiments described in section 3-051. This abnormality was not apparent after 12 hours treatment followed by 12 hours recovery.

3-09 Evidence for the Quiescent Centre

Radioactivity was fed continuously to two plants for 44 hours (2 uc/ml H^3-CdR). At the end of this time tissue was fixed then either squashed or embedded and sectioned at 6u for examination after autoradiography, of silver grain distribution. Heavy label over most cells was expected but was not seen. Whether these happened to be two plants that did not incorporate label as Davidson (1968) has found can occur, or whether my experimental mehtod was at fault is difficult to explain. Rasch et al (1967) could show a LI of 95% after a 24 hours pulse. These authors used H^3-TdR and renewed the radioactive solution twice daily. This latter point could explain the lack of label in my tissue. The existence of the quiescent centre was

evident after only 30 minutes pulsing with H^3-TdR in tissue treated for 3 hours in 5AU (Figures 18 and 19).

3-10 Cell Cycle Duration

There have now been several studies on the duration of the cell cycle in Vicia faba. However all these studies (Howard and Pelc, 1951; Howard and Dewey, 1960; Wimber and Quastler, 1963; Evans and Scott, 1964; Grant and Heslot, 1966; Van't Hof, 1967; M^{c}Leod 1968) have provided us with a large variation in cycle times. This preliminary study using caffeine was designed to add to our present knowledge, but needs refining after the initial data have been obtained, due to the arbitrary times chosen for pulsing which were derived as averages of data from the literature mentioned above.

Caffeine was used in parallel with H^3-TdR and H^3-CdR pulse labeling in an attempt to define the duration of the G_1, S and G_2 phases of the cell cycle in Vicia.

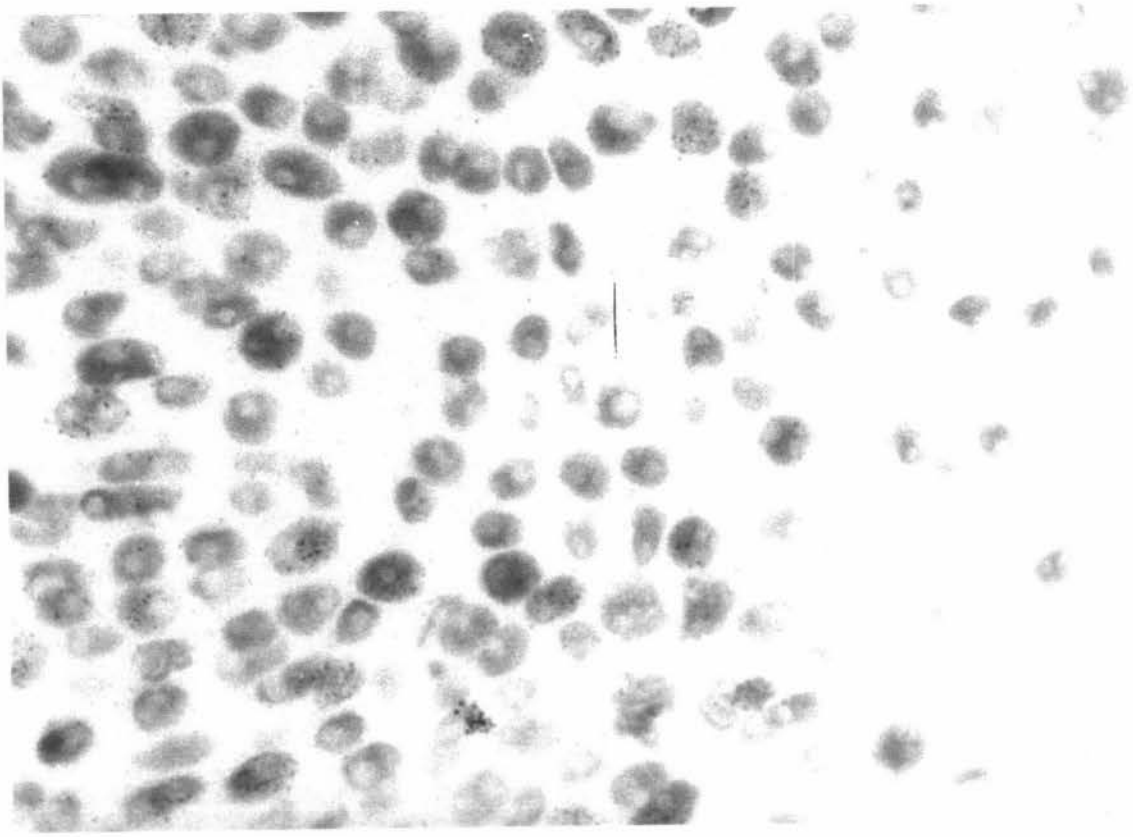
A) Plants were treated with caffeine for one hour then returned to nutrient solution and sampled 16, 17, 18 and 19 hours later.

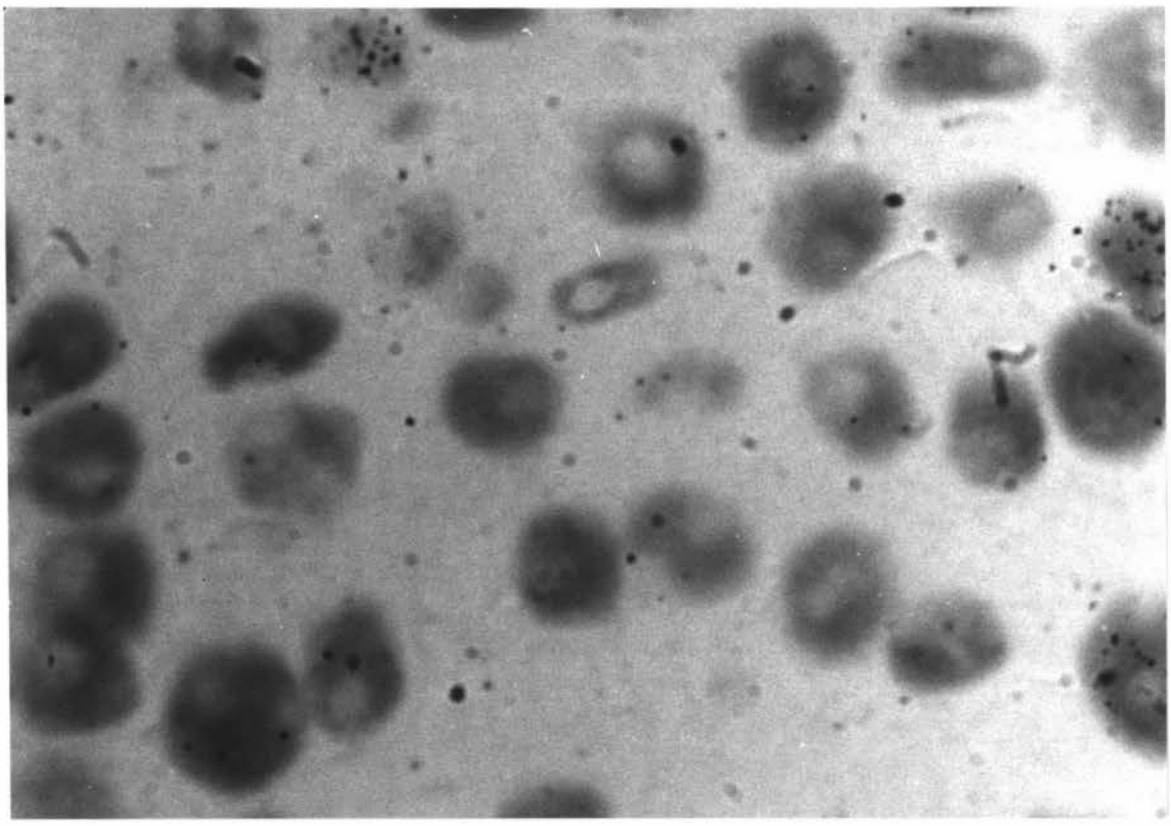
B) Plants were pulsed 4, 4½ and 5 hours after caffeine treatment to catch the beginning of the S phase of the binucleate cells and thus determine the length of the G_1 phase.

C) Plants were pulsed 12½, 13 and 13½ hours later

FIGURE 18 : Longitudinal section of pulse labeled tissue (4 uc/ml H^3-TdR) showing the unlabeled cells of the quiescent centre behind the root cap. (x 640)

FIGURE 19 : Close up of the quiescent centre showing a few labeled cells in the corners surrounding the quiescent cells. (x 1600)





(assuming G_1 to be 4.5 hours) in order to find the end of the S phase of the binucleate cells (ie when no more cells are labeled).

D) The plants treated in C) were sampled into mitosis 4, 4½ and 5 hours later in order to determine the length of the G_2 phase.

The times gained in B), C) and D) can then be added together and compared with that obtained in A).

Data could not be obtained from this experiment as it became very dubious as to whether one was seeing binucleate cells or not. The use of Nomarski Interference Microscopy with Feulgen stained preparations did not make the cell walls any clearer to distinguish. Other authors,(Diez et al 1976) used acetic orcein preparations but this was avoided due to the time factor of preparation, and possible interaction with the nuclear emulsion. It will have to be investigated further if more definite results are to be obtained.

3-11 Growth Inhibition

It was noticed during the course of 5AU treatment that if the lateral roots of Vicia faba were only just 2 cm long when transferred to 5AU solution their growth was stunted compared with control roots, and the plants did not look very healthy. Was 5AU preventing cell elongation as well as lowering the MI?

Longitudinal sections of root tips treated for various times in 5AU were examined and cortical cell

size measured near the quiescent centre and further back (1.5 to 2 mm) from the root cap. It was found in all treatments, (Control and 3½ up to 48 hours continuous 5AU), that cells averaged 13.6 microns long near the quiescent centre but enlarged to an average of 21.8 microns long, 1.5 to 2 mm further up the root.

When average cell lengths for each time are compared (Table 9) the cell size near the root tip increases gradually, then declines and gradually gets larger again suggesting that 5AU is preventing cell

Table 9: Average cell lengths in microns at two positions in the root tip during different lengths of 5AU treatment.

TIME (hrs)	CONTROL	3½	9½	12	15½	18½	21½
TIP	11.4	14.7	14.1	12.5	13.1	13.6	14.1
TOP	19	19.6	20.4	25.6	24.5	21.8	20.1

TIME (hrs)	24½	30	34	38	40	42	48
TIP	13.6	13.6	13.1	13.6	13.6	13.6	12.2
TOP	24.5	26.7	24.5	24.5	27.2	27.3	27.2

division in this region for a few hours, then cells divide and become smaller again. It is interesting to note that in all cases 5AU treated cells were longer than the controls. Looking further back the root tip

a similar trend is evident. All cells are longer than cells in the same area of control tissue but cell size fluctuates with length of 5AU treatment. By 24½ hours the cells are 10.9 microns longer than controls. This could be explained by an extended treatment of 5AU such that the cells continue enlarging. This helps confirm that the effect of 5AU is specific to division rather than general inhibition of metabolism which would also include elongation. This needs to be tested further. Figure 2 of a longitudinal median section through a root grown in nutrient solution shows the different cell size and gradual increase in length as the cells move away from the meristematic region.

The absence of growth that I observed in young lateral roots must have been due to cells being affected by 5AU further back in the root in the true elongation region. Consequently these observations need not be considered as possibly affecting the meristem cell cycle in the first 2 mm of root tip.

Duncan and Woods (1953) found that roots grew less than half as fast in a solution of 50 ppm 5AU compared with distilled water over 48 hours. Addition of equimolar thymine resulted in growth only slightly slower than in distilled water, while folic acid enabled the plants to grow faster than controls. However these compounds did not improve the MI.

DISCUSSION

5-aminouracil was first found to have a biological effect by inhibiting growth of different species of bacteria (Puleston et al, 1955; Hitchings et al, 1950). The inhibition could be reversed by the addition of thymine or uracil to the growth medium. It was concluded that 5AU halted DNA synthesis. Woods and Duncan (1952) and Duncan and Woods (1953) published the first reports on the effect of 5AU on higher organisms using Allium cepa root tips. The most apparent cytological effect was severe inhibition of cell division in the presence of a range of 5AU concentrations up to 3.93×10^{-3}M. The inhibition was not immediate after the immersion of roots in the thymine uracil analogue. The MI gradually declined until it reached almost zero after 12 hours treatment. These authors concluded from photometric determination and MI that 24 hours of 5AU treatment prevented doubling of DNA content, whereupon divisions ceased.

It is with this background that Smith et al (1963) using Vicia faba were able to show that a partial synchronization of the cell cycle occurred after a 24 hour treatment with 5AU (700 ppm), with a peak of divisions occurring 14 hours after removal from 5AU. They suggested that this synchronous division depended on the removal of roots from 5AU to nutrient solution to allow DNA synthesis to recommence after it had been halted during treatment. Pulse labeling with H^3-TdR at intervals after 5AU treatment resulted in higher

incorporation in those tissues that had been removed longest from 5AU. These authors suggested this as evidence for the progressive recovery of the DNA synthetic rate following release from the inhibiting effects of the chemical. However they were mislead as label incorporation is only an indication of DNA synthesis and density of label does not indicate rate of synthesis. In their view all the cells that were held up at the beginning of the S phase all go through DNA synthesis together and subsequently enter division together 14 hours after removal from 5AU. If this explanation is correct I would have expected a MI higher than the 42% observed after a 24 hour 5AU treatment, if DNA synthesis of all cells was halted.

My experiments (Figure 6A) confirmed the partial synchronization effect of 5AU but my results and those of others (Jakob and Trosko, 1965; Mattingly 1966a) lead to a different interpretation and will be mentioned in more detail later.

In 1965 Jakob and Trosko examined the phenomenon of partial synchronization caused by 5AU. They found that a 12 hour, 4mM treatment and a 2 hour, 12 mM treatment were similarly effective in inducing synchrony, even though the MI had not been affected by the 2 hour treatment and was still identical to the control but dropped to zero by 3 hours after removal from 5AU. Therefore it did not appear that 5AU worked by only accumulating cells at say the beginning of the S phase during treatment. Consequently these authors designed

an experiment to investigate if 5AU even blocked the cell cycle. Peaks of synchronous mitoses were observed in the presence of continuous 5AU which indicated that 5AU did not completely block the cell cycle and the synchronization effect was not completely dependant on the removal of roots from 5AU.

My duplicate experiment (Figure 6B), also confirmed that cell division could occur in the presence of 5AU, but because the peak of mitoses occurred at the same total time as that after the 24 hour 5AU treatment, I did another experiment (12 hours 5AU treatment) to investigate how significant the removal of the plants from 5AU was, to the result obtained. The shift of the peak of divisions by 12 hours and the occurrence of a second peak at 26 hours after removal from 5AU (Figure 6C) clearly indicated that the removal of the plants from the chemical definitely stimulated the cells to enter mitosis 12 hours later, but they would have overcome the effect of 5AU anyway in another 12 hours, (the reason for the second peak), showing that synchronization is not completely dependant on removal from 5AU as earlier workers thought, but this removal does stimulate the observed synchronization.

Because cell divisions occurred in the presence of 5AU, DNA synthesis must have also continued at some stage, if not all the time. My results indicated that DNA synthesis occurred throughout 5AU treatment (pulse labeling experiments section 3-05) which confirmed the results of Jakob and Trosko (1965).

It is of interest to note at this point that 5AU definitely halts DNA synthesis in mammalian cells with a block of the majority of cells at the onset or very beginning of the S phase, although the actual mode of action is unknown, (Chu 1965).

The partial synchronization of mitoses obtained by pretreatment with 5AU has been utilized by Prensky and Smith (1964) to study chromosomal proteins through successive cell division cycles in Vicia faba. These authors were prompted to further investigate the effects of 5AU on cellular activity because of its practical value in utilizing such information for other studies of cellular metabolism. By using a double labeling procedure involving a H^3 label followed by a C^{14} label, they were able to show that very few cells left the S phase during 5AU incubation. Prensky and Smith (1965) had no evidence that 5AU affected the G_1 phase but they did find a delaying effect on the G_2 phase as was shown in section 3-032.

Socher and Davidson (1971) postulated an S-G_2 transition where cells were held up during 5AU treatment, but they stated that it would be difficult to identify a cell as being at the transition. The 'transition' time was decided upon after following the frequency of cells entering and leaving mitosis over 6 hours of 5AU treatment as the progression of cells in G_2 at the time of treatment was not inhibited.

The experiment concerning the effect of 5AU on G_2 carried out by Wolff and Luipold and confirmed by

Mattingly (1966a) discussed in section 3-032 has been interpreted as a slowing down of cells in G_2 due to the 5AU. Socher and Davidson (1971) only followed the progress of cells for 6 hours. Possibly this was not long enough to examine cells that were less than half way through G_2 at the beginning of treatment. Cells that had only been in G_2 30 minutes to 1 hour could have been affected by 5AU but the duration of the experiment was not long enough to find if this had in fact occurred. A new interpretation of the Wolff and Luipold experiment could be that 5AU only affects cells very early in the G_2 phase rather than the G_2 phase generally. To test this a longer pulsing time would be required to allow labeled cells to progress well into the G_2 phase before treatment.

More recently Scheuermann and Klaffe-Lobsien (1973) have compared 5AU with FUdR as it was thought that these two chemicals had a very similar effect. They both caused chromatid aberrations and FUdR was known to inhibit DNA synthesis by blocking the thymidylate synthetase. The above authors confirmed the work of Jakob and Trosko (1965), that 5AU does not completely inhibit DNA synthesis and cannot be reversed by thymidine, as well as make a more precise study of the influence of 5AU on the S and/or G_2 phases.

As has been previously mentioned these authors found a 5AU-induced accumulation of cells approximately in the last third of the S phase. They associated these results with late replicating heterochromatin and

postulated different DNA polymerases and/or synthesis erzymes for euchromatic and heterochromatic DNA which could be influenced by 5AU in different ways. This idea will be discussed in more detail later.

In the pulsing experiments using H^3-CdR and H^3-CdR (section 3-051) where four and six hour pulses were used to detect DNA synthesis, the expected peaks of divisions did not occur during the sampling times. 'Cold' thymidine similarly effected the cells. A few dividing cells were present in the 42 hour samples along with a large number of preprophase cells suggesting that a peak of divisions was going to occur in the next hour or so. The low number of mitotic figures compared with the control in the reversal experiment 12 hours 5AU plus 12 hours in nutrient (Table 8) was interpreted as an inhibitory action of thymidine. If sampling had continued, a shift in the peak of divisions may have been found. It seems that the thymidine and deoxycytidine administered for several hours delayed the progress of cells.

Yang et al (1966) working with Chinese Hamster cell lines found that thymidine caused synchrony of cells, even at low concentrations of 1 to 2 mM. The degree of synchronization varied with different cell lines. These authors also observed chromatid aberrations and concluded that these must have been due to growth in an unbalanced pool of deoxyribonucleotides. This could have been the cause of the misshapen cells in my experiments (Figure 17) after extended treatment with thymidine. Bostock et al

(1971) confirmed the work of Yang et al (1966) finding that during the thymidine block, cells apparently move slowly through the S phase, with cells entering the G_2 phase at a low rate.

Independantly Xeros (1962) and Barr (1963) studied the effect of deoxyribosides on mitosis. They found that thymidine caused a block and synchronized the cells; Xeros used 2 mM thymidine, and Barr commented that concentrations greater than 10^{-5}M were sufficient. The latter author stated that it appeared that a phosphorylated derivative of thymidine inhibits the conversion of the ribotide, cytidine-5'-phosphate, to the corresponding 2'-deoxy-compound, which resulted in partial synchronization, by affecting the progress of cells into division.

These findings make it clear that H^3-TdR should not be used for long periods of pulse labeling in conjunction with 5AU synchronization studies, so that in later experiments only short pulses of H^3-TdR (15 to 30 minutes) were used. The action of H^3-CdR needs closer examination.

Prensky and Smith (1965) found that TdR reversed the effect of 5AU and consequently used H^3-CdR in all their labeling experiments to avoid any possibility of this occurring. My results from both pulse labeling and reversal experiments have shown that it is extended treatments of these pyrimidine bases that prolong the appearance of expected cell divisions, rather than reverse any effect of 5AU that the above authors found.

The measurements obtained using the Vickers M85 Integrating Microdensitometer showed that there was a gradual buildup in the number of cells with a 4C DNA content by 36 hours of continuous 5AU treatment. Because the G_1 phase is not affected by 5AU (section 3-03) this indicated that the rate of DNA synthesis must be slowed down, causing a buildup of cells. This slowing down of cells in the S phase results in the partial synchronization of mitotic figures after removal from 5AU.

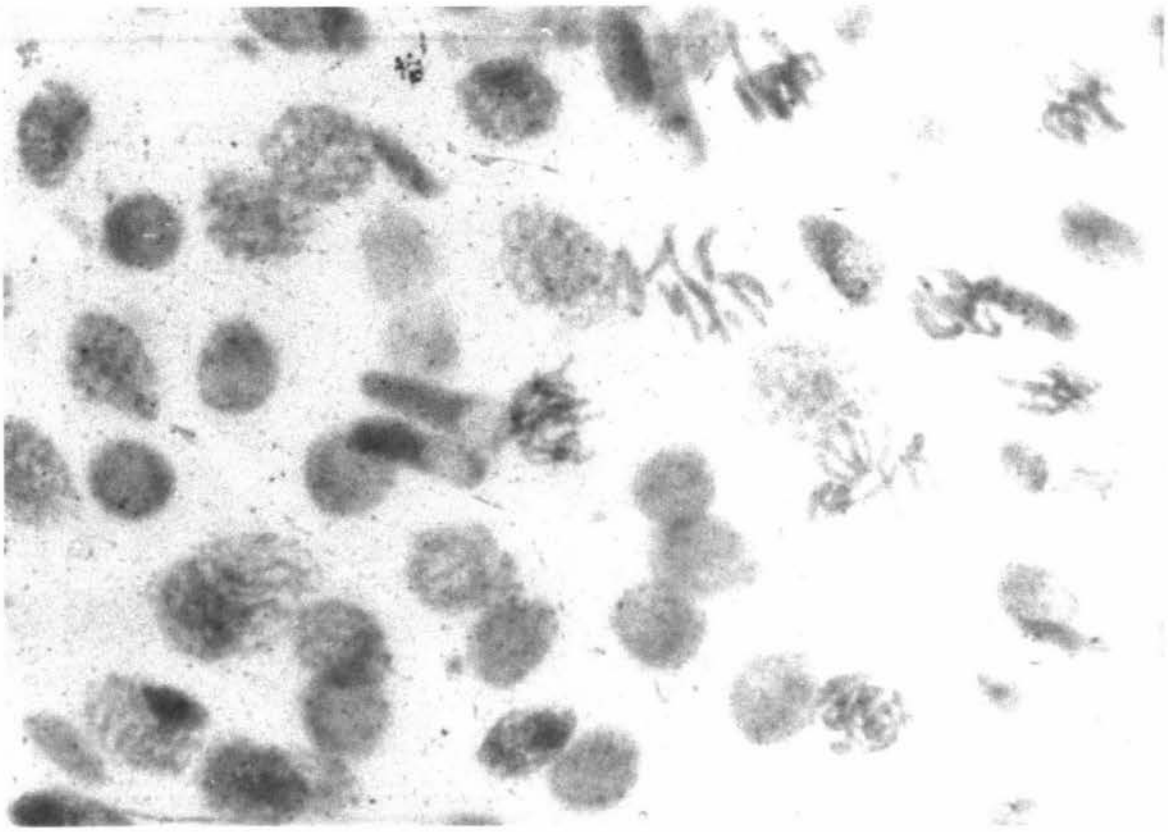
The buildup of cells in the latter third of the S phase in the presence of 5AU tends to contradict the results gained in section 3-032 examining the effect of 5AU on the G_2 phase. During an hours H^3-TdR pulse all cells in the S phase would have been labeled, as well as those that passed from S to G_2 during the hour, but after 11 hours sampling no labeled mitotic figures were seen. This could be explained by 5AU halting the cells in early G_2 during the 11 hours of sampling. It is not known whether these cells would have eventually entered mitosis. DNA density measurements taken of unlabeled G_1 and G_2 cells gave no indication of arrest of cells in the G_2 phase. However the samples were very small and possibly a more detailed study using this method could provide further important information. Scheuermann and Klaffe-Lobsien (1973) who found a build-up of cells towards the latter third of the S phase during continuous 5AU treatment could not reconcile the evidence of Wolff and Luipold concerning G_2 arrest with their results.

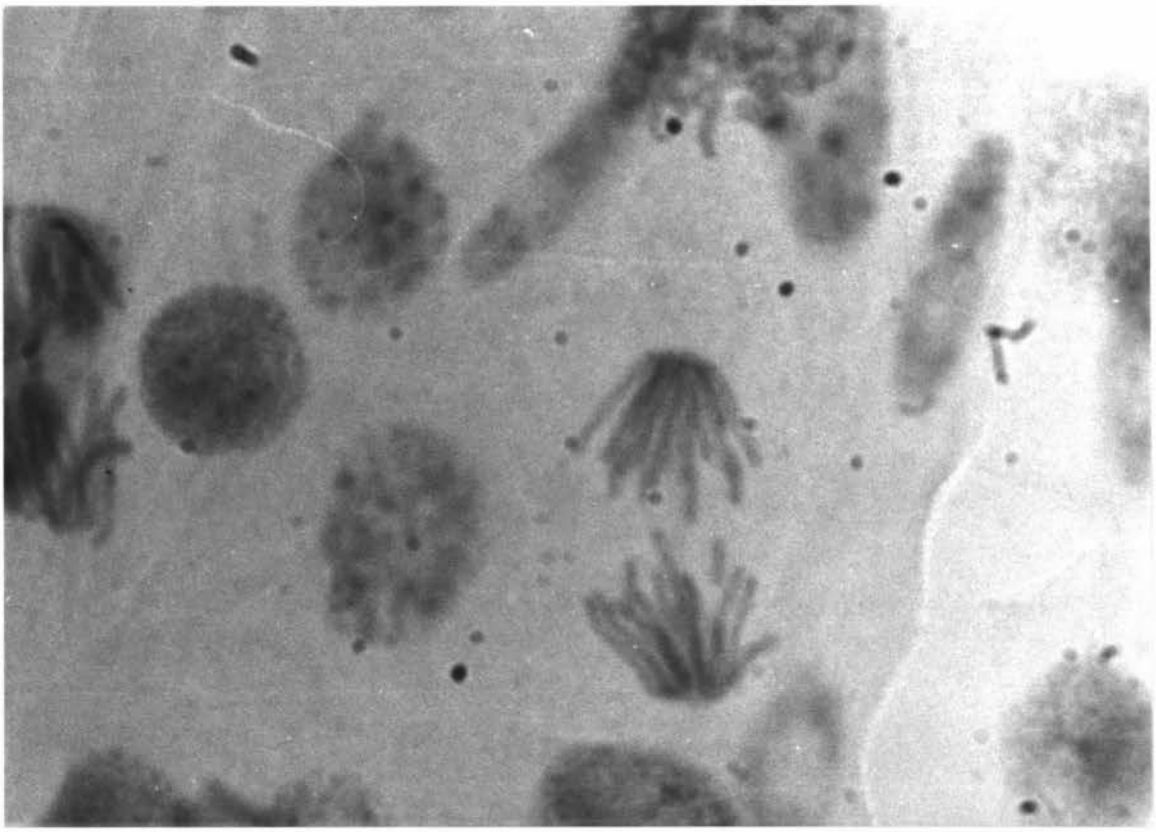
Not long after 5AU had been found to prevent DNA synthesis (as was thought at that time), Martinez-Pico and Duncan (1955) reported Feulgen-negative regions visible in anaphase chromosomes and in some metaphases after treatments of up to 24 hours with this thymine analogue (50 ppm). The number of regions was much higher than observed in control tissue but their frequency declined after a reasonable recovery period. I also observed Feulgen-negative regions in tissue treated with 500 ppm 5AU at a frequency considerably higher than controls. Examples of these regions are illustrated in Figures 20 and 21.

To explain these observations we must consider what is known about how DNA synthesis occurs in chromosomes. Stubblefield (1973) calculated times of DNA replication for mammalian cells. For Chinese Hamster diploid fibroblasts which have 22 chromosomes the length of the DNA ranged between 22 cm in the largest chromosome to 22 mm in the smallest. With DNA replication proceeding at 2 um per minute Stubblefield calculated that it would take 110,000 minutes (76.4 days) to replicate one DNA molecule of a large chromosome if DNA synthesis proceeded from one end to the other. But DNA synthesis only takes approximately 400 minutes and must occur simultaneously at many sites in one chromosome. This means there must be a large number of sites and it is known that these operate in sequence. Taylor (1974) has suggested that these smaller functional subunits of DNA (replicons) are first duplicated

FIGURE 20 : Feulgen negative regions visible in chromatids after 15 hours continuous 5AU treatment. Present in at least 50% of the nuclei. (x 640)

FIGURE 21 : Feulgen negative regions of chromatids visible in control tissue in much smaller numbers. (x 1600)





then joined together by ligase activity and that this joining is possibly delayed until the chain growth of each unit is essentially finished.

FUdR causes chromosome aberrations and 5AU has been compared with this chemical (as was mentioned earlier) because of this. Kihlman (1966) has stated that the breakage of DNA is not associated with DNA synthesis occurring during the S phase. Kihlman (1966) suggested that 'if DNA synthesis was involved at all it could be of a quantitatively and qualitatively different type occurring during G_2. It may be relevant that two different DNA synthesizing enzymes, the replicative and the terminal nucleotidyl transferases have been found in nuclei of mammalian cells (Krakow et al, 1962; Keir and Smith, 1963).' He continued to say that 'perhaps chromosome replication is completed in G_2 by a limited DNA synthesis of the terminal type, too small to be detected by autoradiographic methods.'

Possibly 5AU affects both the latter S phase and early G_2 phase but because cells are followed through the S phase in the DNA density measurements any hold up in G_2 would be disguised. Perhaps only an experiment specifically examining the G_2 phase as in section 3-032 is able to expose this phenomenon.

The Feulgen-negative regions or 'gaps' in DNA observed in chromatids after 5AU and FUdR treatment have been described by Taylor et al (1962) as representing single strand breaks in the DNA double helix produced whenever a replicative unit is unable to

finish DNA synthesis which could be due to the presence of an analogue. Complete fragmentation of these gaps could result from the 'torsions and tensions produced by chromosome coiling at prophase and anaphase.' The recent new theory of DNA not being a helical structure (Rodley et al, 1976) reduces the requirement for a large amount of tension in the condensing of the chromosomes, and therefore may not explain these 'gaps'.

Martinez-Pico and Duncan (1955) stated that the Feulgen-negative regions could be considered segments of chromosomes where interference in pyrimidine metabolism was critical. They also stated that these regions had definite locations - in or near regions which other authors had identified as heterochromatin.

It is very interesting to note that Scheuermann and Klaffe-Lobsien (1973) reached the same conclusion that 5AU was affecting the synthesis of the heterochromatin region of DNA. This was because heterochromatin is synthesized in the latter stages of the S phase in Vicia faba (Evans 1964; Taylor 1974), and they found the buildup of cells treated with 5AU occurred at this same time in the S phase.

Deufel (1950) (quoted by Oehlkers, 1952) studying the specific effect of $AlCl_3$ on Vicia faba DNA found that all breaks in a certain region of the two large chromosomes had taken place in such a way that the nucleolar organizing body in all cases remained connected with the fragment, and in telophase each fragment organized a new nucleolus 'which it never does under

any other conditions.'

Resch and Schroeter (1969) did a study of the interphase nucleus when Vicia faba tissue was treated with 5AU. They observed that the nucleus lost its normal density and was comparable to the behaviour of normal chromatin in early prophase. They thought that the G_2 phase may have been affected by 5AU.

The 42 hour preparations of continuous 5AU treatment in my experiments were noticeably blotchy which seems to be a similar effect to the observations of the above authors. The nuclei had more than one nucleolus which were hardly stained due to the Feulgen DNA sensitive stain used. Could 5AU be causing the same phenomenon as Deufel (1950) observed using $AlCl_3$? Evans (1964) stated that the heterochromatin in Vicia faba DNA is distributed between all 12 chromosomes but the largest blocks being located on either side of the centromere of the single pair of metacentric chromosomes (1 pair) and just proximal to or at the midzone of the acrocentric chromosomes (5 pairs). Martinez-Pico and Duncan (1955) observed the Feulgen-negative regions most commonly proximal to the centromere.

On considering these different aspects of the effects of 5AU it appears that this chemical definitely effects DNA synthesis and particularly heterochromatin synthesis, but does not completely halt cells in the S phase. The continued DNA synthesis in the presence of 5AU indicated by pulse labeling experiments (section 3-052) could be the unaffected synthesis of euchromatin

which occurs early in the S phase in a number of places at once even within a single chromosome, (Evans 1964).

If all the requirements to complete DNA synthesis including joining, are extended into the G_2 phase as Kihlman has suggested, this could feasibly be where 5AU is acting. This could be by preventing some ligase activity (possibly only one enzyme) which results in Feulgen-negative regions when the cells eventually enter mitosis. Possibly a different longer pathway has to be followed to get the same end result which would explain the delayed peak observed in continuous 5AU treatment. The suggestion that 5AU affects the $S-G_2$ transition put forward by Socher and Davidson (1971) can also be encompassed in the above explanation.

The results from the reversal experiments have indicated that some of the bases (especially uracil and deoxyuridine) could be important as positive effectors for specific enzyme mobility or function important in the final stages of DNA synthesis. 5AU somehow prevents their action but when the bases are in excess they are able to overcome this effect and in fact stimulate cells to divide at a much higher frequency than controls. Further work is required before these findings can be elaborated upon.

The effect of 5AU on DNA synthesis has been discussed but does this uracil analogue affect RNA synthesis? Jakob (1968) asked this question after previously studying 5AU's effect on DNA synthesis as

a thymine analogue. He found no evidence that 5AU is incorporated into RNA. There was a reduction in the rate of RNA synthesis during 5AU treatment but Jakob thought that this may have reflected a generally reduced rate of metabolic processes. These quickly returned to normal when 5AU was removed. Woodard et al (1961) reported G_1 to be a period of relatively high rate of RNA synthesis which is presumed necessary for the beginning of DNA synthesis. A complete block in RNA synthesis would be incompatible with the normal progress of cells from G_1 to S which has been confirmed by Mattingly (1966a).

All my investigations into the appearance of 2 peaks of cell division after a 12 hour 5AU treatment indicate that it does not seem to be the same cells dividing twice causing the second peak, although there is no clear evidence, such as different geographic distributions within the root. The delay between the appearance of mitotic figures could be enough time for the first group of dividing cells to have moved out of the first 2 mm such that the second peak is due to a new group of cells distributed through the same area as the first cells were initially. There could possibly be different 'states' of cells that move through the root away from the apex and it is the characteristics of these 'states' that are difficult to decide.

However other workers have found evidence for more than one population of cells in the meristem. Webster and Davidson (1968) pulsing with H^3-TdR, followed the

appearance of labeled mitotic figures and found evidence for one population which consisted of 75% of the cells with a mean cycle time of 14 hours. There was a second population of cells (the remaining 25%) that had a longer cycle time that could not be measured directly. The overall cycle time is much longer. It seems that it is this average time of the two populations that most people measure and would explain why the literature varies so much on this point, (Table 10).

Table 10: Variation in cell cycle times for Vicia faba in the literature. (from Pollister, 1969)

TOTAL	G_1	S	G_2	M	Published by:
-	-	6-8	-	-	Howard and Pelc 1951
30-18	12-2	14-4	8	4-2	Howard 1956 Howard and Dewey 1960
17	1	10.5	2.5	3	Wimber and Quastler 1963
13	-	-	-	-	Van't Hof and Sparrow 1963
19.3	4.9	7.5	4.9	2	Evans and Scott 1964
18	4	9.0	3.5	1.9	Van't Hof 1967
16.6	3.6	8.3	2.8	1.9	MacLeod 1968

Gray and Scholes (1951) showed the mean cycle time for the first 3 mm of root was 25 hours, but that for the apical 1.5 mm was 19 hours. My work has only examined the first 2 mm of root tip and this could be why a distinct second population of dividing cells was never found. The use of the thymine analogue

5AU could affect the cells in some unknown way thus altering the cell cycle compared with studies using H^3-TdR which are more common.

Socher and Davidson (1971) using 5AU were able to demonstrate the presence of subpopulations of dividing cells that differ in the G_2 duration. 85% of the population were fast dividing cells and the other 15% were slow dividing with a G_2phase in excess of 12 hours. These authors found that their results were similar to those obtained from percentage labeled mitoses after incorporation of H^3-TdR, (Webster and Davidson 1968), which suggests that my treatments with 5AU made no difference to the cell cycle (other than the observed effect on the S and early G_2 phases). In addition the 5AU treatments have shown up more clearly these two populations by causing two peaks of division 12 hours apart after the 12 hours of 5AU treatment.

Possibly my graphs with two peaks do show different pooulations: one fast and the second slower dividing: but these cells are randomly distributed within the meristem, ie they cannot be distinguished apart using longitudinal sections as in section 3-02. The small peak at 10 hours in the 24 hour 5AU treatment (Figure 6A) and the second small peak at 42 hours in the continuous 5AU treatment (Figure 6B) could be evidence for these fast and slower dividing components of the total population.

There has recently been criticism of the experimental design used to determine cell cycle times,

(Green 1976). Much of the work has consisted of pulse labeling and then examination of tissue for the appearance of labeled mitotic figures as in Webster and Davidson (1968). Gray and Scholes (1951) have shown that the cell cycle slows down a little as the cells move further away from the root tip. It must be remembered that pulse labeled cells being studied are also continuously moving away from the root apex and depending on their destination, the cell cycle changes at different rates. Therefore any cell cycle studies can only give average values for a selection of tissue types as previously the continual movement of cells has been ignored. This could be the reason for some of my unusual results involving pulse labeling followed by examination for labeled mitotic figures over the peaks of division. Possibly quite a proportion of cells originally labeled had moved outside the 2 mm meristem region by the time the tissue was fixed and were consequently not examined. I did not consider this possibility in analysing my results.

Green (1976) has put forward a new concept for cell cycle studies. Cells can be studied at a site-specific duration, which is the time required to complete the cycle if the cells involved could remain at that position for a whole cycle. The alternative is the cell-group specific cycle time which follows the duration for a specific group of cells as they move down the axis. This new concept of cell cycles in the root may in the future help explain the anomalies of

different populations of cells in the root tip.

It was found that 5AU affected root growth but that cell elongation in the first 2 mm of root tip was not affected. Duncan and Woods (1953) found that inhibition of growth could be reversed by thymine and folic acid but the MI did not change. They concluded that the reversal of inhibition of growth in length must only be temporary and only concerned with cell enlargement.

Clearly 5AU affects more than DNA synthesis but this inhibition of cell enlargement does not affect cells in the first 2 mm of the root tip. The prevention of cell enlargement could slow down the cell cycle but only in the elongation zone where cells enlarge and DNA doubles without cell division occurring.

This work forms a basis for further investigation in which a more detailed examination of how 5AU causes partial synchronization is going to require a more biochemical approach. Work is needed at the electron microscope level to try and understand the significance of the Feulgen-negative regions and proposed interference with heterochromatin synthesis. Enzyme studies may also help provide answers, although further understanding of events within the cell cycle is a prerequisite. As has been previously mentioned, a more detailed approach to the reversal experiments may provide further clues.

CONCLUSION

The aim of this thesis was to answer the question 'How does 5AU induce cell synchrony?' In order to achieve this aim, several avenues of investigation had to be followed.

It was firstly confirmed that 5AU did induce synchrony with both 12 and 24 hour treatments, with peaks of division occurring approximately 14 hours after removal from 5AU, and a second peak at 26 hours after removal in the 12 hour 5AU treatment.

5AU was originally thought to have prevented DNA synthesis and caused a buildup of cells at the G_1-S border. It has been found that DNA synthesis continued in the presence of 5AU at a reduced rate and a peak of mitotic figures was observed between 37 and 42 hours of treatment. Prior to this it was thought that the synchronous peak of divisions was stimulated by the removal from 5AU. This was clearly not correct. It was concluded that removal from 5AU did stimulate the cells to continueinto mitosis, but the cells were capable of doing this anyway after a considerable time lag. Attention then focussed on DNA synthesis and how 5AU could be affecting it.

Results from DNA density measurements and Feulgen-negative regions present in chromatids of 5AU treated tissue suggested that it could be the heterochromatin synthesis that was affected. Further interpretation of experiments suggested that early G_2 was also affected. It has been postulated that DNA synthesis could overlap

into early G_2 where enzyme activity could be important in completing the final DNA structure by the joining of previously synthesized subunits of DNA. If this was so, 5AU could somehow be interfering with this mechanism, but in a way that a certain percentage of cells can eventually overcome the action or find an alternative pathway.

These requirements could then be used to explain the original observations of synchrony. The interference with synthesis in a heterochromatin region results in a buildup of cells in the S phase. If 5AU is removed these cells are free to complete DNA synthesis and continue through to mitosis. If the tissue is left in 5AU the cells over a period of time find an alternative pathway (DNA repair?), or synthesize the affected enzymes at a very slow rate, such that eventually a peak of mitotic figures is seen, but the number of cells dividing is fewer than when 5AU has been removed.

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APPENDIX 1

STAINING PROCEDURES

Acetocarmine Squash Technique.

(modified from Johansen 1940)

Widely used as a chromosome stain especially suitable for root tips.

Fixation: Acetocarmine is a fixative and staining reagent at the same time, though better results will be obtained with some species of root tips if they are first fixed in 1:3 glacial acetic acid to absolute alcohol for at least half an hour. Store after fixation in 70% alcohol if necessary.

Staining: Slowly bring the material down to water, cover in a vial with a small quantity of iron aceto-carmine plus a drop of 1N HCl (iron acts as a mordant, the acid helps break up the tissue). Leave prestaining as such for as long as convenient (up to two hours). Proceed from here with single root tips.

Place a root tip with a liberal quantity of iron aceto-carmine and a drop of 1N HCl in a watch glass. Heat over a spirit lamp flame for 3-5 mins keeping the liquid just below boiling point. DO NOT BOIL. Replen-ish evaporated stain otherwise the carmine will precipitate and ruin the preparation. After heating, transfer the material to a clean slide and cover with a drop of fresh stain. Cut off the meristematic region of the root tip (not more than 1/4"-this region will be more deeply stained than the rest of the root),

and disregard the remaining tissue. Cut up and macerate the tissue as much as possible and disperse the cells over the approximate area of a coverslip. Cover with a coverslip and reheat gently for a further ½-1 minute. Then place the slide between blotting paper and press hard avoiding any lateral movement. Heat gently over the spirit lamp. The chromatin should be stained a deep red, the cytoplasm almost colourless.

If difficulty arises with maceration, hydrolyse the root tips in 1N HCl at 60°C for 5-6 minutes prior to staining.

Gomori's Haematoxylin

(Melander and Wingstrand 1953)

Excellent stain for nuclei and chromosomes - leads to better contrast and suitable for squashed material.

Preparation of Stain: Mix equal parts of 1% aqueous haematoxylin and 3% aqueous solution of chrome alum. Add to each 100mls of mixture 2 mls of 5% aqueous solution of potassium bichromate (= dichromate) and 2 mls of 0.5N H_2SO_4. May be used after 1 day and works for 14 days if refrigerated.

Staining: 1) Treat tips at 20°C for 25 mins with the following mixture: 4.5 parts volume glacial acetic
4.5 parts volume 1N HCl
1 part 25% formalin (formaldehyde)

(this hydrolysis can be varied depending on requirements for softening of tissue)

2) Wash tips rapidly in distilled water.

3)Stain in haematoxylin at 60°C for 40 mins

4) Place in 45% acetic acid 30 mins or more

5) Disintegrate root tips on a slide in a drop of 45% acetic acid by grinding it with the convex side of a watch glass.

6) Put on a coverslip and press so cells are successfully flattened.

The preparations can be made permanent by removing the coverslip with dry ice and passing the slides through 95-100%ethanol. (Avoid xylene). Mount in eukitt.

Haematoxylin $C_{16}H_{14}O_6$ MW 302.272

Feulgen Stain

(modified from Darlington and La Cour 1942, in Ruthmann 1970)

Preparation of Stain: Dissolve 0.5 g basic fuchsin and 0.5 g Na/K metabisulphite in 100 mls of 0.15N HCl. Shake mixture at intervals for 2-3 hours or until the dye is converted to fuchsin-sulphurous acid. Add 300 mg of fresh decolourizing charcoal and shake for at least 5 mins. Filter through hard filter paper. The filtrate should be clear and colourless - if not, repeat the last two steps. Store in the refrigerator in a light proof container.

Staining Procedure: 1) Put roots in a 1N HCl 60°C water bath and incubatefor 12 mins, then rinse in distilled water. (Hydrolysing roots in HCl removes the

RNA ,and forms the necessary aldehyde linkages).

2) Stain in leuco basic fuchsin for approximately one hour.

3) Rinse in two changes SO_2 water.

4) Transfer to running tap water for 30 mins.

Squashing Procedure: 1) Put root meristem towards one end of a slide. 2) Add a drop of 45% acetic acid.

3) Gently tease out the tissue with needles and squash carefully under a coverslip.

4) Remove the coverslip with dry ice. 5) Transfer the slide through 50 to 100% ethanol and mount permanently in eukitt.

(Step 5 is modified for autoradiography by passing the slides through to water then air drying.)

SO_2 water: 1 part metabisulphite, 3 parts 1N HCl, 3 parts distilled water. Dissolve metabisulphite in water then add acid in the fume hood.

(dry ice technique from Conger and Fairchild, 1953)

Safranin - Fast Green

(Jensen 1962)

Tissue must be prepared as described in Appendix 2.

1) Slides are put through an alcohol series to 70% EtOH then placed in Safranin (1% in 95% EtOH diluted 50:50 with distilled water before use) for 24 hours.

2) Rinse quickly in acid alcohol (1 ml 1N HCl in 100 mls 70% EtOH) then put quickly through 95% and

absolute alcohol.

3) Then immerse slides for 30 seconds in fast green (0.5% in 50% absolute alcohol, 50% clove oil).

4) Put through two washes (50% clove oil, 25% xylene, 25% absolute EtOH) then into

5) xylene - each for at least 15 mins.

Finally mount permanently in Canada balsam.

BAO Fluorescence Stain

(Ruch 1966)

1) Fix tissue in 4% formalin (½ to 6 hours) or EtOH-acetic acid (3:1, 1/4 to 3 hours).

2) Wash in water for 10 minutes.

3) Hydrolyse with 1N HCl at 60°C (conditions for maximum fluorescence must be observed).

4) Rinse with water for 5 minutes.

5) Stain in freshly prepared stain solution for 2 hours. (Mix 10 mls BAO solution 0.01% in distilled water, 1 ml 1N HCl and 0.5 ml $NaHSO_3$ solution 10%) shake and filter.

6) Wash in sulphite water, 3 changes each 2 minutes. (180 ml distilled water, 10 ml 1N HCl and 10 ml $NaHSO_3$ solution 10%)

7) Wash in water 10 minutes.

8) Embed in fluorescence free glycerine under cover-slip. Can be sealed if desired.

APPENDIX 2

DEHYDRATION, INFILTRATION AND EMBEDDING OF TISSUE

Dehydration

Procedure of Johansen (1940) using ethyl alcohol/ tertiary butyl alcohol (TBA).The process must be a gradual one as over dehydration causes brittleness and difficulty in sectioning material. Erythrocin is introduced at the 75/25% alcohol step to enable the material to be seen more clearly during embedding and sectioning. It will be displaced again when removing the wax and stepping slides to water after the material has beensectioned and mounted.

Procedure: 1) from 70% EtOH transfer tissue to 50% then 70% TBA/alcohol for 30 mins in each.

2) transfer to 85%, 95%, 100% for 2½ hours in each. 3) transfer to pure TBA - 3 changes over 24 to 36 hours.

50% TBA :	50 ml distilled water:	40 ml 95% EtOH:10 ml TBA
70% TBA :	30 ml distilled water:	50 ml 95% EtOH:20 ml TBA
85% TBA :	15 ml distilled water:	50 ml 95% EtOH:35 ml TBA
95% TBA :		:45 ml 95% EtOH:55 ml TBA
100% TBA :		:25 ml 95% EtOH:75 ml TBA

Infiltration

Passing through paraffin oil to wax being sure to allow infiltration to completely displace the alcohol in the tissue without leaving air spaces.

APPENDIX 2 Continued

1) Transfer material to TBA/paraffin (50:50) for 1 hour.

2) Half fill a vial with molten wax (56 or 60°C melting point) and allow the wax to solidify. Pour off the bulk of the TBA/paraffin from 1) and then pour the remaining liquid plus the tissue onto the solid wax in the prepared vial. Place the vial in the oven (63°C). The oil covering the tissue will protect it from heat damage, and as the wax remelts the material will sink and become progressively infiltrated with wax.

3) When wax is molten, transfer the material to fresh molten wax; repeat this transfer three times over a period of 24 hours. It is essential to remove all traces of TBA. This can only be done by changing the infiltrating wax over a minimum 24 hour period. After 3) the material is ready for embedding when convenient.

Summary: day schedule

1st day: step to 70% EtOH; leave overnight in fridge.

2nd & 3rd days: step to TBA; leave overnight; renew TBA next morning, midday, afternoon; leave overnight.

4th day: infiltrate, change wax midday and afternoon. Leave overnight.

5th day: change wax in morning; embed if desired in the afternoon.

Embedding

Embed material in 56 or 60°C melting point wax, while working near a bunsen flame.

1) Almost fill boat with molten wax and allow around

sides and bottom of boat to begin solidifying while keeping the bulk of the wax in the centre completely molten with a hot needle.

2) With a pair of warm tweezers remove the tissue, one piece at a time from the infiltration wax and place in the embedding wax . The tissue will sink to the semi solid wax and can be oriented with hot needles, keeping the surrounding wax molten. The tissue must be orientated with care - horizontal and parallel to the bottom and sides of the boat to assist with sectioning.

3) Allow the surface of the wax to cool then carefully immerse the whole boat horizontally under iced water. Allow the block to thoroughly solidify before removing from the water.

N.B. If blocks are cooled too slowly the paraffin will crystallize and the tissue will need re-embedding.

The tissue is now ready for sectioning on a microtome. Blocks of wax containing the tissue are melted to the microtome stage then neatly trimmed into a cubic shape. The stage is mounted on the microtome ready for cutting the ribbons which are in turn mounted on microscope slides.