000--1625-26

RECEIVED BY DTIC MAR 7 1969

Research Report

U. S. A. E. C.

MASTER

MITOTIC CYCLES IN MERISTEMS

D. DAVIDSON

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

Report Number: (AT(11-1) 1625-26

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

leg

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

I. Technical Progress Report	Page 1
1. Heterogeneity in Cycle Times in Lateral Root Meristems	1
Conclusions	3
2. Effects of IAA and Colchicine on Cell Cycle Times	3
i. IAA	3
ii. Colchicine	4
iii. Metaphase Accumulation: Errors in Estimates of Cycle Times Following Colchicine	4
3. Development of Primordia	6
i. Heterogeneity in Small Primordia	6
ii. Response of Large Primordia to Colchicine	8
iii. Phenotype in Large Primordia and Lateral Roots	9
iv. Loss of Polyploid Cells from Large Primordia	10
4. Anatomy of Developing Primordia	10
5. Mixoploid Primordia	12
i. The Location of Tetraploid Cells in Mixoploid Primordia	14
Summary	15
6. Microspectrophotometry of Large Primordia	16
7. DNA Synthesis in Diploid and Tetraploid Cells	17
8. The Effect of IAA on Mixoploid Meristems	18
i. Mixoploid Lateral Roots	19
ii. Mixoploid Primordia	20
9. Response to Base Analogs	21
i. 5-Aminouracil	21
ii. Chromosome Aberrations Following 5-AU Treatment	23
iii. 5-FUdR	23
10. Abnormal Nodal Outgrowths on <u>Brassica</u>	25
i. Culture Studies	26
ii. Growth in Culture	27
iii. Physiological Studies of Cultured Cells	27
iv. Auxin Levels in Nodal Tumors	28
11. Apical Dominance: Colchicine Effects	29
Summary	31
12. Effects of X-rays	31

I. Technical Progress Report

Root meristems have been used extensively in studies of the responses of proliferating cells to ionizing radiations and radiomimetic chemicals. The responses of these cells to disruptive treatments have been studied at several levels, for example, the tissue level, cell level, chromosomal level and molecular level. Vicia faba roots have been used extensively in these studies (see books by Read and by Kihlman) and in related studies of chromosome replication and cell cycles.

1. Heterogeneity in Cycle Times in Lateral Root Meristems

At an early stage of the present study it became clear that the lateral roots of V. faba are not as simple a system as had been assumed. In last year's report (AT(11-1)1625-15) our results reported in part were as follows:

Roots were exposed to solutions of ^3H -thymidine (1.0 $\mu\text{c/ml}$ Spec. Act. 3.0 c/mM) for 1 hour. Percentage labeled mitoses curves were determined over the 27 hour period following exposure to ^3H -TdR. Roots were fixed every 3 hours and 3000 cells were scored for each determination. From the curves for percentage labeled prophases, metaphases and mitoses it was found that the mean cycle time was 14.0 hours. The durations of the phases of the mitotic cycle were estimated to be:

S = 6.2 hr.

G2 = 3.3 hr.

mitosis = 2.0 hr.

G1 = 2.5 hr.

These estimates hold, of course, only for the cells that incorporated ^3H -TdR. Cells that do not become labeled do not contribute to the percentage labeled mitosis (PLM) curve. We found a significant fraction of the mitotically active

cells were unlabeled, with the result that the maximum peak of the PLM curve, which would be expected to approximate 100%, in fact never exceeded 75%.

From this result we concluded that the meristem of lateral roots of Vicia faba consist^s of one sub-population with a cycle time of 14 hours and a second sub-population with a cycle time considerably in excess of 14 hours. The ratio of these two populations is approximately 3:1; this was calculated from the ratio of the areas under the ideal and observed PLM curves (AT(11-1) 1625-15 page 5).

There is, in addition, a third sub-population of cells; these cells do not divide at all. They make up about 16% of the meristem. If we consider the meristem as a whole, mean cycle time comes out to 20 hours. This value should be compared with the mean cycle time of the fast dividing cells, which is 14 hours.

The cycle time of the slowly dividing cells is more difficult to estimate but their presence can be demonstrated. If roots are treated with colchicine (0.025%) as well as ³H-TdR, anaphase is inhibited for 27 hours and cells are induced to become tetraploid. Cells of the fast dividing sub-population appear as tetraploid cells within the 27 hours following treatment with colchicine and can be distinguished from cells entering their first mitosis after the colchicine treatment. From 18-27 hours after treatment with ³H-TdR and colchicine we found both labeled and unlabeled diploid cells in mitosis. At 24 and 27 hours, for example, 33% and 20% respectively of diploid metaphases are labeled. These cells have taken 24 and 27 hours to pass from S through G2 to mitosis. Thus, there are cells in the meristem whose cycle times clearly exceed 27 hours. The G2 of these cells, about 21-24 hours, should be compared with the

G2 of 3.3 hours in fast dividing cells. These results have been published in J. Cell Biology (Encl. 1).

Conclusions

Meristems of lateral roots of Vicia faba are heterogeneous with respect to cycle time. We suggest that the differences in mitotic cycle times of sub-populations of cells within a meristem indicates, with regard to this particular property of cells, a marked functional organization within meristems. It is also evidence of closely controlled programming of cells.

This heterogeneity within lateral roots should lead to a re-evaluation of earlier experiments in which the estimated time at which the first, second and third metaphases would appear after some treatment was based on a mean cycle time of 20-24 hours. It is now clear that some cells will complete two mitotic divisions in 24 hours while others will take perhaps 48 hours to undergo one cell cycle. These timings are particularly important for the analysis of patterns of chromosome labeling after exposure to $^3\text{H-TdR}$. Iso-labeling of sister-chromatids in diploid cells could be seen 36-48 hours after $^3\text{H-TdR}$ treatment, i.e. in cells with long cycle times in their first metaphase after labeling: non-isolabeling could be seen in diploid cells 24 hours after $^3\text{H-TdR}$ treatment, i.e. in cells with short cycle times in their second metaphase after becoming labeled.

2. Effects of IAA and Colchicine on Cell Cycle Times.

i. IAA

The results described in the previous section have enabled us to study the effects of IAA and colchicine on a population of cells with a known cycle time of 14 hours. The results were reported in detail in last year's report

(AT(11-1)1625-15 pp 7-15). They have now been submitted to press (Encl. 2). The results from experiments in which cells labeled with ^3H -TdR were also treated with IAA (10^{-5} M) for 3 hours are summarized here because they are important for the results from primordia treated with IAA (see below, section

1. Cells in G2 enter mitosis: they appear to be unaffected by IAA.
2. Cells in S at the time of treatment are delayed: the result is a fall in mitotic index.
3. The IAA-induced delay wears off by 12 hours and the S cells enter mitosis: this is shown by the rise in the percentage labeled mitosis curve.
4. Cells that pass from G1 into S during treatment are also delayed.

We had shown previously that IAA extended the duration of interphase; these results show that IAA affects the duration of the S phase.

ii. Colchicine Effects

The changes in cell cycle times induced by colchicine have been shown to occur in the interphase of treated diploid cells. This results in a rise in the number of cells entering mitosis 6-8 hours after a 3 hour treatment. We have shown that G1 cells have been stimulated; they complete interphase and enter mitosis faster than untreated cells (Encl. 2; MacLeod and Davidson, 1968, Expt. Cell Res. 52). These results and our observation that restitution nuclei appear about 2 hours after the beginning of a colchicine treatment also led us to reconsider the rate of entry of cells into mitosis following colchicine treatment.

iii. Metaphase Accumulation: Errors in Estimates of Cycle Times

following Colchicine Treatment

It has been reported that in colchicine treated roots of V. faba the rate at which metaphases accumulate is regular for 4-6 hours (Evans, et al., 1957, see Encl. 3). This was taken as a basis for an estimate of the rate at which cells normally enter mitosis. The rate observed in treated roots, however, is a true value only if: 1) colchicine has no effect on the passage of cells through interphase and 2) no cells are leaving metaphase and entering interphase. Our results show that neither assumption is valid. The evidence that colchicine speeds up the progress of cells through interphase has been described above and in press. The evidence that restitution nuclei are forming long before 4-6 hours after the beginning of a colchicine treatment is as follows:

Restitution nuclei are seen in lateral roots 2 hours after the beginning of a colchicine treatment (Encl. 1). With 0.025% colchicine we find 4.7 restitution nuclei per 1000 cells at 3 hours and 10.1 per 1000 cells at 6 hours. At 3 hours, this represents a loss of 8.25% of metaphases. Thus, estimates of cycle times based on the rate of metaphase accumulation are in error by at least 8%.

Roots treated for 3 hours and allowed to recover for 2 hours had 11.5 restitution nuclei per 1000 cells. This is a ratio of 36 restitution nuclei to 200 metaphases. Thus, 15.2% of restitution nuclei, and an error of 15% in estimates of cycle times.

The combination of a rise in the numbers of cells entering division and loss of metaphases due to restitution indicates that the rate of accumulation of metaphases is not an accurate basis for estimating cycle times. These results

are important for our understanding of the range of changes that occur in root meristems treated with colchicine. These results have been submitted to press (Encl. 3).

3. Development of Primordia

The results from studies of lateral roots (section 1) had led us to examine the sequence of morphogenetic events that occur between the initial cell divisions in the pericycle and the final emergence of a large primordium as a lateral root. Certain changes have already been described (AT(11-1)1625-15); they concern the ability of primordia to utilize exogenous ^3H -TdR. It has been found that a) small primordia (of 1500 cells or less) show little immediate incorporation of ^3H -TdR but they can store the precursor and use it over the following 10-12 hours; b) large primordia (>1500 cells) do not utilize exogenous ^3H -TdR. They do not become labeled.

The inability of cells of large primordia to incorporate ^3H -TdR has made an analysis of cell cycles by the percentage labeled mitosis curve impossible. We have studied the uptake of ^3H -deoxycytidine by large primordia and find that again labeling indices are low; they vary between 0.5 and 4%. Although little can be said of the make up of the cell populations of large primordia, our studies of small primordia have shown that even at the 1000 cell stage, heterogeneity in cell cycle time has already been established.

i) Heterogeneity in Small Primordia

Whole root systems of V. faba were treated with ^3H -TdR and some were then treated with colchicine (0.025% or 0.5%) for 3 hours. In small

primordia treated only with ^3H -TdR labeling indices, the percentages of labeled cells, increased from ~5% to ~50% over a period of 10-12 hours. However, in spite of both the ability of primordial cells to maintain a reservoir of ^3H -TdR supplied exogenously and the division of labeled cells, the labeling indices shown by small primordia never exceeded 53%. Thus, at best, only 50% of the cells in the small primordia acquire the ability to incorporate ^3H -TdR over the 10-12 hours following treatment. Furthermore, the remaining 50% of cells, which do not become labeled, must be dividing as often as the labeled cells since the labeling indices for the small primordia never exceed 53%. We conclude from this that there is heterogeneity in these cell populations with respect to the ability to use ^3H -TdR.

Small primordia also show variation with regard to cell cycle time. In roots treated with colchicine, the first tetraploid cells were seen 12 hours after treatment, showing that some cells complete one cycle in 12 hours. But diploid cells were still entering mitosis, though anaphase was suppressed, 30 hours after treatment; these cells have a cycle time somewhat in excess of 30 hours. It had previously been reported that dividing diploid cells were present in small primordia 48 hours after colchicine treatment (Davidson, 1965). Thus, with regard to cycle time, there is variation even in primordia of only a few hundred cells. This indicates that the type of heterogeneity seen in fully emerged lateral roots is established early in their morphogenesis. Such heterogeneity in the physiology of these primordial cells suggest programming that is established very early in development; the control mechanisms at work and the developmental

significance of this programming remain to be determined. The results on small primordia are now in press (Encl. 4).

ii. Response of Large Primordia to Colchicine

If whole root systems of V. faba are treated with 0.025% colchicine for 1-3 hours lateral roots will continue to emerge from the primary root. These newly emerged laterals, unlike the laterals present at the time of treatment a) do not develop C-tumors, b) continue to grow and c) contain few or no polyploid cells. From this observation it was concluded that cells of large primordia, from which the newly emerged lateral roots are descended, show a differential response to colchicine; they appear to be less sensitive to colchicine than lateral roots or small primordia (Davidson, 1965). The response of large primordia to colchicine has been examined immediately after treatment and one and two days later.

Roots were treated with colchicine (0.001-0.05%) for 3 hours and then fixed. The responses of lateral roots, large and small primordia were determined.

The two effects that are rapidly induced by colchicine are a) a suppression of anaphase and b) a rise in the number of metaphases. From the frequencies of anaphases and metaphases it is clear that large primordia, in comparison with small primordia or lateral roots, do exhibit a differential response to colchicine. This is clearly seen after treatment with 0.025% or 0.0125% colchicine. With 0.0125% solutions, for example, (Encl. 5; Table 1) large primordia show little increase in the frequency of metaphases per 1000 cells (24.25/1000 cf 22.4/1000 after treatment with 0.001% colchicine) while lateral roots show a distinct increase (100.6 metaphases per 1000 cells

cf. 18.8/1000 cells after 0.001% colchicine). Similarly, large primordia still contain anaphases, though they are reduced in frequency, when they have been suppressed in lateral roots (Encl. 5; Table 1).

The differential response of large primordia is also demonstrated by changes in mitotic index induced by colchicine. Increasing the strength of the solution from 0.001% to 0.0125% results in an increase in MI in lateral roots from 7.35 ± 0.93 to 14.48 ± 3.24 . In large primordia, the MI is fairly constant with 0.001% to 0.025% (7.11 to 9.72) and does not increase till 0.05% colchicine is used; then it rises from 9.72 ± 2.89 (0.025% colchicine) to 15.52 ± 2.67 (0.05% colchicine).

iii. Phenotype in Large Primordia and Lateral Roots.

The changes that occur as a primordia emerges as a lateral root are: a) a fall in MI of about 2% (Encl. 6; Table 1); b) the onset of cell elongation in the base of the primordium; c) a change in response to colchicine (Encl. 5 and 6); d) a change in the pattern of uptake of exogenous $^3\text{H-TdR}$ (AT(11-1)1625-12); e) an increase in sensitivity to treatment with IAA (AT(11-1)1625-2). Clearly, large primordia and lateral roots are phenotypically different. The point at which the switch from one phenotype to the other occurs has been determined by examining the various stages of large primordia.

Large primordia that were just about to emerge at the time of treatment with colchicine (Fig. 1; stage 4-5) respond in the same way as lateral roots: their MI is 1.53 ± 0.7 compared with the control value of 9.3 ± 1.2 at 48 hours (Encl. 6; Tables 1 and 2) and they have developed C-tumors. Furthermore, approximately 75% of metaphases seen at 48 hours are

Figure 1

polyploid, just as in lateral roots with C-tumors. Thus, the lateral root phenotype is fully expressed in very large primordia before they actually emerge through the epidermis of the primary root. The nature of the change that determines this phenotypic switch and the mechanism by which it is induced are not known. Since so many changes that are basic to cell replication and cell growth occur at about the same time, as the primordia emerge, it appears that a crucial change occurs in the systems that control morphogenesis of the apical meristem. These changes and their underlying causes would be worth investigation: the changes are pronounced and the primordia are accessible for experimental manipulation.

These results are now in press (Encl. 6).

iv. Loss of Polyploid Cells from Large Primordia

Tetraploid cells are induced in large primordia by colchicine and they have been seen, in division, 14 hours after treatment (Encl. 6). These tetraploid cells fail to survive, however. When the large primordium emerges as a lateral root, few or no tetraploid cells are seen in division. From this observation, we have concluded that a population of cells present in large primordia and capable of responding to a colchicine treatment, does not survive: it makes little or no contribution to the meristem of the fully emerged lateral root. This implies a very marked differential cell replication or actual cell death, leading to a change in the composition of a primordium as it becomes a lateral root.

4. Anatomy of Developing Primordia

Sections were made of primary roots that contained developing primordia. The pattern of growth is as follows:

i) mitoses occur in the pericycle and endodermis; they extend over 1,000 μ or more and give rise to elongated bumps of mitotically active cells.

ii) small primordia are seen (Fig. 1. 1); divisions are oriented periclinally and anticlinally; cortical cells over the primordia are abnormal.

iii) mitoses in the apical region of the primordium are anticlinally oriented, giving rise to a cap of cells around a central core (Fig. 1. 3); the cortical cells are breaking down and the epidermis begins to look abnormal.

iv) there is a conspicuous dome of cells, 8-10 layers thick, that forms a cap; the cortical cells around the primordium have disappeared leaving a sinusoid (Fig. 1. 4). At the same time, the inner core of cells begin to divide again; these cells form the meristem of the emerged lateral root.

v) mitoses are absent or scarce in the central mass of cells but are frequent in the 8-10 outer layers of cells (Fig. 1. 4). The central cells remain dormant for about 24 hours (Fig. 1. 4-5) and do not recommence mitotic activity until the primordium begins to emerge (Fig. 1. 6).

vi) as the primordium emerges, the frequency of mitosis in the peripheral cells falls; thus these cells make little contribution to the group of dividing cells seen in the emerged lateral root (Fig. 1. 7). At the same time, the inner core of cells begin to divide again; these cells form the meristem of the emerged lateral root.

These results indicate that two discrete populations of cells occur in primordia. One population is most active about 24 hours before the primordium emerges as a root and this coincides with a depression in the mitotic activity of the second population. The connection between this changing pattern of

mitotic activity and the fate of the polyploid cells induced in large primordia is obvious. This connection was tested by determining the location of arrested metaphases and later of tetraploid cells in large primordia treated with colchicine. After a 3 hour treatment, metaphases were seen in peripheral layers of cells in large primordia (Fig. 1; stage 4). Twelve, 18 and 24 hours after treatment (Fig. 1; stages 4, 5 and 6) tetraploid cells were seen, in division, also in the peripheral layers of cells. Occasionally, cells were seen to be dividing in the central core and sometimes tetraploid cells were later in division. As the primordium emerged, the peripheral layers of cells become vacuolated and no longer divide: the tetraploid cells, induced 24 and 30 hours earlier, are no longer dividing. Then the central cells re-enter mitosis, and a few show a colchicine effect. Thus, the organization of the large primordium in terms of the proliferative ability of its sub-populations, determines the pattern of response to colchicine; it explains the failure of some cells to survive as part of the meristem when a primordium emerges as a lateral root.

These results are further evidence of an unusual switch mechanism at work during the morphogenesis of primordia.

5. Mixoploid Primordia

The second line of approach to the problem of primordial morphogenesis was to use primordia that were mixoploid chimaeras. It had already been shown that the lateral roots that develop from C-tumors induced by colchicine retain the mixoploid condition for up to 15 days after treatment (Davidson, 1965); this degree of stability contrasts sharply with instability of chimaeras induced late in primordial development (see section 4).

Whole roots were treated with 0.003-0.05% colchicine for 3 hours. The roots developed C-tumors and when lateral roots emerged from the C-tumors their composition was determined. It was found that polyploid cells were present in high frequencies after cell treatments (Table 1). Even with 0.003% colchicine $68.7 \pm 25.3\%$ of mitoses were tetraploid (mean from 16 roots). With 0.0125%-0.05% solutions the frequency of tetraploid cells remained constant but the octoploid cells increased and the diploid cells decreased in frequency. The average ploidy of a meristem has been expressed as a G-index, i.e. the number of genomes per 100 cells. This is taken as the sum of the percentage of diploid cells, twice the percentage of tetraploid cells and four times the percentage of octoploid cells. The G-index increases as the concentration of colchicine increases from 0.003% to 0.0125%; at higher concentrations of colchicine there is no further increase in G-index (Table 1). With increasing G-index there is also an increase in root width (Table 2). The relationship between width and G-index is linear for roots 1.2 to 1.6 mm wide, but with wider roots there is no clear relationship.

Because of the changes that occur in the relative rates of proliferation of the peripheral and central cells in large primordia (see section 4) the composition of mixoploid primordia at different stages of development was determined. Primordia and lateral roots from C-tumors were observed in whole roots treated with 0.003% or 0.0125% colchicine. Their composition changes as they develop. However, after all treatments the same pattern of change has been observed.

After 0.003% colchicine, for example (Table 3), as the primordium

Table 1.

Effects of a 3 hour exposure to colchicine. Primary roots were treated and the results scored in tumor laterals. Mean length of laterals was 0.7 cm. a) and b) represent two separate sets of treatments.

	colchicine % concentration	M.I.	% of mitoses			genome (G) index	Number of roots
			2n	4n	8n		
a)	0.003	5.2 $\pm$ 1.3	30.6 $\pm$ 26.1	68.7 $\pm$ 25.3	0.7	170.8 $\pm$ 28.2	16
	0.006	5.6 $\pm$ 0.8	18.7 $\pm$ 16.0	74.1 $\pm$ 16.6	7.2 $\pm$ 11.0	195.7 $\pm$ 30.8	19
	0.0125	5.2 $\pm$ 1.8	2.7 $\pm$ 2.9	76.8 $\pm$ 11.3	20.5 $\pm$ 12.7	238.5 $\pm$ 27.5	10
b)	0.0125	7.2 $\pm$ 1.9	6.4 $\pm$ 7.1	73.5 $\pm$ 15.9	20.1 $\pm$ 18.8	234.1 $\pm$ 41.7	32
	0.025	7.0 $\pm$ 2.0	6.2 $\pm$ 6.1	62.1 $\pm$ 18.6	31.8 $\pm$ 21.4	257.4 $\pm$ 46.6	13
	0.05	6.2 $\pm$ 3.7	4.3 $\pm$ 5.9	74.8 $\pm$ 18.4	20.8 $\pm$ 16.2	241.3 $\pm$ 28.7	6

Table 2.

Degree of polyploidy, as indicated by G-index, and root width in lateral roots from tumors induced by treatment with colchicine (0.0125-0.05%). Mean length of roots was 0.7 cm.

	Width (mm)	M.I.	G-index	No. of roots
*	1.2	3.6 ± 0.8	149.3 ± 29.9	6
*	1.3	4.8 ± 2.8	178.5 ± 21.3	6
	1.5	5.8 ± 2.3	211.5 ± 15.6	4
	1.6	8.0 ± 1.6	241.0 ± 34.1	6
	1.7	8.5 ± 2.4	212.2 ± 52.5	5
	1.8	7.2 ± 2.3	224.8 ± 24.6	16
	1.9	7.5 ± 1.1	245.4 ± 20.2	5
	2.0	6.0 ± 2.7	273.8 ± 37.3	9
	2.1	7.4	319	1
	2.2	5.5 ± 0.5	325.0 ± 12.7	2
	2.5	4.7	294	1

* Includes lateral roots from the regenerated primary root.

Mean length of these roots was 3.0 cm, cf. 0.7 cm of tumor laterals.

Table 3.

Composition of developing mixoploid roots induced by colchicine. Experiments a) and b) were carried out on the same sample of beans.

Treatment	days after colchicine	primordial stage/root length (cm)	M.I.	% mitoses			frequency of divisions per 1000 cells			No. of roots examined
				2n	4n	8n	2n	4n	8n	
a) 0.003% colchicine 3 hrs.	4	medium	10.1	3.7	85.3	10.7	3.7	86.2	10.8	7
	5	medium- large	10.4	27.3	66.5	6.2	28.4	69.1	6.5	6
	6	newly- emerged laterals	6.8	45.2	53.7	1.2	30.8	36.5	0.8	6
	8	1.1	8.1	2.0	94.5	3.5	1.6	76.5	2.8	6
	10	2.6	6.8	0.5	97.5	2.0	0.3	66.3	1.4	6
	12	3.3	4.9	6.3	92.3	1.3	3.1	45.2	0.6	6
b) 0.0125% colchicine 3 hrs.	4	medium- small	9.0	3.0	57.3	40.0	2.7	52.2	36.4	6
	5	large	9.1	2.0	37.3	60.7	1.8	34.3	55.8	7
	6	emerging- laterals	9.4	2.3	82.0	15.7	2.2	77.1	14.7	7
	8	0.4	6.5	0.9	53.5	45.7	0.6	34.8	30.4	6
	10	1.3	7.2	2.9	31.5	65.6	2.1	25.7	47.2	8
	12	1.8	5.6	1.1	44.5	54.4	0.6	24.9	30.5	8
	18	3.0	5.5	1.0	44.0	55.0	0.6	24.2	30.3	2

developed from a medium size (Fig. 1. 3) to large (Fig. 1. 4) to a newly emerged lateral root (Fig. 1. 6-7), the percentage of mitoses that were tetraploid decreased: 85.3 to 66.5 to 53.7 (Table 3). At the same time the percentage of diploid mitoses increased from 3.7 to 45.2 (Table 3). In a repeat experiment, tetraploids fell from 68.7 to 9.4%, diploids increased from 29.0 to 83.4% (Table 4). However, once the root has emerged a reverse of this change occurs. Tetraploid mitoses increased from 53.7% (Table 3; day 6) to 94.5% (Table 3; day 8) and diploid mitoses decreased from 45.2% to 2.0% from day 6 to day 8 (Table 3). In the repeat experiment the tetraploid increase was from 9.4 to 67.4% (days 5 to 6; Table 4) and diploid mitoses fell from 83.4 to 28.8% between days 5 and 6 (Table 4).

Once the primordium has emerged and is growing as a lateral there are no further changes in the composition of its meristem (Table 3a, cf. roots on days 8 and 12; Table 3b, cf. roots on days 8 and 18). Thus, the changes in the relative frequencies of diploid and tetraploid cells in mitosis occur within about a day before the primordium emerges and 36-48 hours after it emerges (Tables 4 and 5).

What is striking about these results is not only that there is a temporary fall in the number of tetraploid mitoses just before a primordium emerges as a root but also that the simultaneous fluctuations in diploid and tetraploid mitoses parallel, so closely, the changes in the frequencies of mitoses in the peripheral and core populations of developing primordia.

i. The Location of Tetraploid Cells in Mixoploid Primordia

Roots were treated with 0.003% colchicine and the development of primordia in the C-tumor was followed. Tetraploid cells, in division,

Table 4.

Composition of developing mixoploid roots induced by treatment with 0.003% colchicine for 3 hours. This is a repeat, using different beans, of experiment a) in Table 3.

days after treatment	primordial stage or root length	M.I.	% mitoses			frequency of divisions/1000 cells			No. of roots
			2n	4n	8n	2n	4n	8n	
2	pericycle thickening		29.0	68.7	2.7				3
3	medium	10.8	35.4	64.0	0.6	38.2	69.1	0.7	5
4	very large	9.3	35.0	59.4	5.6	32.6	55.2	5.2	5
5	newly- emerged lateral	5.9	83.4	9.4	7.2	49.2	5.6	4.3	5
6	0.5	6.2	28.8	67.4	3.8	17.9	41.8	2.4	5
7	1.9	8.1	19.0	78.2	2.8	15.4	63.3	2.3	5
8	2.6	7.8	2.6	93.6	3.8	2.0	73.0	3.0	5

Table 5

Changes in composition of tumor laterals during growth.

Treatment	hours after first observation	root length	MI	% mitoses			% polyploid mitoses	No. of roots examined
				2n	4n	8n		
0.003% colchicine	0	just out lcm	7.6	90.2	9.8	0	9.8	5
	26	1.9	6.3	21.0	78.2	0.8	80.0	5
	78		4.8	8.3	83.3	8.5	91.8	4
0.003% colchicine	0	0.9	6.8	24.0	68.8	7.2	76.0	6
	48	1.5	6.1	12.3	85.3	2.5	87.7	4
	96	3.3	7.1	3.5	85.3	10.1	96.5	8

were seen in the pericycle a day after colchicine treatment. By day 2, the cells of the primordia were predominantly tetraploid, though occasional diploid mitoses were seen at the base of the primordia and on their periphery. As growth occurs, the peripheral cells increase in number and produce an outer sheath; these cells are diploid. The central core of cells also increases in number; these are mainly polyploid but some diploid cells are present, scattered throughout the core region. From stages 3 to 4 (Fig. 1 and 1a) there is a gradual decline in the frequency of mitoses in the control core cells. This corresponds with the stage present 24 hours before emergence, i.e. the stage when the relative proportion of polyploid cells fall. The peripheral cells, however, are still actively dividing. These are diploid cells; therefore they increase in relative frequency. As soon as emergence takes place the peripheral (diploid) cells stop dividing and the central core (tetraploid) cells begin to divide again. There is a spatial and temporal organization of the pattern of cell proliferation in the developing primordium.

Summary

Two discrete populations of cells have been identified in developing primordia. They can be distinguished on the basis of spatial distribution and that they differ in the times when they undergo active proliferation. One population, the central core of cells is actively dividing for all but the last 24 hours of primordial growth and in this period is quiescent. The second population, the peripheral cells, are really active only for the last 24 hours of primordial growth; after this period they are not active and are eventually sloughed off as cap cells.

These populations have also been experimentally identified by

Figure la. 3-4

treatment with colchicine. Treatment early in primordial growth labels the central core cells by inducing polyploidy. These marked cells survive primordial morphogenesis and contribute most of the meristem of the fully emerged root. Treatment late in development labels the peripheral cells. These cells divide once or twice and then are lost. Thus, tetraploid cells appear in the peripheral region of the large primordia but do not survive when the root emerges.

These results provide further evidence that the morphogenesis of primordia in Vicia faba presents a unique system for the study of control mechanisms in development.

6. Microspectrophotometry of Large Primordia

The mixoploid large primordia from C-tumors induced with 0.003% colchicine were examined by microspectrophotometry. (We are grateful to Dr. Joseph Grasso of the Department of Anatomy for his kindness in allowing us to use his laboratory for this study.) These large primordia contained diploid and tetraploid cells. A 2C peak of DNA was seen. A 4C peak was prominent, this would include G1 tetraploid cells and G2 diploid cells. An 8C peak was also seen, G2 tetraploid cells, and cells with values intermediate between 4C and 8C were seen; these would be tetraploid cells in S. At the time of emergence the primordia examined showed a rise in the frequency of 2C + 4C cells, i. e. diploid cells in S and a rise in the 4C peak, but a fall in the 8C peak and little evidence of tetraploid cells in S, i. e. 4C + 8C cells. It appears that the tetraploid cells have been arrested in G1.

One day later, i. e. after emergence, there are again cells intermediate between 4C and 8C and the 8C peak reappears. These results taken

with the temporary suspension of division in the tetraploid cells show that DNA synthesis is delayed, i. e. the effect on the tetraploid is largely in G1.

7. DNA Synthesis in Diploid and Tetraploid Cells.

The changes in the composition of mixoploid meristems that occur at about the time of emergence could be due to differences in mitotic cycle times of diploid and polyploid cells. But since large primordia do not incorporate ^3H -TdR a percentage labeled mitosis curve determination of cycle times cannot be made in primordia. However, it can be done once the roots have emerged.

Roots with C-tumor laterals 0.5 to 1 cm long were treated with ^3H -TdR and fixations were made over the following 27 hours. The percentages of labeled diploid and labeled tetraploid mitoses were determined. With the exception of the peak of labeled tetraploid mitoses at 9 hours (Table 6) the values for diploid and tetraploid cells are remarkably similar (Fig. 2). Using the percentage labeled mitosis curves, the following values have been calculated:

	2n cells	4n cells
cycle time	14.4 hours	14.0 hours
S	7.9 hours	7.7 hours
$G2 + \frac{\text{mitosis}}{2}$	3.8 hours	3.8 hours

We have no estimate yet of the duration of mitosis in tetraploid cells and, therefore, G2 and, by subtraction, G1 cannot be determined. The crucial result, however, is quite clear. In growing mixoploid roots, taken about 7 days after treatment with colchicine, diploid and tetraploid cells

Table 6

Percentages of labeled 2n and 4n mitoses in tumor laterals at various intervals after a 35 minute treatment with ^3H -TdR. These laterals were from roots treated with 0.003% or 0.006% colchicine. Their average composition was 35.8% 2n, 62.9% 4n and 1.3% 8n cells. MI = 6.0.

<u>Hrs. after ^3H-TdR</u>	<u>% labeled mitosis</u>		<u>No. of roots</u>
	<u>2n</u>	<u>4n</u>	
2	0.7	1.7	3
4	43.3	49.3	3
6	74.3	78.5	4
9	79.6	93.0	5
12	34.3	34.0	3
15	18.7	14.7	3
18	26.7	25.3	3
21	34.7	45.7	3
24	35.4	45.2	5
27	23.3	33.3	3

Figure 2

Percentage labeled mitosis curves in diploid and tetraploid cells.

have identical cycle times and spend the same time in S. Thus the rate of DNA synthesis in the tetraploid cells must be double the rate in the diploid cells, though the diploid and tetraploid cells are present in the same meristem.

The roots used in these determinations included at least 18% diploid cells (mean frequency was 35.8% diploid cells) and these would be expected to make some contribution to the physiological factors that determine the kinetics of cell proliferation. It may even be that they are imposing their kinetics on the tetraploid cells since the values we report here for tetraploid cells are very similar to those previously found for normal diploid cells in wholly diploid roots (Encl. 1).

We have also determined the percentage labeled mitosis curves and the cycle times for tetraploid cells taken from mixoploid roots with less than 10% diploid cells (mean frequency = 2.5% diploid cells) (Table 7). A comparison of the curves for the two groups of tetraploid cells, i.e. taken from mixoploid with either < 10% or > 10% diploid cells, shows that they are essentially similar (Fig. 3). There is no evidence from these results that the kinetics of tetraploid cells are influenced by diploid cells present in the same root. These results also demonstrate that the duration of the S phase does not necessarily increase as ploidy of the nucleus increases.

These results are now being prepared for publication.

8. The Effect of IAA on Mixoploid Meristems

In normal diploid roots, treatment with IAA leads to a temporary fall in mitotic index (see last year's report and Encl. 2) that results from a delay induced in the S phase (Encl. 2). The effect of IAA on mixoploid

Table 7

Percentages of labeled 4n mitoses in tumor laterals with a) less than, and b) more than 10% diploid cells. Roots had been treated with 0.003%, 0.006% or 0.0125% colchicine. Average composition: a) 2.5% 2n, 83.9% 4n and 13.6% 8n: MI = 5.6. b) 35.8% 2n, 62.9% 4n and 1.3% 8n: MI = 6.0. The roots used in this experiment were the same as in Table 5.

Hrs. after $^3\text{H-TdR}$	(a) Roots with < 10% 2n mitoses					(b) Roots with > 10% 2n mitoses				
	% mitoses 2n	% mitoses 4n	% mitoses 8n	% labeled 4n mitoses	No. of roots	% mitoses 2n	% mitoses 4n	% mitoses 8n	% labeled 4n mitoses	No. of roots
2	0.7	84.7	14.7	2.3	3	40.3	59.7	0	1.7	3
4	1.7	72.3	26.0	38.3	3	34	65	1	49.3	3
6	4.0	82.8	13.2	69.8	5	22.3	77.3	0.3	81.7	3
9	3.7	87.7	8.7	77.0	3	28.8	68	3.2	92.5	6
12	1.3	92.3	6.3	33.7	3	69.3	30.0	0.7	34.0	3
15	0.3	84.3	15.3	11.3	3	32.7	67	0.3	14.7	3
18	2.8	93.3	4.0	24.5	4	20.7	78.3	1.0	25.3	3
21	1.6	84.4	14.0	38.4	5	18.0	78.7	3.3	45.6	3
24	3.8	75.4	20.8	49.2	5	39.5	59.2	1.3	45.2	6
27	5.0	83.3	11.7	35.0	3	54.3	45.7	0	33.3	3

Figure 3

Percentage labeled mitoses curves in tetraploid cells.

lateral roots and developing primordia has now been tested.

i. Mixoploid Lateral Roots

Whole roots were treated with 0.003% colchicine and 7-8 days later fully emerged laterals were present. They were treated with 10^{-6} or 10^{-7} M IAA for 3 hours and examined for 1-12 hours after the end of the treatment.

A comparison of the numbers of diploid and tetraploid mitoses per 1000 cells after the various treatments (Table 8) shows that the different cell types within the meristems responded differently to IAA. The mean frequencies, in all 5 fixations, of 4n and 2n cells per 1000 cells show this clearly (based on Table 8):

	Frequency of mitoses per 1000 cells		No. of roots
	2n	4n	
Control	22.5	45.5	31
10^{-6} M IAA	10.4	44.8	37
10^{-7} M IAA	6.4	40.02	20

Tetraploid cells are reasonably constant in frequency in all three sets of meristems. The diploid cells, however, are only about half as frequent in IAA treated roots as in controls.

The data from all fixations suggests that the frequencies of diploid and octoploid cells are much more variable in IAA treated roots than in control roots. Three points stand out from these results:

a. The variations are probably real, since it seems highly unlikely that in a total sample of 88 roots, a chance distribution would have resulted in roots with lower frequencies of diploid cells only in IAA treated roots.

Table 8

Effects of 3 hour IAA treatments on tumor laterals formed after exposure
to 0.003% colchicine. (75 metaphases scored from each root)

Treatment	hrs. after end of treatment	MI	% mitoses				Frequency of mitoses per 1000 cells		No. of roots examined
			2n	4n	8n		2n	4n	
Control	1	7.3	32.0	66.3	1.8		23.4	48.4	4
	3	5.7	42.3	50.0	7.6		24.1	28.5	7
	5	6.9	34.2	65.7	0.2		23.6	45.3	6
	8	8.6	29.0	69.0	2.1		24.9	59.3	7
	12	6.9	26.9	72.6	1.7		18.6	50.1	7
IAA 1×10^{-6} M	1	5.6	20.0	76.0	4.8		11.2	42.6	5
	3	3.7	15.8	82.8	0.9		5.9	30.6	8
	5	5.1	16	81.6	2.0		8.2	41.6	8
	8	6.7	9.3	87.8	5.9		6.2	58.8	8
	12	6.2	33.3	64.3	2.2		20.7	39.7	8
IAA 1×10^{-7} M	1	6.6	12.3	80.5	7.3		8.1	53.1	4
	3	3.5	8.3	89.3	2.5		3.0	32.2	4
	5	4.5	6.0	79.0	14.8		2.7	35.6	4
	8	5.6	24.0	62.8	13.3		13.4	35.2	4
	12	5.5	8.8	80.2	11.0		4.8	44.1	4

b. The fall in the frequency of diploid cells is consistently found in the period 3-8 hours after IAA. This is the period in which IAA delays the arrival at mitosis of cells labeled with ^3H -TdR in S and then treated with IAA (Encl. 2).

c. The case in which octoploid cells also change in frequency, though they increase rather than decrease in frequency, also occurs after the period in which one would expect cells affected in S to take to arrive at mitosis (i. e. 5-8 hours).

It appears, therefore, that in mixoploid lateral roots, the duration of cell cycles and of the S phase is identical in diploid and tetraploid cells (section 7) but that these two cell types respond differently to IAA. The results (Table 8) indicate that diploid cells are inhibited by a 3 hour treatment with IAA, while tetraploid cells show little or no effect.

ii. Mixoploid Primordia

Whole roots were treated with 0.003% or 0.0125% colchicine for 3 hours. Some roots were then treated with IAA, either for one day or continuously; the treatments are given in the legends to Tables 9 and 10. The effects from both types of treatment are similar.

Continuous treatment with IAA changes the pattern of mitotic activity in the 2 sub-populations of a developing primordium. The tetraploid and diploid populations do not show such marked fluctuations in mitotic activity as they do in the absence of exogenous IAA. The tetraploid cells do not decrease and the diploid cells do not increase in frequency to the extent shown in control roots (cf. Tables 3a and 9; Fig. 4). Similarly, in primordia that are made up mainly of tetraploid and octoploid cells, there is less

Figure 4

Figure 4. Percentage frequencies of diploid and tetraploid cells in mitosis in mixoploid primordia and lateral roots from C tumors induced by treatment with 0.003% colchicine. Some roots were also treated with 10^{-7} M IAA continuously from 24 hours after the end of the colchicine treatment.

● ——— ●	tetraploid mitoses; colchicine treatment
○ ——— ○	diploid " " "
■ - - - ■	tetraploid mitoses; colchicine and IAA
□ - - - □	diploid " " " "

Table 9

Effects of continuous exposure to IAA on the composition of mixoploid primordia at various developmental stages. The chimaeras were formed in two separate experiments after treatment with 0.003% or 0.0125% colchicine. Control values for (a) and (b) are given in Table 3

Treatment	days after colchicine	primordial stage/root length	MI	% mitoses			Frequency of divisions/1000 cells			No. of roots examined
				2n	4n	8n	2n	4n	8n	
a) 0.003% colchicine										
IAA 1×10^{-7} M continuously 24 hours after colchicine	3	small	9.4	20.6	76.4	3.0	19.4	71.8	2.8	5
	4	large very large	9.0	29.2	62.6	6.2	26.3	56.3	4.2	5
	5	emerging laterals	8.1	30.6	68.0	1.2	24.8	55.1	1.0	5
	6	0.3	7.3	15.2	72.4	12.4	11.1	52.9	9.1	5
	7	1.2	7.9	16.2	76.2	7.6	12.8	60.2	6.0	5
	8	2.1	8.0	6.8	89.8	5.2	5.4	71.8	4.2	5
b) 0.0125% colchicine for 3 hours										
IAA 1×10^{-4} M continuously 48 hours after colchicine	4	medium- large	8.7	1.0	49.8	49.2	0.9	43.3	42.8	6
	6	large	8.1	1.6	65.0	33.4	1.3	52.7	27.1	7
	8	0.4	6.3	0.5	55.4	44.3	0.3	34.9	27.9	7
	10	1.5	6.5	0.5	56.5	43.0	0.3	36.7	28.0	6
	12	2.4	8.6	0.9	60.2	39.0	0.8	51.8	33.5	

fluctuation in their frequencies than in roots treated only with colchicine (cf. Tables 3b and 9).

Treatment with IAA for only 1 day was less effective than continuous treatment. When given for 1 day, 24-48 hours after colchicine, there was also little change in the frequencies of $2n$ and $4n$ mitoses (Table 10a). And when given 48-72 hours after colchicine, 10^{-7} M IAA appears to change the pattern of mitotic activity much more haphazardly (Table 10b).

Conclusions

The pattern of mitotic activity in lateral roots and developing primordia is influenced by IAA. We have shown that, at ²least in lateral roots, some event in the S phase is temporarily inhibited. In primordia the phase affected by IAA has not been defined but the normal changes in levels of mitotic activity of the sub-populations are altered by IAA. It also appears that mitotic activity in primordia is less sensitive to IAA than it is in laterals. However, it would be premature to connect changes in endogenous levels of IAA with changes in mitotic activity. Such a possibility should nevertheless be borne in mind when considering the response of primordia to X-rays (see section 12).

9. Response to Base Analogs

Analogues of DNA bases are useful tools in studies of cell proliferation. In these studies 5-aminouracil (5-AU) and 5-bromouracil (5-BU) have been used, also 5-fluorodeoxyuridine (5-FUdR), an analog of thymidine.

i. 5-Aminouracil

Treatment with 250-750 ppm 5-AU inhibits mitotic activity in lateral roots and large primordia. With continuous treatments for 24 hours, lateral

Table 10

Effects of 1 day treatments with IAA on the composition of developing mixoploid primordia. The Chimaeras formed after treatment with 0.003% colchicine. The beans used in this experiment were from the same batch as those in Table 3 (a). The control values for these treatments are given in Table 4.

Treatment	days after treatment	primordial stage/root length	MI	% mitoses			Frequency of divisions/1000 cells			No. of roots examined
				2n	4n	8n	2n	4n	8n	
a) IAA 1×10^{-7} M 24-48 hrs.	3	medium	10.2	36.8	55.8	7.6	37.5	56.9	7.8	5
	4	very large	9.6	21.2	75.2	3.6	20.4	72.2	3.5	5
	5	emerging laterals	9.5	28.2	64.8	7.0	26.8	61.6	6.7	5
	6	0.4	9.0	26.2	62.8	11.0	23.6	56.5	9.9	5
	7	1.3	9.5	19.2	71.2	9.6	18.2	67.6	9.1	5
	8	2.1	8.6	8.2	83.4	8.4	7.1	71.7	7.2	5
b) IAA 1×10^{-7} M 48-72 hrs.	3	medium	9.5	49.0	47.8	3.2	46.6	45.4	3.0	5
	4	medium large	9.5	6.4	84.2	9.4	6.1	80.0	8.9	5
	5	emerging laterals	6.6	8.6	85.8	5.6	5.7	56.6	3.7	5
	6	0.5	8.3	24.8	66.0	9.2	20.6	54.8	7.6	5
	7	1.4	7.3	0.6	92.6	6.8	0.4	67.6	5.0	5
	8	2.2	8.0	14.2	85.0	0.8	11.4	68.0	0.6	5

roots remain inhibited for the whole period. Large primordia on the other hand are inhibited for 12 hours, but show signs of recovery by 18 hours, and at 24 hours have higher mitotic indices than control primordia. We have not been able to establish whether the onset of mitotic activity occurs in the peripheral cells or the central core cells of the large primordia. We would expect, however, that since the microspectrophotometric study reported earlier (section 6) showed that the core cells are quiescent in G1, when the roots are removed from 5-AU then the core cells would show no delay in undergoing S and entering mitosis. These studies of the response to 5-AU will be continued in order to determine where the affected cells are located.

The fact that some cells do enter mitosis after continuous exposure to 5-AU suggest that they have completed S even in the presence of the analog. We cannot, of course, demonstrate this by using ^3H -TdR since large primordia do not incorporate this labeled precursor. Instead we exposed whole roots to colchicine for 3 hours and then treated with 5-AU. The appearance in division of tetraploid cells will then provide evidence that cells have completed a whole mitotic cycle in the presence of 5-AU.

After 3 hours in colchicine and 26 hours in 5-AU (500 ppm) lateral roots and large primordia were examined. In 10 lateral roots, mean MI was 0.8 ± 0.2 ; in large primordia the value was 13.9 ± 3.1 . The lateral roots were seen to have a few polyploid mitoses. In 5 large primordia, of 1503 cells in division, 50.6% were diploid and 49.4% were tetraploid. Thus, we can conclude that a large number of cells in the large primordia have completed DNA in the presence of 5-AU.

ii. Chromosome Aberrations Following 5-AU Treatment

The diploid and tetraploid cells that come into mitosis in large primordia after continuous exposure to 5-AU (500 ppm) were seen to show 2 kinds of chromosome effects--unstained gaps and chromosome breakage and reunion. In 114 diploid cells, for example, only 4 had normal chromosomes. In 23 cells there were gaps in the nucleolar chromosomes (M chromosomes) and in 2 cells there were gaps in the non-nucleolar chromosomes (S chromosomes). Seventy-six cells had gaps in M and S chromosomes. The gaps were in, or very close to, known heterochromatic regions.

Chromosome breaks were scored in 144 cells. 20.8% of the cells had undergone chromosome breakage.

B^1	B^{11}	R^1	C_1SR	C_0SR	Total breaks	Total cells
42	12	37	6	10	54	144

Chromosome breakage in diploid cells of large primordia. 26 hours treatment with 500 ppm 5-AU. B^1 -chromatid break; B^{11} -isolocus break; R^1 -chromatid reunion; C_1SR -sister reunion in centric fragment; C_0SR -sister reunion in acentric fragment.

There were 0.37 breaks per cell ($\frac{54}{144}$). 5-AU is not only delaying cells, but in some cells it induces chromosome aberrations and in over 90% of the cells it induces the formation of unstained gaps.

iii. 5-FUdR

5-FUdR can inhibit DNA synthesis because it prevents thymidylate synthetase from converting uridine monophosphate (UMP) to thymidine

monophosphate (TMP). The result of a 5-FUdR treatment is an inhibition of DNA synthesis and, in proliferating cell systems, a fall in mitotic index. This effect is seen in lateral root meristems. Primordia of Vicia faba treated with 5-FUdR respond somewhat differently. Some large primordia show a fall in MI 3 to 6 hours after treatment with 10^{-5} , 10^{-6} and 10^{-7} M 5-FUdR. Associated with this effect we also find an effect on chromosome contraction and organization. Some cells have very sticky chromosomes and in others we find unstained gaps. In some primordia, however, we find the effect on chromosome structure without any detectable effect on MI.

The basis for the variation in the response of large primordia to 5-FUdR is now being examined. It presents an interesting problem for the following reason. 4-FUdR is not effective as an inhibitor of thymidylate synthetase until it has been phosphorylated and the enzyme responsible for the conversion of 5-FUdR to 5-FUdR-monophosphate is thymidine kinase. In bacterial cells and some tumor cells it has been shown that strains resistant to 5-FUdR lack thymidine kinase. Since some large primordia respond quickly to FUdR it is reasonable to suggest that these cells have thymidine kinase. One must then wonder why, if a cell can respond to FUdR and appears to have thymidine kinase, is the same cell unable to utilize exogenous ^3H -TdR? As we have seen, large primordia do not incorporate ^3H -TdR.

This inability to use ^3H -TdR could result from a) failure of the precursor to enter; b) immediate destruction; c) absence of thymidine kinase; d) utilization by the cell of a pathway that does not involve the steps

TdR $\longrightarrow$ TMP $\longrightarrow$ DNA synthesis. The obvious alternative pathway is UMP $\longrightarrow$ TMP $\longrightarrow$ DNA synthesis and this could occur even in the presence of thymidine kinase if the TdR pool was not drawn upon. Whatever the reason, it can be stated that the developmental steps that take place in the morphogenesis of a primordium involve marked changes in the pathways leading to DNA synthesis. This is shown by the evidence already presented that relates to: 1) uptake of ^3H -TdR and delay in incorporation in small primordia; 2) failure of incorporation in large primordia; 3) differential response to 5-AU; 4) insensitivity of some large primordia to 5-FUdR. It is also shown by the response of large primordia to ^3H -cytidine. Labeling indices in large primordia are low; they range from 0.5% to 4%.

It seems unlikely that ^3H -TdR cannot penetrate to the large primordia since so many other molecules that are chemically similar do penetrate and since, once the cortical cells have broken down, the only physical barrier between the large primordia and the outside is the epidermis. Primordia present an interesting problem in terms of changes in the biosynthetic pathways that produce DNA.

10. Abnormal Nodal Outgrowths on Brassica

It was reported last year that when primary shoots of the cabbage (Brassica oleracea) were treated with colchicine in lanolin paste two effects were observed: i) apical dominance was suppressed and the plants developed lateral branches; ii) outgrowths resembling calluses grew from stem nodes. Decapitated cabbages responded in a similar way and it was concluded that the abnormal growth is not a direct consequence of the colchicine; rather, it was suggested, the abnormalities result from an imbalance (or lack) of

growth factors that are normally supplied by growing apical regions.

The nodal outgrowths that first appeared 3 months after treatment with colchicine have continued to form on shoots that have gone on growing for the period of 18 months that have elapsed since the original treatments. Thus, whatever the change, it has been maintained and transmitted to newly arisen cells over a period of 18 months.

i. Culture Studies

The nodal outgrowths have been termed tumors because they have shown continuous, though slow growth for a long period, and because they lack organization into recognizable tissues. In those cases in which shoots have grown from the nodal outgrowths, the latter should be termed teratomas.

The nodal tumors, from colchicine treated and decapitated plants, have been grown in culture on a modified Murashige and Skoog medium (Encl. 7). Cultures have also been maintained of cells derived from pith from treated and control plants. Tumors and pith from colchicine treated plants are referred to as "C-tumors" and "C-pith", those from decapitated plants as "D-tumors" and "D-pith".

"C-" and "D-tumors" in vitro show consistently better growth than either their pith counterparts or control material (Encl. 7; Figs. 1 and 2). This demonstrates not only that the in vivo change that leads to tumor formation is perpetuated over many months, but also that a second change, revealed in vitro under culture conditions, is also perpetuated over many months. The different growth patterns shown by the various cell types should be emphasized. Thus, both nodal tumors and their neighboring pith

cells are descended from the same apical meristem yet in vivo and in vitro they behave differently. In vivo, the "C-tumors" proliferate and the cells grow, though slowly, while "C-pith" does not proliferate or grow. In vitro, the "C-tumors" grow at rates that exceed those of "C-pith". Similar results have been found with "D-tumors" and "D-pith".

ii. Growth in Culture

The "C-" and "D-tumors" grown in culture have been examined in order to determine the type of cell present, levels of mitotic activity and the chromosome number of the dividing cells. Mitosis is rare in culture and it has not been possible to make chromosome counts. All the mitoses seen were in cells about $10\mu^3$ in volume. These small cuboidal cells are similar in size to meristematic cells examined in normal seedling roots; both types of cells also have nuclei of identical diameters. It appears that proliferation is taking place only in cells that are like meristematic cells. These small cuboidal cells, however, constitute about 10-15% of the total cell population. The other cells range in size from $15-20\mu^3$ to $20\mu \times 20\mu \times 60-100\mu$ in size. Thus, the small cells make a small contribution in terms of cell number and an even smaller contribution in terms of cell volume. This should be borne in mind when considering the growth and the physiology of the various cell types in culture.

iii. Physiological Studies of Cultured Cells

The "C-" and "D-tumors", "C-" and "D-pith" and control pith cells grown in culture have been grown on a medium containing $3.75\mu\text{C}$ of ^{14}C -sucrose per 10 ml of medium. Their respiration and intermediary metabolism have been studied. The methods are given in Encl. 8.

"C-tumor" cells are clearly different from all other cultures. Their respiration rate, expressed as $\text{mc } ^{14}\text{C}$ per gm fresh weight per hour is 0.122, compared with a value of 0.597 in "C-pith", and 0.598 in control pith cultures (Encl. 8, Table 1). In keeping with this low respiration rate in "C-tumors" is the observation that these cells accumulate more ^{14}C -sucrose than "C-pith" cells (Encl. 8, Table 3).

Our results show that "C-tumor" and "D-tumor" cells differ from the other cultures. We have pointed out that in growth rate, respiration rate and intermediary metabolism "C-tumors" resemble completely transformed tumors of tobacco, while "D-tumors" are like partially transformed tumors. The only difference in the origin of these two tumors is that in one case the lateral branches which eventually give rise to the tumors formed while the plant was still influenced by colchicine, and in the other case the lateral branches arose after removal of the primary shoot apex. The differences that result from these conditions are expressed months later and after many sub-culturings. But there are differences between pith and tumor cells from one plant as well as between tumors from different plants; so differences must arise between different cell lineages from a single meristem. These differences are probably not mutational in origin since shoots and roots can arise from the tumors. The system is closer to a Dauer modification or a paramutation; it appears to be a long-lived change in the synthetic abilities of the cells or rather in the control mechanisms. These observations are suggestive of methods of approaching the problems of the control of normal and neoplastic growth.

iv. Auxin Levels in Nodal Tumors

Using thin layer chromatography we have studied the growth factor status of normal and colchicine treated shoot apices and also of nodal tumors in vivo and of nodal tumor cells grown in vitro. Normal apices were removed from the plants. About 2 mm of the apex were taken. IAN and IAE were identified on the TLC using Salkowski, Prokhaska and nitrite-nitric reagents. Two days after treatment with colchicine the amounts of these two growth factors had fallen (AT(11-1)1625-15).

We have now examined "C-" and "D-tumors" in vivo and cultures of these tumors in vitro. In neither case have we been able to detect IAE and IAN.

The reason may be that these compounds are present in undetectable amounts; or it may be that they are present, but only in those cells that are capable of proliferation and are similar to the meristematic cells. And, as we have seen, these cells constitute only 10-15% of cell numbers and on a volume basis they constitute a small fraction of total volume of a culture. A rough calculation, taking $30\mu^3$ as the mean volume of the larger cells, gives a value of only 0.5% for the volume of small cuboidal cells in a culture. All that can be said at present is that the tumor cells cannot be shown to possess IAE and IAN.

11. Apical Dominance: Colchicine Effects

The results reported last year showed that apical dominance could be suppressed by treatment of the primary shoot apex with colchicine in lanolin paste. The experiments were originally undertaken in an attempt to find support for the suggestion that the long-term changes seen in roots that had been treated with colchicine were the results of induced changes in

endogenous levels of growth factors. The results have supported this suggestion. Since last year's report was submitted, Dr. Sharp and I have extended our study of tumors to tobacco. The results agree with the idea that colchicine affects growth factor levels in plants.

Nicotiana glauca-langsдорфii is an amphidiploid that naturally produces tumors when flowering occurs. This then takes place in mature plants. We treated seedlings at the 5-7 leaf stage with colchicine in lanolin paste. Shoot growth was inhibited and the plants began to develop tumors within 7 days. The outgrowths remained green and were teratomas; they bore small leaf-like outgrowths (Fig. 5). A normal and a treated plant are shown in Fig. 6.

These results show that a disruption of shoot growth, in this case with colchicine, will lead to tumor inception long before flowering occurs. The speed with which the plants react suggest that the effect on the shoot apex is rapid and that the consequences within the plant are expressed at once.

Though the effect of colchicine is often one of inhibition of growth, this is not always so. Tobacco plants were treated with colchicine paste (2%) 2 or 3 days before they were about to flower. The plants developed lateral branches within 4 days; these branches elongated and flowered. In some plants, the lateral branches were very close to the apex and when shoot elongation occurred, the lateral shoots were carried up. These branches also flowered. The result is a modification of the structure of the inflorescence (cf. Figs. 7 and 8). The obvious result from this experiment is that the colchicine has changed the morphology of the plant but did

Figure 5. Teratoma on a tobacco plant treated with a colchicine in lanolin paste. The shoot apex was covered with the paste. This teratoma would not normally appear until the plant had flowered.

Figure 6. Normal and colchicine treated plants. This shows the inhibition of shoot growth induced by the drug and the development of a teratoma.

Figure 7. Tobacco plant. Untreated.

Figure 8. Tobacco plant treated with colchicine in lanolin paste just before flowering. Note the lateral branches with flowers.

not prevent elongation or flowering. It appears that the response of the plant to colchicine depends upon its physiological condition at the time of treatment.

Summary: Sections 10 and 11

The effects induced by colchicine have been shown in these studies to extend beyond the commonly held view that this drug arrests metaphase and induces the formation of polyploid cells. We have shown that in roots there are short-term effects, which lead to a shortening of cell cycle times, and long-term effects, lasting 2-5 days, which reduce mitotic activity almost to zero. In shoots we have shown that apical dominance can be suppressed and that the whole growth pattern of the plant is changed for as long as 18 months after treatment; furthermore, additional effects on the cells are expressed when they are grown in vitro. Several of these effects and their underlying causes would be worth further investigation.

12. X-ray Effects

Whole root systems of V. faba were irradiated with 50, 100 and 150 rads of X-rays. Fixations were made up to 8 hours after irradiation and the roots were treated with 0.025% colchicine for the two hours immediately before fixation in order to facilitate scoring chromosome aberrations. A longer treatment with colchicine was not used as we knew that it would lead to marked increases in mitotic index.

We have examined lateral roots, roots that were just emerging and large primordia. Mitotic indices have been scored; each value is based on 3,000 cells. Six and 8 hours after irradiation a clear difference can be seen between the different meristems. Lateral roots show the typical pattern of mitotic inhibition. Scoring of the roots fixed at 4 hours suggests an

inhibition in earlier fixations, but the data are still incomplete. In large primordia there is little evidence of an inhibition of mitosis and roots that were just emerging show much less effect than lateral roots (Table 11).

These results are not complete; it may be that large primordia respond to X-rays but that our fixation times have not covered the period in which the response occurs. As they stand, these results suggest that primordia are less sensitive to X-rays than fully emerged and growing lateral roots. It is yet another example of the differences between meristematic cells in primordia and lateral roots. Chromosome breakage in the various meristems is now being scored.

Table 11

Mitotic indices in lateral roots, just emerging roots and large primordia of *V. faba* following X-irradiation. Roots were treated with 0.025% colchicine for 2 hours before fixation.

	Hours after irradiation					
	6			8		
	control	100 rads	150 rads	control	100 rads	150 rads
lateral root	8.2	4.2	2.1	8.8	5.3	2.5
just out-root	9.5	7.8	6.2	8.7	10.1	9.8
large primordium	12.1	9.4	10.9	12.3	12.2	13.1