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Factors Affecting Mitotic Cycles in
Meristems

MASTER

Effects of IAA on Colchicine Induced
Changes in Mitotic Activity
and
Root Growth

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Technical Progress Report:

The study outlined in the original project abstract concerned the processes by which damaged tissues undergo repair. It was proposed to determine the "extent to which growth factors are involved in both the initial cessation of growth and its eventual recovery".

Analysis of populations of growing cells can be made at several different levels and, using colchicine and X-rays as tools for disrupting normal events, it was proposed to study events at these levels:

- i) chromosome replication
- ii) chromosome coiling and cell division
- iii) the regeneration locaus in previously disorganized meristems.

The ultimate goal of these experiments is to provide evidence on the factors that control mitotic rates in different parts of a meristem.

1. Colchicine Treatments

Changes occur in mitotic index in root meristems treated with colchicine. It was well known that mitotic index rises within a few hours of treatment and this rise was generally attributed to the arrest of cells at metaphase. This conclusion was based on the increase in numbers of metaphases and it was suggested that colchicine induced metaphase delay and did not alter the entry of cells into prophase.

Two results led us to suspect that this was an oversimplification:

- a) the presence of tetraploid metaphases in meristems with high mitotic activities and b) the long term changes in mitotic index (MI) seen in colchicine treated roots.

a) Tetraploid Metaphases in Roots with High Mitotic Indices.

In roots treated with 0.025% colchicine for 3 hours and examined 24 hours later MI was 13.5 (cf. control value of 5.8). This increased MI is not based only on diploid cells. Thus in a sample of 1,873 cells in mitosis, 1077 were tetraploid metaphases; they increase the MI and without them it would have been only 6.73 (cf. control 5.8). These results show that 24 hours after treatment a high MI is not due solely to metaphase delay of diploid cells. The presence of tetraploid cells in division along with a near normal number of diploid cells suggests that some stimulation of mitotic cycles occurred following treatment with colchicine and that the increased mitotic indices generally found within a few hours of treatment do not result solely from metaphase delay (Davidson, MacLeod and O'Riordan. Nature. 213. 1541-1542. 1966. Encl. 1).

The suggestion that colchicine could change the rate at which cells enter division is obviously important: it relates to the long term changes in MI that we have described (Encl. 1) and to short term changes, seen with 12 hours of treatment (section 1C).

b) Long Term Changes in Mitotic Index

Roots of Vicia faba were treated with 0.025% colchicine for 3 hours and fixed at intervals up to 87 hours. Mitotic index was high in the first 24-36 hours but then fell to values below normal, e.g. at

87 hours MI was 1.0 (Encl. 1). Mitotic activity remains low for several days. We have followed it for 7 days; MI falls to 4.0, 48 hours after treatment, and remains around 0.5 - 1.0 for the following 4-5 days (MacLeod and Davidson. New Phytologist, 65: 532-546. 1966. Encl. 2).

The fall in mitotic activity coincides with the period when the roots stop growing and we have suggested that the meristems lose their ability to maintain normal levels of mitotic activity because of changes in the levels of endogenous growth factors. (Evidence supporting this suggestion comes from treatments with colchicine and IAA: see Encl. 1, 2, 3, 4 and section 2).

c) Effects of Colchicine on The Entry of Cells into Prophase

Colchicine has been used to determine the rate at which cells arrive at metaphase and has been an important tool in studies of mitotic activities in different parts of a meristem. The basis of such studies is that colchicine does not alter the entry of cells into prophase. This assumption, however, does not appear valid. Results from studies of MI lead us to suggest that colchicine does have an effect somewhere during the mitotic cycle; we have tested this hypothesis by examining the response of meristems to a series of concentrations of colchicine. Lateral roots were treated with colchicine (0.000125, 0.00125, 0.0125, 0.025, 0.5 and 1% solutions) for 3 hours and fixed 3, 5, 7, 9, 11, 13, 15, 17, 28 and 30 hours later. The results have been written up for publication (Encl. 5).

With the exception of 0.000125% colchicine all the treatments we used led to changes in numbers of prophases. The two strongest solutions, 0.5 and 1.0% colchicine produced an initial decrease (Encl. 5; Tables 6,7), but this was followed by a subsequent rise. 0.00125% -- 0.025% solutions appeared to stimulate some cells in interphase at the time of treatment to enter mitosis (Encl. 5; Tables 2-5). A typical example of this response is seen (Encl. 5; Fig. 1) following treatment with 0.00125% colchicine. Within 9 hours of treatment the number of prophases per 5000 cells scored is 432 compared with a control value of 269 prophases. This rise in prophase numbers makes a significant contribution to the high mitotic indices induced by colchicine; the rise itself makes a contribution and it is then magnified by the subsequent arrest of these cells when they reach metaphase. This confirms our view that while delay at metaphase does ~~not~~ contribute to increases in MI it is not the sole cause. When metaphase delay follows a rise in prophase numbers then MI reach high values (Encl. 5; Table 4; Fig. 2), e.g. the largest number of metaphases, 933 per 5000 cells occurred 11 hours after treatment with 0.0125% colchicine. This value was found 2 hours after a peak in prophase numbers.

The changes in MI found with a 15-17 hour period of treatment depend upon numbers of cells entering prophase and the duration of the period of metaphase delay. The changes we have demonstrated

in prophase numbers are clear evidence that colchicine induces many changes in roots and, in particular, that it disturbs the pattern of entry of interphase cells into mitosis. The changes seen within 6 hours of treatment are further evidence that colchicine affects the growth factor status of meristems and thus affects mitotic cycles. Confirmatory evidence for this suggestion comes from treatments with colchicine and IAA.

2. Treatments With Colchicine and IAA

We have shown (Davidson, MacLeod and Taylor. New Phytologist 64, 393-398. 1965) that when primary roots inhibited by colchicine regenerate they grow faster if treated with IAA. Those results on primary roots have been extended by studies on lateral roots and lateral root primordia (Encl. 1, 2, 3, 4; Table 1).

a) Effects on Mitotic Cycles

Treatment with IAA together with colchicine alters the MI of meristems. Roots given joint treatments for 3 hours and examined 24 hours later show a reduction in MI. The reduction is slight with 0.329×10^{-4} M IAA and severe with $4.7-6.26 \times 10^{-4}$ M IAA. At 4.2×10^{-4} M IAA, the inhibitory effect of IAA balanced the stimulatory effect of colchicine and the MI was not significantly different from that of control roots (Encl. 6).

The interaction of the effects induced by colchicine and IAA is seen many hours after treatment. As with the effects on prophase numbers seen after treatment with colchicine alone it is clear that these compounds are inducing changes during interphase -- and these changes are later seen as changes in MI. IAA has this effect when given alone (Encl. 2, Table 1). 10^{-4} M IAA reduces MI in roots of V. faba. The effect is seen 5 hours after a 3 hour treatment and is significant after 14 hours. These results, and the changes in prophase numbers induced by colchicine (section 1c), are clear evidence of an effect during interphase. This effect could be operative during the G1, S or G2 phases of interphase.

To demonstrate directly that IAA alters the duration of a mitotic cycle we have treated roots with i) 0.025% colchicine, ii) 0.025% colchicine and 10^{-4} M IAA, iii) colchicine and 10^{-6} M IAA. Roots were treated for 3 hours and fixed at intervals: mitotic index and the time of appearance of tetraploid cells at metaphase was determined (Table 1).

Tetraploid metaphases were first found, in roots treated only with colchicine, 15 hours after the beginning of treatment. They did not appear till 18 hours in roots also treated with IAA; with 10^{-6} M IAA they were frequent at 18 hours (5.4% of all metaphases) but with 10^{-4} M IAA, though they were present at 18 hours, their frequency remained very low till 23 hours. The restitution nuclei from which these tetraploid

metaphases are descended were actually formed during, or soon after, the treatment with colchicine and IAA; therefore, most of the period of 12-15 hours in which they could be affected by the drugs was spent in interphase. This indicates that IAA is prolonging the mitotic cycle time of a marked population of cells that passes from mitosis into interphase soon after treatment. 10^{-4} M and 10^{-6} M both delay the entry of tetraploid cells into mitosis, the delay increasing with an increase in the concentration of IAA, but eventually, by 28 hours, the delayed cells come into mitosis -- then the frequency of tetraploid metaphases is higher in roots treated with IAA and colchicine than in roots treated only with colchicine.

This effect of IAA on tetraploid cells is exactly what was to be expected from previous results (Encl. 1, 2, 6). They, together with the effects of colchicine or colchicine and IAA mixtures on diploid cells (Encl. 1, 2, 5, 6; Table 1), suggest the following conclusions:

1. 0.025% colchicine (3 hours) affects interphase cells, stimulating some to enter mitosis. This combines with metaphase delay to increase MI.
2. Treatment with IAA reduces MI.
3. Treatment with IAA and colchicine leads to an initial reduction in MI (in about 4-8 hours) but this is followed by a rise in MI.

Table 1. 0.025% colchicine. 3 hours.

a)

t	3	9	15	18	23	27	31
MI	11.9	17.7	21.9	18.5	16.4	15.1	16.8
P	41	66	40	43	32	32	35
M	78	111	178	141	132	120	132

0.025% colchicine + 10^{-6} M IAA.

b)

t	3	9	15	21	27	31
MI	13.9	11.0	29.6	16.0	15.9	15.8
P	40	20	92	35	30	41
M	98	90	204	125	129	117

0.025% colchicine + 10^{-4} M IAA.

c)

t	3	9	15	18	21	23	27	31
MI	12.2	6.6	13.0	19.9	23.6	19.7	15.6	31.1
P	35	10	39	61	42	56	31	47
M	87	56	91	138	193	142	125	69

Frequency of 4n metaphases (per 100 M)

d)

t treatment	3	9	15	18	21	23	25	27	31
col	0	0	4.3	15.0	21.2	17.2	26.4	41.7	47.5
col + 10^{-6} M IAA	0	0	0	5.4	17.1	20.2	23.0	30.1	53.7
col + 10^{-4} M IAA	0	0	0	<0.5	<0.5	1.7	18.8	19.3	55.0

Mitotic index and frequencies of diploid metaphases and metaphases and tetraploid metaphases at different times after treatment, for 3 hours, with 0.025% colchicine, colchicine and 10^{-4} M IAA or colchicine and 10^{-6} M IAA.

4. It appears that the colchicine induced stimulation of interphase is reversed by IAA. With time, however, the effect of IAA wears off and the colchicine effect is then expressed.

5. The effect of colchicine appears to be due, at least in part, to an alteration in the levels of endogenous growth factors -- and that alteration can be reversed by addition of exogenous IAA.

This interaction between IAA and colchicine on mitotic cycles and mitotic index agrees with the results from studies of their interaction during root growth.

b) Effects on Root Growth

Roots of V. faba were treated with 0.025% colchicine for 1 hour and, 24 hours later, treated with IAA (3×10^{-4} M, 3×10^{-6} M or 3×10^{-8} M) for 24 hours. They were grown for up to 6 days. Other roots were treated only with IAA.

Treatment with IAA for 24 hours was found to kill 98% of primary and 86% of lateral roots. Pretreatment with colchicine, 1 day before IAA treatment, protected the roots and the numbers killed were reduced by 14% (primaries) and 62% (laterals). Thus pretreatment with colchicine increases the tolerance of roots to a subsequent exposure to a lethal concentration of IAA.

Though primary and lateral roots are seriously affected by IAA, primordia are not. These grow out and form laterals even after

treatments with IAA that kill fully-formed roots. This could be due to a failure of the IAA to penetrate to the primordia; that it is not so, however, was shown by measuring the growth of emerging laterals after treatment with IAA.

Roots were treated with colchicine. Laterals and small primordia emerge as growing laterals within 24-36 hours. A day after treatment with colchicine the roots were treated with IAA for 24 hours and their length measured 4 and 6 days later (Encl. 4, Table 3). Newly emerged roots treated with colchicine and then IAA produced the following growth in 6 days.

10^{-4} M IAA	-	4.8 ± 2.5 cms
10^{-6} M IAA		8.4 ± 3.4 cms
10^{-8} M IAA		8.9 ± 3.3 cms
colchicine alone		8.8 ± 3.0 cms

It is clear that 10^{-4} M IAA penetrates the primordia and delays their subsequent growth as lateral roots; it is equally clear that IAA does not inhibit primordia as severely as it does laterals and primary roots.

The results led us to conclude that colchicine treatment results in a fall in the levels of endogenous auxins. As a consequence i) there is a stimulation and then a fall in mitotic activity, ii) the sensitivity of roots to exogenous IAA changes. The time when mitotic activity falls (about 24 hours after treatment) coincides with the time when

roots lose their sensitivity to IAA and are actually stimulated by exogenous solutions. This reduction in the levels of endogenous auxin is important for the subsequent behavior of colchicine treated roots.

3. Studies of Primordia

It has been shown that large primordia are relatively insensitive to solutions of colchicine that inhibit primary and lateral roots (Davidson, 1965. Ann. Bot. 29: 253; Encl. 4). The results described in the previous section indicate that large primordia are also relatively insensitive to IAA. The observation that large primordia are not inhibited by concentrations of IAA that affect lateral roots provides us with a further system in which to study the systems that control cell division and cell growth. We have tested other methods by which we could compare the responses of lateral roots and primordia. The treatments we are now using are: i) exposure to 5-aminouracil, ii) labelling with H^3 -thymidine and iii) X radiation.

i) 5-Aminouracil...

Whole growing root systems of V. faba were treated for up to 24 hours with 500 ppm 5-aminouracil. Mitotic indices were determined in primary and lateral roots and large and small primordia at 6, 12, 18 and 24 hours. MI has fallen in all meristems by 6 hours and

remains low (1.0% or less) at 12 hours. In primary and lateral roots it continues to be low in 18 and 24 hour fixations. However, in large primordia it is rising at 18 hours and by 24 hours it is higher (15.9%) than in untreated large primordia (7.68%). 5-aminouracil also induces chromosome aberrations and their frequency is being scored in cells of laterals and large primordia.

ii) H^3 -thymidine studies

The recovery of cells of large primordia from the mitotic inhibition imposed by 5-aminouracil suggests that the cells become able to deaminate the base or that they can synthesize and utilize thymidine even in the presence of the analog. We, therefore, exposed root systems to solutions of H^3 -thymidine (1 μ c/ml) in order to study their incorporation patterns after treatment with 5-aminouracil and to determine mitotic cycle times. Four independent runs have been completed; at no time have we been able to label nuclei of cells of large primordia. It appears that they possess pools of DNA precursors that are larger or organized differently from the pools of lateral roots. Whatever the underlying mechanism, this failure to incorporate exogenous H^3 -thymidine is further evidence that large primordia are physiologically different from other meristems (cf. responses to colchicine, IAA and 5-aminouracil).

Small primordia do incorporate H^3 -thymidine, showing that the molecule can penetrate primordia. Labelled cells have been studied and estimates made of mitotic cycle times (see section 4).

iii) X-rays

Work has begun on the response of large primordia to X-rays. The initial experiment was designed to obtain an estimate of the duration of G1 in induced tetraploid cells. Roots were treated with colchicine (0.025%) for 3 hours and irradiated 2 hours later. Serial fixations were made and breakage was scored in diploid and tetraploid cells. The first tetraploid cells to enter division were found to have only chromatid aberrations - the aberration that is induced in S or G2. This indicates that tetraploid cells that take 12-15 hours to complete a mitotic cycle have completed G1 within 2 hours of the end of colchicine treatment. Until a method of labelling large primordia with H^3 -thymidine can be found, treatments with X-rays provide one of the possible means of timing the entry of interphase nuclei into S. These experiments are being pursued.

4. Determination of Mitotic Cycle Time

Other basic parameters that must be defined in the experimental system used in these studies concern the durations of mitotic cycles and the various phases of interphase. Determinations of these parameters have been made using H^3 -thymidine. Whole root systems of

V. faba were exposed to H^3 -thymidine ($1 \mu\text{C}/\text{ml}$) for 1 hour. The roots were washed and grown on. Growth was at 20°C . Serial fixations were made every 2 hours for 36 hours. Slides were dipped and exposed at 5°C for 14 days.

Mitotic cycle time has so far been determined for small primordia and primary root meristems.

Phase	Duration in Hours	Meristem
G1	4.9	Primary root
S	9.5	
G2	2.2	
mitosis	<u>1.16</u>	
Intermitotic time	17.8	
G1	< 0.89	Small primordium
S	6.6	
G2	> 3.2	
mitosis	<u>0.71</u>	
Intermitotic time	11.4	

The estimate for small primordia is close to the intermitotic time of 12 hours calculated from colchicine treated roots (Davidson, 1965. Ann. Bot. 29: 253). These new and more accurate estimates confirm that the development of primordia into lateral roots consists of an increase in cell number and a concomitant decrease in MI, lengthening of mitotic cycle time, change in sensitivity to colchicine, an auxin (IAA) a base analog (5-aminouracil) and a failure to incorporate exogenous H^3 -thymidine.

The results reported here, briefly, and more extensively in the reports of this work that have been submitted to press (Encl. 4) or are in preparation (Encl. 5) show that the developing root systems of V. faba are yielding important new data on the morphogenesis of meristems.

② Outline of Proposed Research

1. Cell cycle studies

Experiments have been started to determine mitotic cycle times in primary and lateral roots and large and small primordia. It is proposed to determine, using H^3 -thymidine, the durations of the G1, S, G2 and mitotic phases of a cycle; these determinations are being made in normal roots and in roots treated with colchicine and IAA. From these experiments it will be possible to define the time of action of IAA and colchicine during interphase; this should provide evidence on the relationship between IAA and chromosome replication. This evidence, it should be emphasized, is being obtained within one mitotic cycle. The effects of IAA can be detected on entry of cells into S, duration of S, duration of G2 and entry of cells into mitosis.

Comparative studies have been started of the durations of mitotic cycles in roots and primordia. These will provide data on the change in mitotic cycles that occur in root development. It is planned to