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# DNA SYNTHESIS IN RELATION TO POLYPLOID MITOSES IN EXCISED PEA ROOT SEGMENTS CULTURED *IN VITRO*[1]

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MITOSES can be stimulated in mature cells of both plant and animal tissues by a variety of procedures including wounding and hormone treatments. The initial response to these treatments may or may not involve DNA synthesis. Thus, Gelfant [10] observed that mouse ear epidermis contains populations of cells both in G1 and in G2; the latter cells can be induced to enter mitosis without any preceding DNA synthesis by wounding the ear. The initiation of mitoses by auxin and kinetin treatment of mature pea root segments cultured *in vitro* provides a convenient system in which to determine whether there are cells in a mature root in G2 which can be induced to divide and whether the action of auxin and kinetin in inducing mitoses involves the stimulation of DNA synthesis.

Both diploid and polyploid mitoses are initiated in 1-mm root segments when they are cut from the mature portion of a seedling pea root and grown on a sterile nutrient medium which contains auxin and a cytokinin such as kinetin [27]. There is a lag period between the time of the excision of the segment and the observed increase in mitotic frequency. For diploid mitoses this lag is about 24 hr; for polyploid mitoses it is about 60 hr [16].

When the segments were cultured on a medium lacking auxin and/or kinetin, no polyploid mitoses are observed; when the missing hormones are added to the culture medium at specific times after excision, one finds that auxin is required during the first 24 hr in culture and that kinetin and possibly auxin are required after the first 24 hr in order to obtain a normal frequency of polyploid mitoses at 74 hr [16].

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In the light of these results it seemed of interest to study whether a preceding period of DNA synthesis is required for the initial diploid and polyploid mitoses to occur in these excised segments and, if so, when such DNA synthesis takes place. It is known from microspectrophotometric absorption measurements that untreated pea roots contain cells with a sufficient amount of DNA to divide as polyploids without a preceding period of DNA synthesis [14]. If no DNA synthesis were required for initial polyploid mitoses, then this would prove that these cells were pre-existing polyploids and not cells which became polyploid as a result of the treatment. Partanen [19, 20] obtained this result studying auxin-stimulated polyploid mitoses in intact onion roots.

In this study, DNA synthesis in mature root cells under hormonal stimulus was analyzed in two ways. One method was to study the incorporation of tritiated thymidine, a specific precursor of DNA. A second method involved the use of an inhibitor of DNA synthesis, the deoxyuridine analogue 5-fluorodeoxyuridine (FUdR), which is supposed to act by competing with deoxyuridylate for the enzyme thymidylate synthetase in the presence of ATP [12] while having no effect on glycolysis or RNA synthesis in these cell suspensions studied [3]. In *Vicia faba* roots Taylor *et al.* [26] found that $10^{-4}$ *M* FUdR completely inhibited all mitoses within 4 hr after it was applied. The results from experiments on pea root cells are reported below.

## MATERIALS AND METHODS

*Preparation of media.*—The standard medium used for the cultivation of the root segments was the S-2 medium of Shigemura [24] with 1 mg/l kinetin added. This medium contains macro- and micronutrient salts, sucrose, vitamins, and amino acids. Liquid filter paper cultures as previously described were used in all experiments [16]. 5-Fluorodeoxyuridine, kindly provided by Hoffman–La Roche, New Jersey, was filter sterilized. Colchicine was also filter sterilized and was added to the culture medium in a final concentration of 0.002 per cent. No anaphase division figures were observed in the presence of this concentration of colchicine. Thymidine was sterilized by autoclaving. Tritiated thymidine ($^3$H-methyl thymidine, 28 mc/mg), purchased as a sterile solution from New England Nuclear Corporation, was included in the culture medium in a concentration of 0.2 $\mu$c/ml.

*Preparation of cultures and scoring of slides.*—Procedures for the culture, fixation and staining of root segments and the preparation and scoring of slides were the same as those described in Matthysse and Torrey [16]. The frequency of mitoses on each slide was determined. Counts were made of diploid division figures ($2n = 14$) and polyploid figures ($4n = 28$ or higher) and an arbitrary scoring scale established from 0 (no mitoses observed in a search of the slide lasting more than 10 min) through

½, 1, 2, 3, 4 to 5 (at least one mitosis in each microscope field using a 40 × objective lens). The use of an arbitrary scale was necessary since the mitoses were not randomly distributed on the slide, but, instead, tended to occur as clusters of diploid and/or polyploid mitoses. The maximum and minimum scores for slides of segments which had received the same treatment at the same time usually differed by not more than two units on the arbitrary scale. Slides were coded to prevent biasing of the results.

The experimental results were compared with the standard using the two-sided *t* test for the difference between means. Unless specifically noted otherwise, each experimental treatment involved about 5 segments and was repeated twice. The separate repetitions were each compared with a standard treatment run at the same time and the results of the comparison were combined using Fisher's method of combining significance tests [17]. The standard treatment used for comparisons in these experiments was 1-mm root segments cultured on the S-2 medium with 1 mg/l kinetin and fixed at 72 hr.

The experimental results are reported using the following categories of mitotic frequency and giving the confidence levels determined from the statistics: 0, none; +/−, no significant difference from none; +, greater than none; s −, less than standard; and s, no significant difference from standard. It was considered that the experimental treatment showed no significant difference from the standard if the probability of obtaining the result by random fluctuations was greater than 10 per cent.

*Procedures for autoradiography.*—Segments cultured in tritiated thymidine were washed in cold thymidine, fixed in 35 per cent isopropanol and stained with the Feulgen procedure. Kodak nuclear track emulsion type NTB3 was used and exposures were usually 1–2 weeks. Most of the $^{3}$H-labelled thymidine incorporation was nuclear although slight incorporation occurred in the cytoplasm as has been observed by other workers on occasion [4, 6].

*Sources of chemicals.*—5-Fluorodeoxyuridine: Hoffman La Roche, New Jersey. Tritiated thymidine: New England Nuclear Corporation. Thymidine: Mann Research Laboratories, Inc. Kinetin: Mann Research Laboratories, Inc. Colchicine: Fisher Scientific Co. Indole-3-acetic acid (IAA): Nutritional Biochemicals Corp. 2,4-Dichlorophenoxyacetic acid (2,4-D): Eastman Kodak Co.

## RESULTS AND DISCUSSION

### *The occurrence of DNA synthesis—studies using FUdR*

The effects of varying the concentration of FUdR, an inhibitor of DNA synthesis, on the frequency of mitoses in pea root segments in culture were determined. It was found that if FUdR was added to the culture medium at the beginning of the culture in a concentration of $4 \times 10^{-6}$ *M* or higher ($4 \times 10^{-5}$ and $4 \times 10^{-4}$ *M* were also tested), no diploid or polyploid mitoses were observed at 35, 46, 48, 50, 55, 72, 74, or 76 hr (Figs 1 and 2). If the concentration of FUdR used was $4 \times 10^{-7}$ some diploid mitoses were obser-

ved at 35 and 48 hr, but no diploid mitoses were found at 55 or 74 hr. A very few polyploid mitoses, less than one per segment, were observed at 35 hr and no polyploid mitoses were seen at the other times (Fig. 2).

To check whether there is a population of cells which can divide in the presence of $4 \times 10^{-6}$ *M* FUdR and whose existence had been missed by

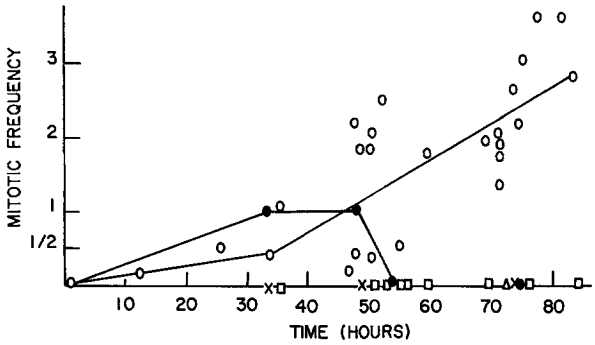


Fig. 1.—The change in diploid mitotic frequency with time after the beginning of the culture period. The mitotic frequency is given in arbitrary units which are defined in the text. The basic medium to which various concentrations of 5-fluorodeoxyuridine were added was liquid S2 medium with 1 mg/l kinetin. Each point represents the average score of 5 root segments. The FUdR concentrations used were: ○, none; ●, $4 \times 10^{-7}$ *M*; □, $4 \times 10^{-6}$ *M*; ×, $4 \times 10^{-5}$ *M*; △, $4 \times 10^{-4}$ *M*.

sampling at the wrong time, 0.002 per cent colchicine was added at 24 or 48 hr to cultures growing in the presence of FUdR. The segments were fixed at 49 and 73 hr, respectively. A very few diploid mitoses were observed in such segments, but no polyploid mitoses were seen.

In view of the data on the effects of varying the concentration of FUdR in other systems, the most probable explanation of these results is that concentrations of FUdR at or about $4 \times 10^{-6}$ *M* completely inhibit DNA synthesis and that $4 \times 10^{-7}$ *M* FUdR does not completely inhibit DNA synthesis. However, it is possible that FUdR inhibits some process other than DNA synthesis which is necessary for mitosis in pea root segments. This possibility may be supported by the observation that the nuclei of segments treated with FUdR had an abnormal, grainy appearance. Control experiments were carried out to test the hypothesis that FUdR does indeed inhibit mitosis by blocking DNA synthesis in pea root segments in culture. These control experiments included the use of uridine to minimize the effects of FUdR on RNA synthesis, the reversal of the FUdR inhibition of mitosis by thymidine, and studies of the effect of FUdR on the nuclear incorporation of tritiated thymidine.

Some investigators (e.g., [2]) have used uridine at the same time as FUdR to minimize the effects of FUdR on RNA synthesis. In one experiment, $10^{-5}$ *M* uridine and $4 \times 10^{-6}$ *M* FUdR were added to cultures of pea root segments 24 hr after the beginning of the culture period. No mitoses, either diploid or polyploid, were observed in these segments which were fixed

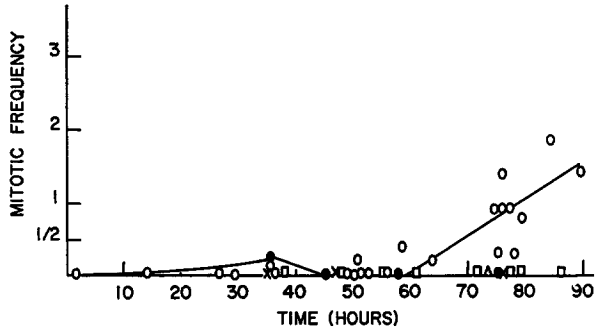


Fig. 2.—The change in polyploid mitotic frequency with time after the beginning of the culture period. The mitotic frequency is given in arbitrary units which are defined in the text. The basic medium to which various concentrations of 5-fluorodeoxyuridine were added was liquid S2 medium with 1 mg/l kinetin. Each point represents the average score of 5 root segments. The FUdR concentrations used were: ○, none; ●, $4 \times 10^{-7}$ *M*; □, $4 \times 10^{-6}$ *M*; ×, $4 \times 10^{-5}$ *M*; △, $4 \times 10^{-4}$ *M*.

at 74 hr. This result suggests that FUdR probably does not act by inhibiting RNA synthesis in pea root segments.

If FUdR acts by inhibiting thymidylate synthetase, one would expect that its effects could be reversed by the addition of thymidine. This has been found to be the case in other systems, for example, in L cells in culture [13] and in roots of *Vicia faba* [26]. Experiments to test this idea were performed with the cultured pea root segments.

If $4 \times 10^{-4}$ *M* thymidine alone was added to the standard culture medium, no effect on diploid or polyploid mitoses at 72 hr was observed. If $4 \times 10^{-4}$ *M* thymidine and $4 \times 10^{-6}$ *M* FUdR were added to the culture medium at the beginning of the culture, the frequencies of diploid and polyploid mitoses observed at 72 hr were equal to the standard. Thus the thymidine reversed the effect of the FUdR. The same complete reversal of the effect of FUdR was observed if $4 \times 10^{-4}$ *M* thymidine was added to the medium at the beginning of the culture, $4 \times 10^{-6}$ *M* FUdR was added at 25 hr, and the segments were fixed at 76 hr. However, if the FUdR was added to the medium before the thymidine, then the thymidine was less effective in reversing the inhibition caused by the FUdR.

Another way of checking whether FUdR does indeed act by blocking DNA synthesis is to determine its effect on tritiated thymidine incorporation. If $^3$H-thymidine (0.2 $\mu$c/ml, $3 \times 10^{-10}$ *M*) was included in the standard medium on which segments were cultured for 72 hr and autoradiographs of the stained and squashed segments were then prepared, about 60 per cent of the nuclei showed some incorporation of radioactivity. If $4 \times 10^{-6}$ *M* FUdR was added to the medium, only 20 per cent of the nuclei showed $^3$H-thymidine incorporation. (These figures remained constant with increasing times of exposure of the autoradiographs.) Thus FUdR decreases, but does not completely block, the nuclear incorporation of $^3$H-thymidine. The fact that some thymidine can be incorporated in the presence of FUdR may be because the site of action of FUdR is at a point preceding the completion of thymidine synthesis. Thus Bosch *et al.* [3] found that $^3$H-thymidine was incorporated into DNA thymine in the presence of a concentration of FUdR high enough to prevent the incorporation of $^{14}$C-formate into DNA thymine [12].

Both the results with thymidine reversal of FUdR inhibition and with tritiated thymidine incorporation support the hypothesis that the main effect of FUdR in this system is to inhibit DNA synthesis. Assuming that FUdR does act primarily to inhibit DNA synthesis in this system one is led to conclude that the majority of the cells in these segments which are stimulated to divide may be in G1 at the time of the excision of the segment from the root. If there were populations of cells in G2, one would expect that these cells would divide in the presence of FUdR except at concentrations high enough to affect such processes as respiration, RNA synthesis, or protein synthesis.

*The occurrence of DNA synthesis—studies using tritiated thymidine*

The study of the incorporation of tritiated thymidine into diploid and polyploid mitoses was used as another way to determine whether DNA synthesis was involved in the initial diploid and polyploid mitoses.

0.2 $\mu$c/ml $^3$H-thymidine was included in the standard culture medium. The segments were fixed at 52 and 73 hr, autoradiographs were prepared, and the per cent of diploid and polyploid mitoses which showed incorporation of radioactivity was determined. The results are shown in Table I. At 52 hr 64 per cent of the diploid mitoses showed incorporation and no polyploid mitoses, labeled or unlabeled, were observed. At 73 hr 58 per cent to 80 per cent of the diploid mitoses and 47 to 53 per cent of the polyploid mitoses showed incorporation of radioactivity. The per cent of mitoses which

showed incorporation of radioactivity was very variable. This was probably due to uncontrolled variations in many factors including the contact between the segment and the medium (this would affect how much $^3$H-thymidine got into the segment), possible differences in the distribution of the $^3$H-thymidine

TABLE I. *Occurrence of DNA synthesis.*

Segments grown on S2 medium and 1 mg/l kinetin and $^3$H-thymidine.

| Fixation time | Additions[a] | Time of additions (hr) | Labeled[b] diploid divisions % | Labeled[b] polyploid divisions % | Labeled[c] nuclei % |
|---|---|---|---|---|---|
| 52 hours | — | — | 64 (78) | — | 60 |
| | Colchicine | 23 | 42 (45) | 50 (4) | 54 |
| | FUdR[e] | 0 | — | — | 19 |
| 73 hours | — | —[d] | 58 (94) | 47 (17) | 62 |
| | | | 80 (84) | 53 (32) | 65 |
| | Colchicine | 23[d] | 49 (72) | 36 (39) | 56 |
| | | | 47 (189) | 52 (122) | 58 |
| | | 48[d] | 60 (35) | 67 (9) | 55 |
| | | | 52 (205) | 57 (103) | 57 |
| | FUdR[e] | 0 | — | — | 19 |

[a] The concentrations used were colchicine—0.002 %, and FUdR—$4 \times 10^{-6}$ *M*.
[b] Numbers in parentheses are total numbers of divisions counted.
[c] Based on counts of more than 500 nuclei.
[d] The results of two experiments are given separately.
[e] No divisions either diploid or polyploid were observed in the presence of FUdR.

in the various tissues of the segment, and variations in the thickness of the squash preparation on the slide and in the thickness of the emulsion (the thicker the squash, the fewer exposed grains; the thicker the emulsion, the more exposed grains).

Colchicine was added to some culture tubes so that the completion of mitosis would be prevented and the $^3$H-thymidine incorporation seen would all be due to incorporation by cells before their initial mitoses in culture. If 0.002 per cent colchicine was added at 23 or at 48 hr and the segments were fixed at 52 and 72 hr, between 35 and 60 per cent of the diploid and polyploid mitoses showed incorporation of radioactivity.

Torrey [28] studied tritiated thymidine incorporation in this same system of pea root segments in culture. He found that at 3 days 38 per cent of the diploid mitoses and 5 per cent of the polyploid mitoses showed incorpora-

tion of radioactivity. He also found that about 35 per cent of the nuclei were labeled. The results reported here agree with his as to the per cent of diploid mitoses labeled. However, a larger per cent (35 to 60 per cent) of the polyploid mitoses and of nuclei (60 per cent) were labled in these experiments than in his experiments. The cause of these differences is not known. The only variation in method in the two experiments is that Torrey's experiments used solid agar medium while liquid medium was used in these experiments.

The above data were determined using exposure times for the autoradiographs such that there were few enough grains in the exposed emulsion for the chromosomes of the mitotic figures to be clearly visible. If the exposure time was increased by a factor of approximately 3, then most of the nuclei which showed incorporation of label were covered with grains so that the ploidy of dividing nuclei or even whether the nuclei were dividing was impossible to ascertain. This longer exposure time resulted in an increase of about 10 per cent in the per cent of labeled nuclei. Since it was not possible to determine the number of labeled mitoses on such slides with any accuracy, the slides were searched for unlabeled mitotic figures. An average of less than 10 unlabeled diploid mitoses and less than 2 unlabeled polyploid mitoses were found in each segment which had received $^3$H-thymidine at the beginning of the culture and colchicine at 24 hr. Data from similar, unlabeled slides suggest that these figures represent less than 10 per cent of the total diploid and polyploid mitoses on each slide.

There were areas on the slides showing heavy or light label which suggests that although the $^3$H-thymidine reached most of the cells, some cells or regions of the segment received much more than others. The fact that the per cent of labeled mitoses increased more with increasing exposure times than did the per cent of labeled nuclei is not understood.

Thus most of the cells which give rise to diploid and polyploid mitoses probably synthesize some of the DNA necessary for these mitoses during the time in culture. This agrees with the results of the experiments reported above using FUdR. There are, however, some dividing cells, both diploid and polyploid, which showed no tritiated thymidine incorporation. These cells were probably in G2 at the time of the excision of the segments, and so were able to divide once without any preceding DNA synthesis.

Partanen [18, 19] did similar experiments on tritiated thymidine incorporation using onion roots which were treated with 2,4-D to induce polyploid divisions. He reported that interphase nuclei in the mature region of the root were labeled sporadically; some diploid divisions were lightly labeled, but none of the polyploid divisions were labeled. He concluded that no DNA

synthesis was necessary for the initial polyploid mitoses. However, he used intact plants. Van 't Hoff [32] working with the incorporation of tritiated thymidine in the meristematic region of pea roots found that the incorporation into roots of intact plants was much less than the incorporation into excised 1-cm root tips. Thus Partanen's failure to show tritiated thymidine incorporation may be due to a lack of uptake of the compound rather than the absence of DNA synthesis. On the other hand, it may reflect a real difference between the stimulation of polyploid mitoses in onions and in peas. That such a difference exists is suggested by the fact that polyploid mitoses can be stimulated in onion roots by auxins alone, while the stimulation of polyploid mitoses in excised pea root segments requires the presence of a cytokinin as well as an auxin.

Pelc and LaCour [22] studied the incorporation of tritiated thymidine into roots of intact seedlings of *Vicia faba*. They observed labeled nuclei both in the meristem and in the region of elongation. Since cells in the region of elongation in the intact root divide very infrequently the incorporation of tritiated thymidine into nuclei of these cells could be due to DNA synthesis associated either with polyploidization or with some form of DNA turnover.

Deeley *et al.* [9] made microspectrophotometric absorption measurements of the amount of DNA per nucleus in *Vicia faba* roots. They observed nuclei which contained more than the diploid amount of DNA in the region of elongation. Thus the tritiated thymidine incorporation by these nuclei could be due to DNA synthesis and polyploidization. This is the interpretation of Tschermak-Woess based on similar experiments with tritiated thymidine incorporation [29].

*Time of DNA synthesis—studies using FUdR*

From the data reported above we know that most of the initial diploid and polyploid mitoses were preceded by DNA synthesis. The time of this DNA synthesis was studied using the same two techniques previously employed—the addition of FUdR to the cultures and the addition of tritiated thymidine and colchicine to the cultures.

The results are shown in Table II. Each value represents the average response for 5 or more segments. In cultures to which no FUdR was added, diploid mitoses were observed after the first 24 hr in culture, but no polyploid mitoses were observed until about 60 hr [16]. Thus, regardless of treatment, all cultures fixed before 60 hr show no polyploid mitoses. If FUdR was added to the cultures more than 30 hr before fixation, no mitoses either diploid or polyploid were observed. If FUdR was added to the cul-

tures less than 10 hr before fixation, the frequency of diploid and polyploid mitoses was not affected and was equal to that found in non-treated cultures. If FUdR was added to the cultures between 10 and 30 hr before fixation, some diploid and polyploid mitoses were observed, but these were less frequent than in untreated controls. These results would indicate that DNA synthesis is not yet complete about 30 hr before mitosis and that DNA synthesis has been completed by about 10 hr before mitosis. These times are only approximate and must be longer than the actual times since they also include the time required for the uptake of FUdR. Even taking into account the relatively crude method of determing the times, it is an interesting observation that, relative to the time of mitosis, the timing of DNA synthesis seems to be about the same for diploid and polyploid mitoses. This

TABLE II. *The effect of the time in FUdR on mitotic frequency.*

| Time of FUdR[a] addition (Ti) (hr) | Time of fixation[b] (Tf) (hr) | Time spent in FUdR (Tf–Ti) (hr) | Category of mitotic frequency[c] | |
|---|---|---|---|---|
| | | | Diploid mitoses | Polyploid mitoses |
| — | 72 | 0 | Standard | Standard |
| 0 | 84 | 84 | 0 | 0 |
| 3 | 74 | 71 | 0 | 0 |
| 0 | 60 | 60 | 0 | 0 |
| 25 | 76 | 51 | 0 | 0 |
| 60 | 98 | 38 | 0 | 0 |
| 85 | 118 | 33 | S−* | 0 |
| 59 | 91 | 32 | S−* | +/− |
| 45 | 75 | 30 | +/− | 0 |
| 74 | 103 | 29 | S−* | +/− |
| 49 | 76 | 27 | S | S−* |
| 74 | 97 | 23 | S | S |
| 75 | 97 | 22 | S−* | S−* |
| 48 | 65 | 17 | S | S |
| 61 | 74 | 13 | S | S |
| 75 | 84 | 9 | S | S |
| 69 | 75 | 6 | S | S |

[a] $4 \times 10^{-6}$ *M* FUdR added to S2 medium with 1 mg/l kinetin.

[b] No data are given for segments fixed before 60 hr since no polyploid mitoses were observed in such segments cultured in the presence or absence of FUdR.

[c] Categories of mitotic frequencies are reported as follows: O, none; +/−, no significant difference from none; S−, less than standard; S, no significant difference from standard.

* These data are significant at the 0.01 level. The statistical methods used are described in the text.

lack of effect of the ploidy of the cells on the timing and duration of DNA synthesis has also been observed in haploid and diploid embryos of *Xenopus laevis* [11].

*Time of DNA synthesis—studies using tritiated thymidine*

Results of experiments on the time of DNA synthesis obtained using tritiated thymidine, shown in Table III, are in good agreement with those obtained using FUdR. Tritiated thymidine (0.2 μc/ml) was added to the culture medium at the beginning of the culture period or at 24 hr or at 48 hr after the beginning of the culture. Colchicine (0.002 per cent) was added either at the same time as the tritiated thymidine or 24 or 48 hr after the addition of the tritiated thymidine. Colchicine was used to prevent any of the cells from going through more than one complete mitotic cycle in the presence of the tritiated thymidine. The segments were fixed at 74 hr.

If the DNA synthesis for the initial mitoses occurred primarily during the first 24 hr in culture, then the segments exposed to tritiated thymidine from 0 time should show a much higher per cent of labeled mitotic figures than those segments which were exposed to tritiated thymidine only after 24 or

TABLE III. *Time of DNA synthesis.*

Segments grown on S2 medium and 1 mg/l kinetin and fixed at 73–74 hr.

| Time of $^3$H-thymidine addition[a] (hr) | Time of colchicine addition[b] (hr) | Labeled diploid mitoses[c] % | Labeled polyploid mitoses[c] % | Labeled nuclei[d] % |
|---|---|---|---|---|
| 0 | None[e] | 58 (94) | 47 (17) | 62 |
| | | 81 (84) | 53 (32) | 65 |
| | 24[e] | 49 (72) | 36 (39) | 56 |
| | | 47 (189) | 52 (122) | 58 |
| | 48[e] | 60 (35) | 67 (9) | 55 |
| | | 52 (205) | 57 (103) | 57 |
| 24 | 24 | 63 (156) | 69 (104) | 43 |
| | 48 | 39 (137) | 42 (100) | 47 |
| 48 | 48 | 58 (152) | 55 (110) | 29 |

[a] Final concentration—0.2 μc/ml $^3$H-thymidine.
[b] Final concentration—0.002 % colchicine.
[c] Numbers in parentheses are total numbers of mitoses counted.
[d] Based on counts of more than 500 nuclei.
[e] The results of two experiments are given separately. These data are also included in Table I.

48 hr in culture. Similarly, if DNA synthesis occurred between 24 and 48 hr, then cultures to which tritiated thymidine was added at 0 or 24 hr should show a higher per cent of labeled mitoses than cultures to which tritiated thymidine was added at 48 hr. However, if DNA synthesis occurred during the last 24 hr before mitosis, then cultures to which tritiated thymidine was added at 0, 24, or 48 hr should all show roughly the same per cent of labeled diploid and polyploid mitoses. This is what was actually observed. None of the figures in Table III are significantly different from each other except for the per cent of labeled nuclei (last column) observed when tritiated thymidine and colchicine were added at 48 hr. Presumably this figure is lower than the others since some diploid cells divide before 48 hr (and thus synthesize DNA before 48 hr) and may not have had time to reach the S phase (DNA synthesis phase) of the mitotic cycle again before fixation at 74 hr.

Thus for the cells which must synthesize DNA in order to divide, the first 6 to 12 hr of the lag period for the initial diploid mitoses and the first 36 to 48 hr of the lag period for the initial polyploid mitoses may be spent in G1. This means that for those cells which synthesize DNA most of the lag period and delay in the time of the increase in the frequency of polyploid mitoses is due to the long time spent in G1.

The times for the various phases of the mitotic cycle in normal pea root meristem cells are as follows: G1—7 hr, S—5 hr, G2—0.8 hr, and M—2.5 hr [30]. The mitotic cycle time for colchicine-induced polyploids is 1 hr shorter than that for normal diploids [31]. Thus the cells in these root segments, both diploids and polyploid, which were stimulated to divide have a greatly lengthened mitotic cycle time and those cells which synthesize DNA also have a very long G1 compared with the cells of the meristem.

This is, of course, assuming that all DNA synthesis occurs in one time interval of the mitotic cycle in pea root cells, which seems reasonable for the cells which give rise to diploid mitoses. Van't Hof's [30, 31] results in making measurements on pea root meristem cells support this assumption. However, Woodard *et al.* [35] observed a biphasic DNA synthesis in cells of the root meristem of *Vicia faba*. Most of the DNA synthesis occurred in mid-interphase, but there was also a small amount of DNA synthesis just before the beginning of prophase.

In the initiation of callus cultures from Jerusalem artichoke tuber tissues, a lag period was also observed between the beginning of the culture and the first mitoses which occurred at 36 hr. Tritiated thymidine was incorporated by these cells only after the first 12 hr in culture and microspectrophotometric measurements showed that all the cells initially contained the 2C

amount of DNA. Thus these cells were all in G1 at the time of excision [23, 26]. (This is a plant which contains only diploid cells and cultures derived from it contain only diploid mitoses [18].) There are other cases in which cells which are not going to divide again for a long time were found to be in G1 in the intact plant. Thus Clowes [5] found that the cells of the quiescent center of the root meristem of the *Zea mays* (these are cells which divide only about one tenth as often as the other meristematic cells) spend most of the extra time required for their mitotic cycle in G1.

The stimulation of mitoses of cells which were initially resting in G2 has been reported in germinating roots of *Triticum* [1], *Zea mays* [25], and *Vicia faba* [8, 34], and in ear epidermis, Gelfant [10].

The assumption that all DNA synthesis occurs in one time interval between the time of excision and mitosis is not necessarily true for the cells which give rise to polyploid mitoses. If the polyploid mitoses come from preexisting polyploid cells, then one period of DNA synthesis before mitosis may be all that is required. However, if the polyploid mitoses come from diploid cells, then two periods of DNA synthesis may occur between the time of excision and mitosis. The present data do not distinguish between these two possibilities. DNA synthesis does occur in the cells which give rise to polyploid mitoses between 24 and 10 hr before mitosis. However, since pulse labeling experiments were not feasible in these pea root segments, it was not possible to determine if another cycle of DNA synthesis occurred earlier.

The results on the timing of DNA synthesis can be combined with the results on the timing of the hormone requirements for polyploid mitoses [16]. The picture which this produces for polyploid mitoses is as follows: There is an initial phase in the culture (the first 24 hr after excision) during which auxin must be added, and no kinetin is required. This is followed by a period in which kinetin is required (from 24 hr until mitosis at 60 to 74 hr). There may also be an auxin requirement for this phase. In many of the cells DNA synthesis occurs after about 48 hr (in these cells there may also be an earlier cycle of DNA synthesis). Mitosis follows about 10 hr after the completion of DNA synthesis. Thus the presence of kinetin and probably auxin may be required for DNA synthesis, but the addition of the hormones probably does not immediately trigger DNA synthesis in these cells. Instead there may be at least two other kinds of events triggered by the hormones which precede DNA synthesis. The first of these requires only auxin. The second requires kinetin and may require auxin as well.

Kinetin and auxin are known to affect DNA synthesis in tobacco pith

tissue. Patau, Das, and Skoog [21] found that the provision of auxin and kinetin together to cultures of tobacco pith tissue stimulated DNA synthesis, but auxin or kinetin alone was also able to stimulate a smaller amount of DNA synthesis [7]. In this tissue DNA synthesis preceded mitosis by about 6 hr. There was also a differential effect on cells of various ploidy levels. The higher the ploidy the less likely the cell was to respond to the hormones by dividing [21]. The increasing difficulty of stimulating mitosis in cells of higher ploidy was also observed in pea root segments. Diploid cells were able to divide in the excised segments cultured on water alone, but tetraploid divisions were observed only in the presence of salts, sucrose, vitamins, auxins, and kinetin [16]. The mechanism of the action of the hormones which produces these polyploid mitoses is not understood, but the evidence given above makes it unlikely that they act by directly stimulating DNA synthesis.

## SUMMARY

Diploid and polyploid mitoses were observed in pea root segments cultured on a medium containing auxins and kinetin for 72 to 74 hr. Initial diploid mitoses were observed as early as 24 hr after the beginning of culture; initial polyploid mitoses were observed only after about 60 hr. The presence of kinetin was not required for the first 24 hr in culture for the appearance of polyploid mitoses at 74 hr. However, kinetin must be present in the culture medium after 24 hr. Auxin was required during the first 24 hr of the culture and its continuous presence may be required for polyploid mitoses.

The question whether DNA synthesis must occur preceding the initial diploid and polyploid mitoses in cultured segments was studied using 5-fluorodeoxyruridine and tritiated thymidine. The results, while not definitive, support the hypothesis that DNA synthesis is required for most of the mitoses, both diploid and polyploid. The same methods were used to study the time of DNA synthesis. In those cells in which DNA synthesis took place it seemed to occur between about 24 and 10 hr before mitosis for both polyploid and diploid mitoses.

The results on the timing of the hormone requirements and the time of DNA synthesis make it unlikely that auxin or kinetin acts in this system primarily by directly triggering DNA synthesis.

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