KINETIN AS TRIGGER FOR MITOSIS IN MATURE ENDOMITOTIC PLANT CELLS[1]

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In root development, cell divisions at the root tip are predominantly localized in the first few millimeters of the typical dicot seedling root. These divisions give rise to the cells which form the mature root structure. By differential enlargement and maturation processes these cells form the distinctive mature tissue systems of the primary root: the epidermis, cortex, endodermis, pericycle, and the xylem and phloem tissues of the central cylinder. Once these tissues are differentiated, their cellular components seldom undergo further mitosis or cytokinesis, but remain as mature differentiated cells of the root. Under appropriate internal stimulus, the pericycle cells may undergo cell division to give rise to lateral roots or at a late stage in root development to vascular cambium or cork cambium.

The dividing cells of the root meristem typically possess the 2n or diploid number of chromosomes, the so-called "somatic" chromosome number [9]. This same chromosome number is usually found also in the pericycle even in mature root tissues, so that cells of lateral root meristems derived from the pericycle possess the same diploid chromosome complement as the main axis tip.

It is now well established from extensive cytological studies (cf. reviews by D'Amato [7], Geitler [11]), that in a very large number, if not in most higher plants, both monocots and dicots, the cells of mature root tissues, notably cells of the cortex, endodermis, and metaxylem, undergo chromosome doubling without subsequent mitosis such that the DNA content of individual nuclei may be double or quadruple that in the diploid cell of the meristem. Such chromosome doubling by endomitotic reduplication is a

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usual concomitant of cellular maturation in many cell types of both the root and the shoot.

The occurrence of chromosome doubling in mature tissues was first detected by artificially stimulating mature cells to divide by wounding (e.g. [6]) or by treatment with externally applied auxin [17]. That the doubling had occurred prior to these artificial treatments rather than being induced by the treatment has been well established [2, 5, 25], but little is known about the factors influencing endomitotic reduplication or, during the normal course of root development, the internal conditions which inhibit these cells from further mitotic division once chromosome doubling has occurred.

In earlier work on the initiation of callus tissue from mature pea root tissues [27], it was observed that certain components of the complex yeast extract–2,4-D medium used to establish the callus tissue apparently selectively stimulated cell division in cells with the tetraploid chromosome complement at the expense of the diploid population such that, over a long period of culture, an essentially tetraploid population was established. In a defined synthetic medium containing auxin (as 2,4-dichlorophenoxyacetic acid) which maintained excellent callus tissue growth, but which lacked yeast extract, only diploid cells underwent cell division. Thus, some component of yeast extract, lacking from the synthetic medium, was responsible for the production of the tetraploid population. It was found at the outset [26] that kinetin, the cell division factor of Miller *et al.* [19] could replace the yeast extract in effecting rapid division of tetraploid cells. The experiments described below are concerned with the nature of the kinetin effect, the type of cells affected, and the mechanism of the action.

MATERIALS AND METHODS

Seeds of the garden pea, *Pisum sativum*, variety Alaska, were surface-sterilized in 0.1 per cent mercury bichloride solution for 20 min, rinsed six times with sterile glass distilled water then placed in sterile Petri dishes with sterile water to germinate in the dark at 25°C for 60 hr. At this time the radicles were about 15 to 20 mm in length. A cylinder of root 1 mm thick was excised from each root by two cuts at 10 and 11 mm behind the root apex. Each segment was transferred aseptically to nutrient agar medium and placed with one of the cut surfaces down on the medium. Usually eight segments were cultured in each 11 cm Petri dish containing 20 ml of medium.

At intervals of 1, 2, 7, and 14 days, several segments from each treatment were removed and fixed with aspiration in acetic acid alcohol (1 part glacial acetic acid to 3 parts absolute ethyl alcohol). The tissues were removed before the end of 24 hr in the fixative, were rinsed in 70 per cent ethyl alcohol and then stored in fresh 70 per cent

alcohol. Squash preparations were best made within a few days as the Feulgen staining was less dependable with increased periods of storage after fixation.

Staining procedures.—The Feulgen staining procedure and squash preparations were made according to methods described by Darlington and LaCour [8]. Segments of a given treatment were transferred from the 70 per cent ethyl alcohol to a small cork-stoppered vial containing 1 *N* HCl preheated in an incubator to 60°C and the vials were quickly returned to the 60°C temperature. The optimum period for hydrolysis for this material was found to be 10 min at 60° C, although variation in hydrolysis time was sometimes necessary. Then the root segments were immediately removed from the acid with forceps and transferred to small Bureau of Plant Industry Syracuse dishes containing 1 ml of Schiff's basic fuchsin reagent, where they were allowed to stain in the dark for two hours. Sets of 6 to 8 treatments each comprising 2 to 4 segments could be handled conveniently in one series of operations.

After the staining period in Schiff's reagent, the pieces were transferred to bleach solution (SO_2 water), which was changed at 10 min intervals three times and then a series of rinses with tap water followed for another period of about 30 min. At the end of this time, the segments were ready for squashing and mounting.

A segment was transferred into a drop of 45 per cent aqueous acetic acid on a clean glass slide and teased apart with needles into four or five small pieces. In many cases, the segment was cut in half and handled on two slides, depending on the size of the tissue piece. A clean glass coverslip of No. 1 thickness was coated very thinly with albumen and dried gently over an alcohol flame. When cool, the coverslip was placed over the tissue pieces with the coated side down. Gentle pressure with the wooden handle of the needle was first applied to spread the tissue pieces, then the coverslip was covered with several layers of filter paper and pressure was applied with the thumb to produce the thinnest possible squash. Excessive liquid under the coverslip was taken up by the filter paper. Each slide was then gently warmed by two or three rapid passes over the alcohol flame.

In these studies, permanent mounts were desired so that the extensive counts which had to be made could be made at convenient time periods. Each slide was inverted in 40 per cent ethyl alcohol and supported at both ends with glass slides so that the coverslip could readily drop off into the alcohol solution. Usually one to several minutes in alcohol effected removal of the coverslip with the adhering tissue which was then transferred with forceps to 80 per cent ethyl alcohol in a Columbia staining dish. Subsequent transfers at about two-minute intervals were made through each of two dishes containing absolute ethyl alcohol and two dishes containing pure xylene. Final mounting of the squash preparation was made with Michrome mountant (B. T. Gurr, Ltd., London). The slides were placed flat in dust-free drawers and allowed to dry for about two days when studies under oil immersion could be made. All slides were coded so that the treatment could not be known at the time counts were made.

In some treatments histological sections rather than squash preparations were made. Tissue pieces were fixed with aspiration in Randolph's modified Navashin fluid (Craf) made up according to Johansen [15]. After 12–24 hr in fixative, they were transferred to 70 per cent alcohol, changed several times, then stored in 70 per cent ethyl alcohol. Paraffin embedding involved the usual tertiary-butyl alcohol series for dehydration and embedding was done in Tissuemat. Sections were cut at

10 μ, mounted with Haupt's adhesive and formalin. The Feulgen staining procedure was used essentially as described above with a period of 10 min hydrolysis followed by staining in Schiff's reagent for 2 hr, bleaching with SO_2 water, rinsing in tap water, a counter stain with fast green, and finally mounting after dehydration through ethyl alcohol and xylene.

The nutrient media used.—In earlier studies [27, 28] it had been found that pea root tissue grown in a nutrient medium containing yeast extract and the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) increased rapidly in fresh and dry weight and in cell number, but showed a considerable complexity in its cytological constitution, containing a mixed population of dividing cells of diploid, tetraploid, octaploid and even higher chromosome numbers. In addition, in long term culture, cytological abnormalities in mitoses became apparent.

Shigemura [24] was able to develop a completely synthetic nutrient medium which was capable of maintaining the continuous growth of pea root callus tissue at a rate almost comparable to that for the complex yeast extract medium. This medium, referred to hence forth as the synthetic medium (S medium) was based on the medium used for the culture of excised pea roots with the addition of components leading to callus tissue development. The S medium contained the following components per liter of solution: 242 mg $Ca(NO_3)_2 \cdot 4H_2O$; 42 mg $MgSO_4 \cdot 7H_2O$; 85 mg KNO_3; 61 mg KCl; 20 mg KH_2PO_4; 2.5 mg $FeCl_3 \cdot 6H_2O$ (freshly prepared stock solution); 0.1 mg thiamin HCl; 0.5 mg nicotinic acid; 1.5 mg H_3BO_3; 1.5 mg $ZnSO_4 \cdot 7H_2O$; 4.5 mg $MnSO_4 \cdot H_2O$; 0.25 mg $Na_2MoO_4 \cdot 2H_2O$; 0.04 mg $CuSO_4 \cdot 5H_2O$; 40 g sucrose and 7 g Difco Bacto-agar. To these were added 10^{-6} *M* 2,4-dichlorophenoxyacetic acid, and 10^{-6} *M* indoleacetic acid and the medium was steam autoclaved for 15 min at 15 lb/in^2. Just prior to solidification of the cooling nutrient agar medium, a solution of organic nitrogen components which had been previously cold-sterilized by Seitz filtration was added by sterile pipette. The final concentration of these components per liter of solution was as follows: glutamic acid, aspartic acid, glycine, asparagine, arginine, and urea all at 10^{-3} *M*. Sterile 0.1 *N* NaOH solution was also pipetted to bring the pH of the final cold nutrient medium to pH 5.5. Additions to this synthetic medium of compounds to be tested were always made with Seitz-filtered solutions and pH adjustment to pH 5.5 made with sterile NaOH solution. Glass-distilled water was used for all solutions.

Nuclear counts.—The following procedure was used in determining the per cent of cells in division and the proportion of the dividing cells of different chromosome numbers. In the latter case, a search for mitotic figures in late prophase, metaphase and early anaphase was made using a low power objective (usually 100 × magnification). Each figure was then examined with the oil immersion objective (approximately 900–1200 × magnification) and a count of chromosomes made. When an actual count could be made, it was recorded together with the coordinates of its location from the mechanical stage. When an accurate estimate of chromosome number was not possible, the figure was not counted. Usually several areas on each of two slides from two separate root tissue pieces were counted. At least 50 nuclei in division were scored for each treatment.

In the determination of the per cent of cells in division, independent counts were made. The oil immersion objective was focussed on the edge of a tissue mass and a band of cells the width of the field was scanned across the tissue mass and a record

made of the stage of mitosis of every cell observed in the band of tissue. This procedure was repeated at random in each tissue mass on each slide until a total of 1000 cells had been counted for each treatment. From these counts it was possible to express the per cent of cells in division and also the per cent of dividing cells in each stage of mitosis.

RESULTS

The effects of complex nutrients and nucleic acid derivatives on mitosis.—It had already been shown [27] that powdered Difco yeast extract contained components which, in the appropriate medium, led to the rapid proliferation of tetraploid cells and cells of higher ploidy in cultured mature pea root segments. In contrast, the synthetic medium devised by Shigemura [24] produced active proliferation of only cells with the diploid number of chromosomes. The contrasting behavior of the tissues on these two media made possible the search for a yeast extract component active in effecting mitosis in polyploid cells. By the addition of known compounds to the synthetic medium, it should be possible to discover substances which act to stimulate cell division of polyploid cells. At the outset of this work, several complex mixtures used in tissue culture were tested as well as a group of compounds known to be involved in nucleic acid metabolism.

In Table I are summarized the results of these preliminary tests. Mature pea root segments were cultured for 7 days on the synthetic medium containing the constituent noted and then tissue pieces were stained, squashed and chromosomal counts of dividing nuclei were made. It is interesting to note that both powdered yeast extract and coconut milk are active in producing mitoses in polyploid cells. Of the other compounds tested at a number of concentrations, only 6-furfurylaminopurine (kinetin), the cell-division factor isolated and described by Miller *et al.* [19] had significant effect and it, at a concentration of 1 ppm (3.2×10^{-6} *M*), produced essentially the same effect in this assay as the complex mixtures. Because of its remarkable activity in this test, further work was concentrated on the kinetin activity.

The effects of kinetin and kinetin analogues on mitoses in mature root segments.—A comparison was made of the distribution of dividing cells of different chromosome number in tissues on the synthetic medium with and without kinetin during the period of initial establishment of callus tissues from mature pea root segments. Chromosome counts made of the two types of tissues after 1, 2, and 7 days are shown in Fig. 1. On the synthetic medium alone a few tetraploid mitoses were observed after 7 days. All other divisions were of diploid cells. In the presence of 1 ppm kinetin, however, in 2 days

over 50 per cent of the dividing cells were tetraploid cells and by 7 days over 70 per cent of the dividing cells were tetraploid. Thus, one can reproduce the effect of yeast extract reported earlier ([27], cf. Fig. 7–10) by the simple addition of a very small amount of the synthetic substance, kinetin.

TABLE I. *Per cent of dividing cells in each ploidy class in mature pea root segments cultured for 7 days on a synthetic medium with added constituents related to nucleic acid components.*

The count in each treatment includes at least 50 dividing nuclei.

Nutrient medium	% of dividing cells in each ploidy class		
	2*n*	4*n*	8*n*
Synthetic medium (S)	100	0	0
S + powdered yeast extract (0.1 %)	30	60	10
S + 10 % coconut milk	28	72	0
S + yeast nucleic acids (0.0025–0.0125 %)	100	0	0
S + adenine sulfate (1, 10, 40 ppm)	100	0	0
S + guanine (0.1, 1.0 ppm)	100	0	0
S + thymidine (0.1, 1.0, 5.0 ppm)	100	0	0
S + 2, 6-diaminopurine (0.1, 1.0, 5.0 ppm)	100	0	0
S + adenosine (50 ppm)	98	2	0
S + 6-furfurylaminopurine (1 ppm)	31	68	1

Kinetin is active in producing this effect at very low concentration. In Table II are presented data showing the effect of a range of kinetin concentrations on mitosis in the tissue (i.e., the percentage of cells in division in the tissue mass as a whole), on the course of mitosis as judged from the proportion of the dividing cells in each mitotic stage, and finally the proportion of dividing cells in each ploidy class. For comparison, observations of tissues grown on the synthetic medium alone and in the presence of 1 ppm adenine sulfate are also presented.

Note that the per cent of cells in mitosis in the control medium is about 6 per cent. Adenine sulfate has no significant effect on this level of mitosis. Low concentrations of kinetin (0.01–0.1 ppm) stimulate a larger percentage of cells into mitoses and still higher concentrations produce nearly double the number of cells in mitosis as compared to the control medium.

The percentage of dividing cells in each mitotic stage is in general a reflection of the time course of mitosis. In the synthetic medium, most of the

dividing cells are in prophase as would be expected from its usually long duration; the other stages are of short duration and fewer cells are found in each of these stages. Kinetin over the range of concentrations tested here appears to cause no significant change in this picture so that one might conclude the mitoses are quite normal.

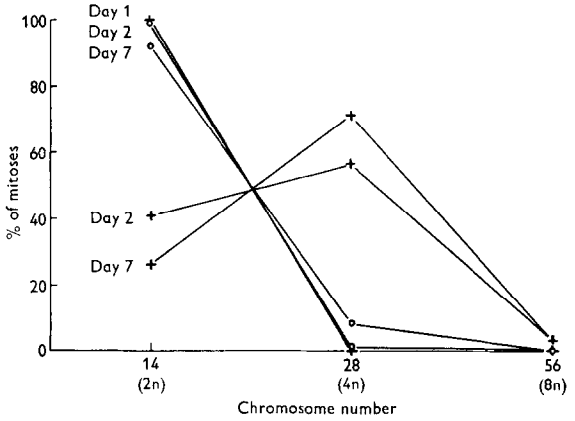

Fig. 1.—The effect of cultivating 10–11 mm mature pea root segments on synthetic nutrient medium in the presence and absence of 1 ppm kinetin on the per cent of mitoses at different ploidy levels. Each point represents a count of at least 50 mitotic figures. ○, minus kinetin; +, plus kinetin 1 ppm.

TABLE II. *The effect of increasing kinetin concentrations on the per cent of cells in mitosis, in the different mitotic stages, and in each ploidy class.*

Mature pea root segments were cultured on the nutrient medium for 2 days.

Nutrient medium	% of cells in mitosis[a]	% of dividing cells in each mitotic stage[a]				% of dividing cells in each ploidy class[b]		
		P[c]	M	A	T	2*n*	4*n*	8*n*
Synthetic medium (S)	5.8	62	16	7	15	100	0	0
S + 1.0 ppm adenine sulfate	6.9	55	28	4	13	100	0	0
S + 0.01 ppm kinetin	8.3	61	15	10	14	100	0	0
S + 0.1 ppm kinetin	8.3	51	15	19	15	76	24	0
S + 1.0 ppm kinetin	13.9	59	23	9	9	10	90	0
S + 5.0 ppm kinetin	12.5	53	18	8	21	35	65	0

[a] Based on samplings of at least 1000 cells for each treatment.
[b] Based on at least 50 dividing cells for each treatment.
[c] P, M, A, T: prophase, metaphase, anaphase, telophase.

Quite striking however is the effect of kinetin in bringing into division an increasing proportion of tetraploid cells. At 0.1 ppm one-quarter of the dividing cells were tetraploid and at 1 ppm which appears to be optimum in this test 90 per cent of the cells in mitosis were tetraploid. The highest concentration tested was apparently supra-optimal. It should be noted that at 1 ppm kinetin, the per cent of cells in division had doubled over that in the control, but at the same time there had been a marked change in the class of cells undergoing mitosis. Nearly all the mitotic nuclei were tetraploid.

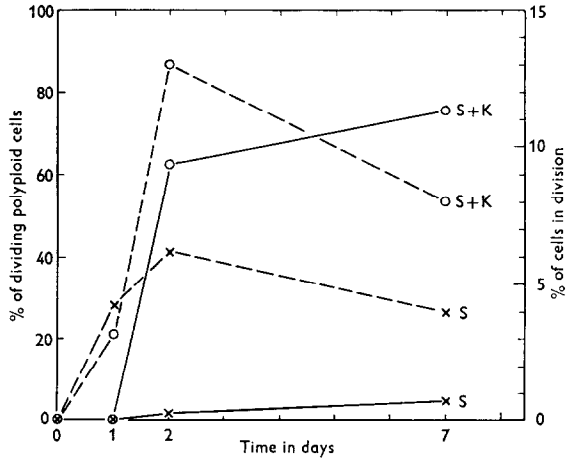

Fig. 2.—Changes with time in the per cent of cells in division and the per cent of dividing cells which were polyploid. Mature pea root segments (10–11 mm segments) were cultured on synthetic medium (*S*) with and without 1 ppm kinetin (*K*) for 7 days. Each point represents a count of at least 50 mitoses. ——, % polyploid; ----, % in division.

It should also be noted that the cytological evidence showed a very high proportion of tetraploid divisions with diplochromosomes, indicating that the tetraploid cells had doubled their chromosomes by endomitotic reduplication [18] and were then brought into mitosis by the kinetin treatment. The same experiment was repeated in the absence of added IAA with only 2,4-D present and the same result was observed. The evidence presented above does not allow us to distinguish between two possibilities: (*1*) that the tetraploid cells had doubled their chromosomes endomitotically in the mature root tissue and were pre-existing in the tissue segments at the time of treatment, or (2) that kinetin induced diploid cells to undergo endomitotic reduplication and, further, brought them into mitosis as tetraploid cells. If the first possibility is the correct one, there is circumstantial evidence that kinetin simultaneously caused inhibition of mitosis of diploid cells.

In Fig. 2 are presented data comparing the occurrence of polyploid mitoses in relation to the per cent of total cells in division during the first 7 days. At the beginning of the experiment, essentially no cells were dividing. Upon excision and transfer of the mature tissue piece to the synthetic medium, mitoses of diploid cells began and by 2 days were about 6 per cent of the cell population. Thereafter the mitotic rate decreased to about 4 per cent at 7 days. On this medium almost no polyploid cells divided (less than 5 per cent of the cells in mitosis at 7 days).

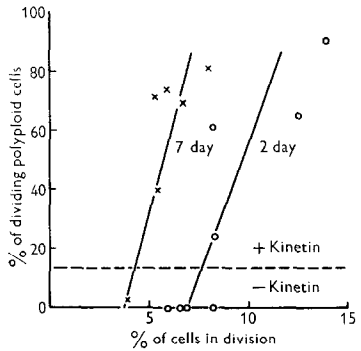

Fig. 3.

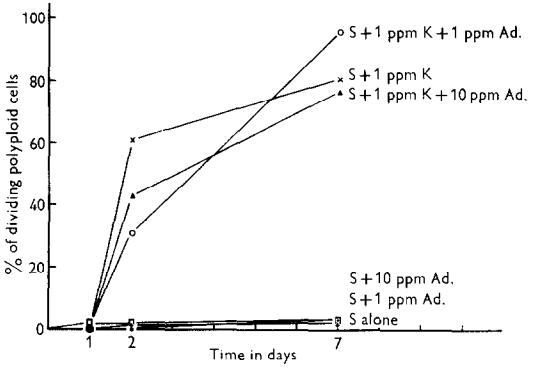

Fig. 4.

Fig. 3.—The per cent of cells in division plotted against the per cent of dividing cells which were polyploid in mature pea root segments cultured on synthetic medium in the presence or absence of added kinetin. Circles represent treatment of two days; crosses represent treatment of seven days. All points above dotted line are for kinetin-treated material at several different concentrations.

Fig. 4.—The interaction between adenosine and kinetin in affecting the per cent of dividing cells which were polyploid in mature pea root segments cultured for seven days on the synthetic medium with additions as noted. *K*, kinetin; *Ad*, adenosine; and *S*, synthetic medium.

On the same medium containing in addition 1 ppm kinetin a marked change was apparent. After 1 day of treatment the situation was comparable to that in the tissues on synthetic medium. By 2 days, however, the per cent of cells in mitosis was approximately double that on the synthetic medium and of these dividing cells, about 65 per cent were tetraploid cells. By 7 days the total number of mitotic cells had dropped to about 8 per cent but the per cent of those in division which were polyploid had increased to almost 80 per cent. Clearly, kinetin had a marked effect in bringing into mitosis nuclei which were at the tetraploid level or higher.

It was interesting to study the relationship between the percentage of cells in mitosis which were polyploid and the total number of cells in division

expressed as per cent. In Fig. 3 are brought together data from several different experiments in which tissues were grown on synthetic medium with or without various concentrations of kinetin. Chromosome counts made at 2 days and at 7 days are plotted separately.

In the first place, it is clear that, under these conditions of culture, only diploid cells undergo mitosis in the absence of kinetin. Upon the addition of kinetin, polyploid cells are brought into mitosis and, in general, there are more cells dividing. In the 2-day treatment, a much higher number of cells were in mitosis than after 7 days. There is, however, no simple direct relationship suggesting that as the per cent of cells in division increases there is a corresponding increase in the number of dividing cells which are polyploid. With respect to the data on the second day, if one were to expect a simple doubling in the number of dividing cells which were polyploid, to match the doubled rate of mitotic division, the slope of the line would be much lower, approximately half the angle which is found. Thus, there is a much greater effect on the percentage of polyploid cells in mitosis than on the mitotic rate itself.

Here again the cytological observations are important. Whereas the incidence of tetraploid division figures showing diplochromosomes was very high in the 2-day treated tissues, their occurrence in 7-day treated material was very low. Thus it would appear that at 2 days the endomitotically reduplicated cells are stimulated to divide by the kinetin treatment. Thereafter, cells with the tetraploid number of chromosomes continue to divide, at a reduced rate, closer to that of the usual diploid cells, but all of these divisons are normal mitoses of tetraploid nuclei.

An anatomical point is of interest here. On the synthetic medium alone, the diploid mitotic figures occurred predominantly in the tissues of the central cylinder of the root segment. In contrast, in the presence of kinetin, while diploid mitoses still occurred in the central cylinder tissues of the root, the tetraploid mitoses were largely located in the root cortex. This anatomical distinction between the origin of diploid and polyploid mitoses further emphasizes that the effect of kinetin is to bring into division a different class of cells than those actively dividing in the synthetic medium alone.

Two analogues of kinetin have been tested in this system. The compounds, 6-hexylaminopurine and 6-phenylaminopurine (kindly provided by F. Skoog), were added to the synthetic medium at concentrations of 0.1, 1.0 and 5.0 ppm and squash preparations of mature pea root segments were made after culture periods of 2, 6, and 14 days. The results of these tests are presented in Table III. It is apparent that over the same range of concen-

trations these compounds act in a fashion essentially similar to the furfuryl derivative.

The effects of kinetin on mitosis in 1-mm root tips.—Experiments were made to determine the effect of the synthetic medium, with and without kinetin, on mitoses of cells in the terminal 1-mm root tip. Theoretically, all cells in this terminal apex, except root cap cells which have never been induced to divide under any nutrient conditions, should possess the $2n$ number of chromosomes.

TABLE III. *Per cent of dividing cells in each ploidy class in mature pea root segments cultured on synthetic media with added kinetin analogs.*

Counts are based on at least 35 dividing cells for each treatment.

Nutrient medium	Days of treatment	% of dividing cells in each ploidy class	
		$2n$	$4n$
Synthetic medium (S)	2	100	0
	6	100	0
	14	100	0
S + 1 ppm 6-N-phenylamino purine	2	71	29
	6	20	80
	14	53	47
S + 5 ppm 6-N-phenylamino purine	2	70	30
	6	25	75
	14	—	—
S + 1 ppm 6-N-hexylamino purine	2	71	29
	6	20	80
	14	26	74
S + 5 ppm 6-N-hexylamino purine	2	71	29
	6	51	49
	14	89	11

In squash preparations made at 1 and 2 days division figures of nuclei above the $2n$ level were exceedingly infrequent whether in the absence of kinetin or when kinetin was provided in the medium at 1 or 5 ppm, as is evident in Table IV. This response is in marked contrast to that shown by mature root segments described above. After 7 days of continuous treatment, no polyploid cells were in division in the tissues on synthetic medium alone or with kinetin at 1 ppm. However, after kinetin treatment at 5 ppm, for

7 days, about 20 per cent of the cells in division were tetraploid, indicating cellular response in terms of chromosome doubling. None of these tetraploid cells at 7 days showed diplochromosomes. It is not inconceivable that one or a few tetraploid cells which were present in the initial tip underwent division selectively, such that at the end of 7 days, they represented about one-fifth of the dividing cell population. Other kinds of evidence than that provided here would be necessary to decide the nature of this effect.

TABLE IV. *The effect on increasing kinetin concentration on the per cent of cells in mitosis in each ploidy class in one-mm pea root tips cultured on nutrient agar medium.*

Counts are based on 50 dividing nuclei in each sample.

	Days of treatment	% of dividing cells in each ploidy class	
		$2n$	$4n$
Synthetic medium (S)	1	100	0
	2	100	0
	7	100	0
S + 1 ppm kinetin	1	100	0
	2	97	3
	7	100	0
S + 5 ppm kinetin	1	97	3
	2	100	0
	7	78	22

In the normal elongating root, cells in the meristem which divide give rise to cells which in 1 day are part of the mature root, many millimeters behind the root apex. Many of these cells would then be mature cortical parenchyma cells not normally subject to further division and already endomitotic and at the $4n$ or higher level with respect to chromosomal constitution. In the excised tip cultures, however, the processes of endomitotic reduplication appear to be inhibited since, even in the presence of added kinetin, except at high concentration, no tetraploid mitoses showing diplochromosomes are observed. Here, the diploid cells are not stimulated to undergo DNA synthesis or to double chromosomes as would occur if they had remained as a part of the intact root. That tetraploid mitoses do not occur in these tip cultures treated with kinetin as they do in cultures of mature segments suggests that

the class of cells responsive to kinetin is lacking in the tips. This fact gives further support to the idea that kinetin specifically triggers mitosis of mature endomitotic cells.

Interactions of kinetin and adenine compounds.—There have been suggestions that kinetin acts by virtue of its structural similarity to adenine. In preliminary experiments, an interaction between kinetin and adenine sulfate was suggested (Table V). In these experiments, the presence of added

TABLE V. *The interacting effect of kinetin and adenine sulfate on the per cent of dividing cells in each ploidy class in mature pea root segments cultured 2 and 7 days in each nutrient agar medium.*

Each sample is based on a count of approximately 50 dividing nuclei.

Treatments	% of dividing cells in each ploidy class				
	2 days		7 days		
EXPERIMENT 1	2*n*	4*n*	2*n*	4*n*	8*n*
Synthetic medium (S)	100	0	94	6	0
S + 40 ppm adenine sulfate	100	0	100	0	0
S + 1 ppm kinetin (K)	54	46	31	68	1
S + 1 ppm K + 40 ppm adenine sulfate	74	26	60	36	4
EXPERIMENT 2					
S medium	98	2	94	6	0
S + 1 ppm K	53	47	16	76	8
S + 1 ppm K + 10 ppm adenine sulfate	73	27	18	76	6

adenine appeared to suppress the effectiveness of kinetin in triggering the mitosis of polyploid cells, especially after 2 days of treatment. A further experiment was set up to test kinetin interaction with the purine in which kinetin and the riboside, adenosine were provided to the tissue simultaneously. This form of adenine was selected to obviate possible problems of permeability and to provide it to the tissue in what may be presumed to be an active combined form. Squashes were prepared at intervals of 1, 2, and 7 days and chromosome counts made as before. Kinetin concentration was maintained constant at 1 ppm and levels of adenosine at 0, 1, and 10 ppm were tested in the presence and absence of kinetin.

In Fig. 4 the data are presented graphically with the per cent of dividing polyploid cells plotted as a function of time in days. As observed earlier,

only diploid division figures occur in tissues cultured on the synthetic medium. Additions of low concentrations of adenosine to the synthetic medium effect no change in this particular over the entire period of 7 days. In all treatments without kinetin, no more than 2 per cent of the dividing cells were polyploid.

One ppm kinetin induces many mitoses in tetraploid cells by the second day. It is interesting to note that after 1 day essentially no polyploid mitoses were apparent in any nutrient medium, and that only after 2 days was the effect of kinetin apparent. This observation confirms that made in earlier experiments (Fig. 2). These mitotic tetraploid cells frequently showed diplochromosomes and were derived largely from cells whose chromosomes had doubled endomitotically. The proportion of tetraploid cells in mitosis in the kinetin medium increased still further by 7 days, at which time diplochromosomes were observed infrequently and mostly normal tetraploid mitoses were seen.

Adenosine at 1 or 10 ppm in the presence of kinetin reduced the number of tetraploid mitoses in the tissue at 2 days, by as much as one-third to one-half that of the kinetin treatment alone. There appears to exist no direct concentration relationship as might be expected if the kinetin effect were that of a metabolite antagonist. However, the limited data and the relatively crude measure of interaction cannot rule out this possibility. By the seventh day, the effect of interaction was no longer apparent and it is clear that the interaction centered on the initial action of kinetin in inducing endomitotic cells into mitosis.

In one experiment, adenosine at 50 ppm was tested in the presence and absence of 1 ppm kinetin in the synthetic medium. It was observed that even at this concentration adenosine alone has no effect on initiating polyploid mitoses. However, with the addition of 1 ppm of kinetin, a high percentage of tetraploid nuclei in mitosis was observed after 2 days, suggesting that at the high concentration of adenosine, the interaction between it and kinetin was no longer of a competitive sort but in fact became synergistic. Further experiments are necessary to establish clearly the nature of this interaction.

DISCUSSION

In earlier studies [27] one of the concomitants of continuous culture of mature pea root segments on a yeast extract—2,4-D medium was the progressive establishment of a tetraploid population of actively-dividing cells. In the light of the present experiments it is clear that the yeast extract contributed, among other things, a component, either kinetin itself or a kinetin-like

compound, which at very low concentrations in the presence of a stimulating auxin, effectively brought into division cells which had doubled their chromosomes by endomitotic reduplication. Once these tetraploid cells had divided, they continued to divide as tetraploid cells to form the major component of the callus tissue population.

In the root segments used in these experiments, there were present in the tissues at the time of excision at least two distinct classes of cells with respect to their capacity to undergo cell division. The diploid cells of the central cylinder tissues, perhaps predominantly pericycle and phloem and xylem parenchyma cells, under the stimulus of excision and treatment with the auxin-containing medium, were stimulated to double their DNA, undergo mitosis and cytokinesis, forming a population of dividing diploid cells. The second general class of cells included those which on the synthetic medium did not divide even though exposed to the stimuli of wounding and auxin treatment. When provided a small amount of kinetin for 48 hr these cells began to undergo mitosis and cytokinesis. The occurrence of diplochromosomes or chromosome pairs in early metaphase with the production of tetraploid cell progeny was clear evidence that a large proportion of such cells had undergone chromosome doubling by endomitosis during interphase. These cells occurred predominantly in the cortex of the root segments and had already undergone endomitosis prior to segment excision as a part of normal processes during cell differentiation. In this second class of cells, kinetin specifically overcame the block to mitosis, a block which in the normal course of events would have persisted for the life of the cells. It is notable that 1-mm-root tip cells did not respond to kinetin in the same way as mature root segment cells and the presumption is that the second class of cells was essentially lacking in the tip.

An alternative interpretation of the effect of kinetin on the production of tetraploid cells might be given. Patau *et al.* [21] showed that in tobacco pith tissues, kinetin induces DNA synthesis and that auxin and kinetin together effect necessary DNA doubling essential for subsequent mitosis which, followed by cytokinesis, results in new diploid cells. Applying their conclusions to these experiments, one might presume that kinetin treatment resulted in DNA doubling from the 2*C* level, not just to the 4*C* level prerequisite for diploid mitosis, but to the 8*C* level, resulting in cells at metaphase which were tetraploid with respect to chromosome number. All the present evidence is against this interpretation of the data. Further, preliminary experiments feeding ^{3}H-labelled thymidine to mature root segments in the presence and absence of kinetin have resulted in autoradiographs which show that DNA

synthesis clearly did not occur during kinetin treatment in those cells stimulated to come into division at the tetraploid level. The results of these experiments will be published separately. It is quite clear in these experiments that kinetin did not induce polyploidy or endomitosis as was suggested for onion roots by Guttman [13].

In the present experiments it seems certain that kinetin acts as trigger to mitosis in mature root cells. There is evidence also that kinetin, while stimulating endomitotic tetraploid cells to divide, inhibits mitosis in diploid cells. Here, the evidence is less direct and further data bearing directly on this fact are needed. The observation is consistent, however, with published reports of inhibition of mitosis by kinetin at certain concentrations [10, 13]. In our own unpublished studies on lateral root initiation, we have found that kinetin at 1 ppm is an effective inhibitor of auxin-induced lateral root initiation in which diploid divisions are inhibited. Other reports of an antagonistic interaction between kinetin and auxin have been made [23, 29].

Of special interest is the time required for the response of endomitotic cells to kinetin treatment. While auxin treatment induced mitosis in diploid cells promptly—in less than 24 hr, no tetraploid cells in mitosis were observed before 48 hr. In these cells, a time lag is required while processes take place in which kinetin specifically plays some role. Since DNA synthesis is not involved, some other aspect of cell metabolism related to mitosis must be limiting. Chouinard [5] also reported that endomitotic cells responded to external stimuli leading to mitosis at a much slower rate than diploid cells.

These experiments are concerned with the early ontogeny of callus tissue *in vitro* and in particular the type of cells which forms the cell population. It is a striking fact that a single component of the medium by its specific stimulating effect on one type of cell in the population can determine the nature of the cell population by a selective mechanism. It is interesting to consider closely related phenomena in nature: wound callus formation and crown gall tumor initiation. With respect to wound callus formation, it is conceivable that kinetin treatment of some regenerating stem tissues would accelerate development of polyploid tissues preferentially producing tissues from which polyploid shoots might be readily initiated.

In the case of crown gall tumor initiation, two facts are suggestive. The very precise timing of the polyploid cell response following treatment corresponds rather well to the precise time for initiation of tumors following bacterial infection [3]. It suggests that one contribution of the effective bacterial infection might be acting like kinetin. The fact that polyploid cells are involved in crown gall tumor tissue is well established and has recently been

reconfirmed [16, 22]. It is of special interest that Braun [4] has found that kinetin, together with certain metabolites, when added to a nutrient medium, allows the proliferative growth of normal tobacco tissue in a manner closely approximating that of fully altered tumor cells of the crown gall type. It will be of interest to see to what degree the specific effects of kinetin described in the present study are parallelled in the onset of crown gall tumor initiation. It is also of interest to refer to the evidence that in the initiation of root nodules by the bacterium, *Rhizobium*, in legume roots, polyploid cortical cells represent the most frequent component of the proliferating tissue mass, suggesting that in some way the bacteria overcome the block to mitosis in the mature cortical cells, perhaps by contributing a kinetin-like factor at the time of infection. The pseudo-nodules of Arora *et al.* [1] produced by kinetin treatment may be more akin to normal nodule structures than would at first seem possible.

Concerning the mechanism of the trigger effect of kinetin on mitosis in endomitotic cells, there is little conclusive evidence presented here as to its nature. The experiments on the interaction of kinetin and adenine or adenosine suggest that kinetin may interfere in some way with the normal role of adenine. Which of the several known roles of adenine is affected is not clear. Judging from observations of Glasziou [12], it seems unlikely that kinetin acts on adenine at the level of its role as component of ATP in high-energy phosphate transfer. The present evidence also tends to rule out the role of adenine as precursor for DNA synthesis *per se* although some indirect effect may be present. Rather, the experiments here, together with reports in the literature, suggest that kinetin action centers on interference with a role of adenine in protein synthesis, perhaps by affecting RNA metabolism. Olszewska [20] found kinetin inhibited adenine incorporation in RNA in onion roots. Guttman [14] also suggested that the effect of kinetin centered in RNA metabolism.

Experiments to explore further the interaction of kinetin and adenine in protein synthesis and its relation to mitosis in mature endomitotic cells have been undertaken.

SUMMARY

Pea root callus tissue cultivated for many months on a nutrient medium containing yeast extract and the auxin, 2,4-dichlorophenoxyacetic acid, is predominantly composed of dividing cells of the tetraploid chromosome number even though the initial tissues cultured from the root were known

to consist in part of cells of the diploid chromosome number. In experiments designed to determine the basis for what appeared to be cell selection by the nutrient medium, 1-mm thick segments of 60 hr germinating seedling roots of the garden pea, *Pisum sativum*, excised 10–11 mm behind the root tip, were cultivated on a synthetic nutrient medium in which the yeast extract was replaced by a mixture of vitamins, amino acids, amides and urea. On such a medium, callus tissues developed, but only diploid cell mitoses were observed. Upon the addition of 1 ppm kinetin (6-furfurylaminopurine) to the synthetic medium, the number of cells in mitosis approximately doubled and a very high percentage of dividing cells possessed the tetraploid chromosome number. Lower concentrations of kinetin were proportionately less effective in stimulating mitosis and in bringing tetraploid cells into mitosis. From these studies it has been concluded that kinetin specifically triggers mitosis in mature pea root tissue cells which have undergone DNA- and chromosome-doubling by endomitotic reduplication before kinetin treatment. This conclusion is based on the following lines of evidence: (*a*) After 2 days of kinetin treatment, the tetraploid cells which enter mitosis showed diplochromosomes, indicating that chromosome doubling had been endomitotic; (*b*) Tetraploid mitoses were largely localized in mature cortical cells of the root while most diploid mitoses occurred in tissues of the central cylinder; (*c*) One-mm root tips cultured for up to 7 days in 1 ppm kinetin showed no tetraploid mitoses, which is expected since few or no endomitotically doubled cells are likely to occur in the apical meristem of the root. Certain experiments in which adenine sulfate or adenosine was fed the mature pea root segments together with kinetin suggest that an interaction between adenine and kinetin does occur in which adenine reverses in part the effect of kinetin on induction of mitoses in mature endomitotic cells.

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