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Effects of 6-dimethylaminopurine, 2-aminopurine, olomoucine and sodium vanadate on DNA endoreduplication in primary roots of *Pisum sativum*

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Abstract

The effects of 2-aminopurine, olomoucine, 6-dimethylaminopurine (inhibitors of cyclin-dependent kinases; CDK), and sodium vanadate (a potent inhibitor of protein phosphatases) on DNA endoreduplication were investigated during elongation and differentiation of the primary roots in *Pisum sativum*. When compared with the untreated control plants, at least one additional round of DNA replication was evidenced to occur within most cells, the majority of which have attained 4C DNA level, and a considerably greater portion of cells represented the endopolyploid state with nuclear DNA content approximating the 8C value. It is concluded that cellular commitment to DNA endoreduplication may appear not only as a consequence of suppression imposed directly upon CDK activity, but also as an indirect output connected with the decreased activity of cdc25 protein phosphatase, an enzyme necessary to turn the switch on for appropriate conformation of the CDK/cyclin B complex. By calculating the absorption profiles of Feulgen-stained nuclei, specific phosphorylation-dependent changes in chromatin condensation of endopolyploid cells have been revealed. It is proposed that acquisition of a certain critical level of chromatin condensation constitutes a prerequisite for additional rounds of DNA synthesis in plants.

Additional key words: cyclin-dependent kinases, pea, protein phosphatases.

Introduction

DNA endoreduplication is believed to serve as a means to enhance the amount of gene templates available for transcription, and consequently, to produce various types of RNAs, ribosomes, and other materials (Nagl 1976). Alternatively, its significance may reside in the properties that are consequent upon the endopolyploid state itself. The role of endopolyploidy may thus consist in creating concentration gradients of metabolic components and regulators, in making economic use of the resources available for cell growth, and in reducing the cell-to-cell barrier, thus providing the more effective and better coordinated exchange of signaling molecules (Barlow 1978).

Although the endocycles follow an incomplete course the cell cycle (without intervening nuclear divisions), they represent an orderly regulated process. The formation of differentiating polyploid tissues in plants is likely to be controlled by the key modular elements of the cell cycle machinery, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (Nagl 1995; similar data for animal cells are reviewed by Grafi 1998). The transition from mitotic to endoreduplication cycles requires exit from the control system that links the completion of DNA replication to the entry into M phase. Endoreduplication in maize endosperm was found to involve both inhibition of $p34^{cdc2}$/cyclin B complex (MPF) and induction of S phase-related CDKs, capable of interacting with human E2F-1 transcription factor and providing activities needed for S-phase entry (Grafi and Larkins 1995, Den Boer and Murray 2000). Extra rounds of DNA synthesis have been found in yeast cells carrying the *cdc2* or *cdc13* mutations of genes encoding kinase Cdc2 and cyclin B proteins, respectively (Broek *et al.* 1991, Hayles *et al.* 1994). Compatible results were obtained in cells expressing the $p21^{waf1/cip1}$ inhibitor of CDK (Niculescu III *et al.* 1998). The staurosporine

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Abbreviations: APC/C - anaphase promoting complex/cyclosome; 2-AP - 2-aminopurine; CDK - cyclin-dependent kinases; 6-DMAP - 6-dimethylaminopurine; MPF - M-phase promoting factor; OM - olomoucine; RLF - replication licensing factor; SPF - S-phase promoting factor.

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analogue, K-252a, was found to inhibit mitotic divisions, but to permit additional rounds of DNA replication in cell suspension cultures of *Phaseolus coccineus* (Nagl 1993). On the other hand, in transgenic *Medicago sativa*, suppression of *ccs52* gene encoding a 52 kDa plant homologue of anaphase promoting complex/cyclosome (APC/C) activator decreased the number of endocycles and the volume of largest cells, presumably, by inhibiting the ubiquitin-dependent degradation of mitotic cyclin (Cebolla *et al.* 1999). Furthermore, it appears that following initiation of DNA synthesis, phosphorylation by S phase kinases marks certain replication proteins (*e.g.* proteins of the Mcm family, some of them composing the replication licensing factor, RLF) for ubiquitination and abrupt degradation mediated by APC/C. It seems thus that the competence of DNA for replication is negatively controlled by the activity of S-, G2-, and M-phase CDKs (Grafi 1998).

Until recently, experimental work on the CDK/cyclin activities and molecular mechanisms underlying the switch from proliferation to endoreduplication in plants lagged far behind similar work with animals. No information is still at hand concerning the influence of CDK inhibitors on nuclear polyploidization controlled by the developmental programme in intact plants. In the present paper we document the effects of 6-DMAP, 2-aminopurine, olomoucine, the protein kinase inhibitors (Veselý *et al.* 1994, Meijer 1995), and sodium vanadate, a potent inhibitor of dual-specificity protein phosphatase, cdc25 (Ajiro *et al.* 1996), on DNA endoreduplication in elongation and differentiation root zones of *Pisum sativum*. Concurrently, by calculating the absorption profiles of Feulgen-stained cell nuclei after selecting the increasing threshold values of absorbance (Maszewski *et al.* 1998, Polizzi *et al.* 1998), we indicate specific phosphorylation-dependent changes in chromatin condensation during endopolyploidization.

Materials and methods

Plants: Seeds of *Pisum sativum* L. cv. Sweet (Center for Seed Production in Sobiejuchy) were sawn on moist blotting paper and germinated at room temperature in the dark. Four days after imbibition, intact seedlings with roots ranging from 2 to 3 cm were selected for treatments with water solutions of kinase and phosphatase inhibitors: 3 mM 6-dimethylaminopurine (6-DMAP), 200 μM 2-aminopurine (2-AP), 200 μM olomoucine (OM) and 200 μM sodium vanadate ($NaVO_3$). Each of these inhibitors were purchased from *Sigma-Aldrich* (Poznań, Poland). Using carbon/water mixture, tiny black lines were drawn to discriminate each root meristem (1.5 mm above the tip). During the next 4- and 7-d incubations the solutions were permanently aerated (*ca.* 500 cm^3 min^{-1}).

Fixation and staining for cytophotometry: 1 mm long root segments were cut below the carbon-marked zone, fixed in cold Carnoy's mixture (absolute ethanol and glacial acetic acid; 3:1, v/v) for 1 h, washed several times with ethanol, rehydrated, hydrolysed in 4 M HCl (1.5 h), and stained with Schiff's reagent (pararosaniline, *Sigma-Aldrich*) according to standard methods (Maszewski *et al.* 1998). After rinsing in SO_2-water (3 times) and distilled water, root segments were placed in a drop of 45 % acetic acid and squashed onto *Super-Frost* (Menzel) microscope slides. Following freezing with dry ice, cover slips were removed and the dehydrated dry slides were embedded in *Euparal*.

Cytophotometry: Cytophotometry was made using *Jenamed-2* microscope (*Carl Zeiss*, Jena, Germany) with the computer-aided *IMAL-512* system for image analysis. The absorbance of Feulgen-stained cell nuclei was measured at 565 nm and calibrated in arbitrary units (a.u.). To evaluate the frequency distribution pattern for DNA contents, 200 readings of individual nuclei were taken for each selected root zone.

Quantitative analysis of chromatin condensation was based on data recorded from 10 endopolyploid nuclei selected according to both morphometric criteria and 2C DNA content (Rembur and Nougerede 1987). Measurements were made by advancing the threshold absorbance values within the limits indicated by minimum and maximum profile areas recorded on the monitor. The mean values were computed according tc analyses presented earlier (Cavallini *et al.* 1996, Maszewski *et al.* 1998; see also Results). To facilitate comparisons, all absorption values were normalized (the total nuclear area being 100 % at the minimum absorbance treshold) for each experimental series and each selected root zone (*cf.* Polizzi *et al.* 1998).

Results

The effect of protein kinase inhibitors and sodium metavanadate on DNA endoreduplication: Feulgen-cytophotometric analyses of nuclear DNA contents made for the selected root segments in the control (untreated) seedlings revealed low levels of endopolyploidy, both after 4 and 7 d of culture (Fig. 1). The DNA distribution

patterns indicated good separation of 2C and 4C nuclei, nearly complete lack of cells characterized by the intermediate 2-4C nuclear DNA values (typical for meristematic S-phase cells), and a small fraction of cells approximating roughly 8C DNA level (Table 1).

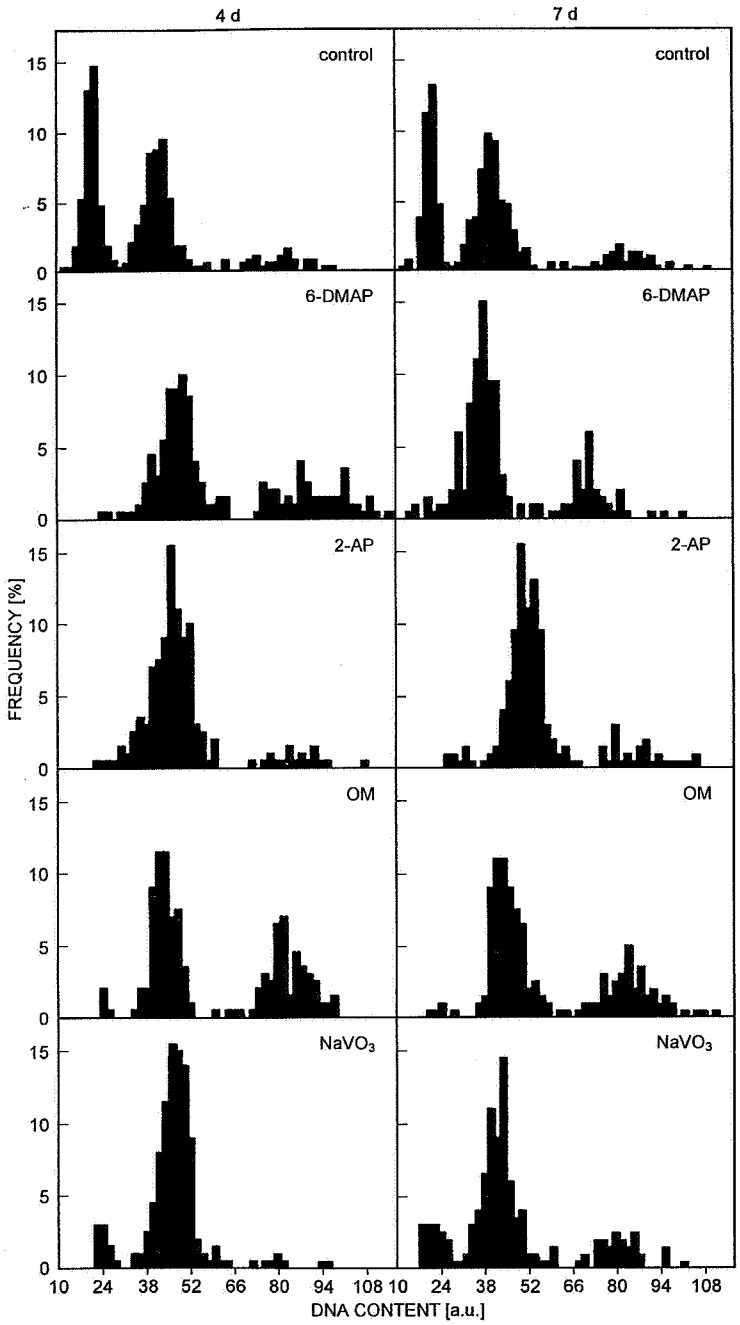

Fig. 1. Frequency distribution [%] for nuclear DNA content (a.u.) in selected root zones of *Pisum sativum* after the 4-d and 7-d culture in the control conditions, following treatment with 6-dimethylaminopurine (6-DMAP), 2-aminopurine (2-AP), olomoucine (OM), and sodium vanadate ($NaVO_3$).

Permanent treatment of roots with 6-dimethylaminopurine (6-DMAP), 2-aminopurine (2-AP), or olomoucine (OM) brought about considerable changes in nuclear DNA profiles (Fig. 1). In each experimental series, only a residual, tiny fraction of cells with 2C nuclear DNA level could be discriminated, with the majority of cells concentrated around the 4C level. In 6-DMAP- and olomoucine-treated seedlings, a substantial fraction of cells indicated endopolyploidy, with nuclear DNA contents ranging from about 6 to 10C value. No

significant differences were found between the frequency distribution histograms plotted for the selected cell populations 4 and 7 d after the start of incubation (see also Table 1).

In general, similar DNA distribution patterns have been evidenced in root segments cut out from sodium vanadate-treated seedlings of *P. sativum* (Fig. 1). However, small portions of 2C nuclei could still be easily discerned both after the 4 and 7 d of incubation. Moreover, the longer period of cultivation in $NaVO_3$ solution resulted in a slight increase in the frequency of endopolyploid cells with nuclear DNA approximating the 8C level (Table 1).

Table 1. Estimated relative shares [%] of cells with 2C, 4C, and >4C nuclear DNA values in selected root zones of *Pisum sativum*, following the 4-d and 7-d culture in the control conditions, after treatment with 2-aminopurine (2-AP), 6-dimethylaminopurine (6-DMAP), olomoucine (OM), and sodium vanadate ($NaVO_3$).

Inhibitor	Treatment [d]	2C	4C	> 4C
Control	4	44.5	45.5	10.0
	7	37.0	50.5	13..5
2-AP	4	4.5	84.0	11.5
	7	4.5	72.0	23.5
6-DMAP	4	2.0	61.0	37.0
	7	15.5	73.0	11.5
OM	4	2.5	55.5	42.0
	7	3,5	64.0	32.5
$NaVO_3$	4	9.0	86.0	5.0
	7	15.5	64.5	20.0

The effect of protein kinase inhibitors and sodium metavanadate on chromatin condensation in endopolyploid cell nuclei: Cytophotometric examination of Feulgen-stained endopolyploid cells from selected root zones revealed significant changes in chromatin condensation, specific for particular series of experiments. In order to quantify the areas occupied by chromatin of various absorbance, a series of microspectrophotometric measurements was done at increasing threshold absorbance. The analysis of obtained curves (cumulative distribution profiles; Fig. 2) allowed for evaluating mean relative shares of four differently condensed fractions of chromatin, termed as highly and moderately condensed, decondensed and totally dispersed (Fig. 3).

Some basic differences in nuclear condensation comes to be noticed depending on whether the roots have been incubated either with any of the protein kinase inhibitors, or with sodium vanadate, an inhibitor of protein phosphatases. When compared with the graphs showing sequentially discerned regions of chromatin in the 8C DNA cell nuclei from the control, or vanadate-treated seedlings, the OD screenings for endopolyploid cell nuclei in the corresponding root zones from 6-DMAP-, 2-AP-, and OM-treated plants start out to increase at relatively lower threshold levels (Fig. 2). Taking the mid-point of this scale (55 a.u.) as a reference, the graphs plotted for endopolyploid cell nuclei from roots incubated with CDK inhibitors attain considerably higher y-axis values, indicating relatively larger nuclear areas occupied by highly and moderately condensed chromatin (Fig. 3). This is in marked contrast to vanadate-treated root cell nuclei, particularly those analysed after the 4-d incubation (Fig. 2).

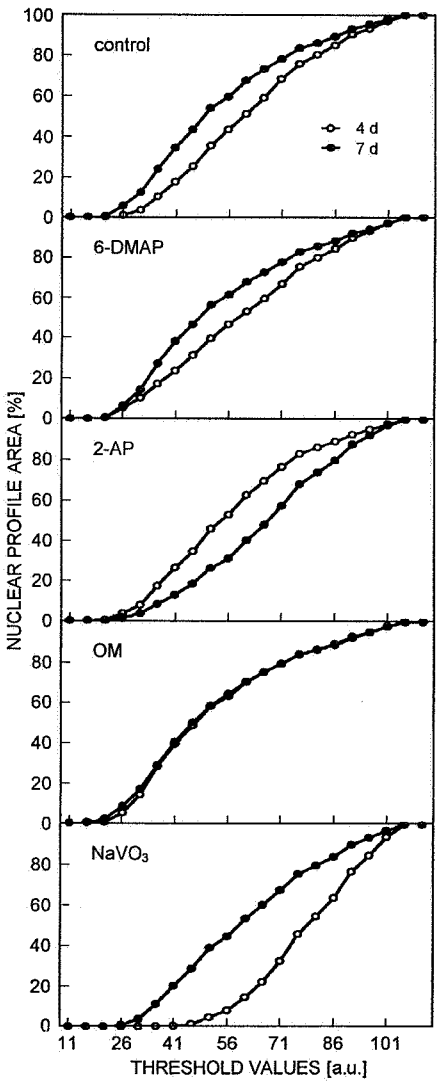

Fig. 2. Fractions of the total profile areas of endopolyploid cell nuclei [%] discerned by consecutive optical density levels (threshold values; a.u.) in selected root zones of *Pisum sativum* after the 4-d (*open circles*) and 7-d (*closed circles*) culture in the control conditions, following treatment with 6-DMAP, 2-AP, OM, and $NaVO_3$.

Depending on the duration of incubation, evident changes in the calculated relative shares of nuclear areas occupied by various fractions of chromatin have also

been found in cell nuclei from roots treated with 6-DMAP and 2-AP. Following the 7-d incubation in 6-DMAP, endopolyploid cells indicate higher proportion of condensed chromatin (Figs. 2, 3). On the contrary, when compared with the 4-d treatment, prolonged incubation in 2-AP solution produces 8C cell nuclei characterized by considerably larger areas of dispersed chromatin. The nuclear OD screenings for OM-treated roots give overlapping distribution profiles (Fig. 2). Hence, the evaluated proportions of particular chromatin fractions (shares of condensed and decondensed areas) remain unchanged irrespective of the duration of incubation (Fig. 3).

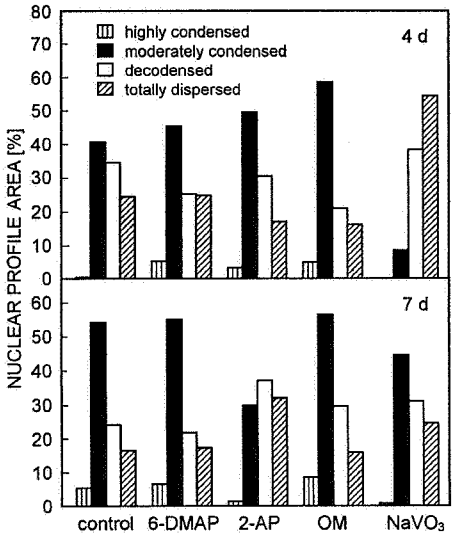

Fig. 3. Relative shares [%] of the total nuclear profile areas occupied by highly and moderately condensed, decondensed and totally dispersed fractions of chromatin; estimations made for endopolyploid cells in selected root zones of *Pisum sativum* after the 4-d and 7-d culture in the control conditions, and following treatment with 6-DMAP, 2-AP, OM, and $NaVO_3$.

Discussion

Nuclear distribution profiles made for selected root segments in the control seedlings of *Pisum sativum* are characterized by a high proportion of cells with 2C and 4C DNA levels, similar to that evidenced earlier by Van't Hof and Kovacs (1972) in meristem cell populations from excised roots starved in carbohydrate-free mineral medium. Both after the 4- and 7-d culture in water, the frequencies of endopolyploid cells (with C value above 4) are low and attain 3 and 5 %, respectively. Continuous hydroponic incubation with protein kinase inhibitors brings about marked quantitative remodeling of cell populations, indicating that at least one additional round of DNA replication must have occurred within most 6-DMAP-, 2-AP-, or OM-treated root cells, the majority of which now have 4C DNA, and a considerably greater portion of cells represents the "truly" endopolyploid state with nuclear DNA content approximating the 8C value. Since an overall character of changes induced in roots treated with chemical inhibitors of CDKs is remarkably similar to those evidenced following incubations with $NaVO_3$, it seems reasonable to assume that there may be a common unifying principle underlying the whole range of effects observed in differentiating root cells of *Pisum*.

DNA replication and mitosis is triggered by the evolutionary conserved class of serine/threonine protein kinases, the catalytic subunits, in association with their regulatory partners, the cyclins, and ATP (Renaudin *et al.* 1998). The ATP-binding site, located in the cleft between N- and C-terminal lobes of the kinase, is a major domain for interactions with a variety of ligands, including exogenous inhibitors containing adenine, such as 6-DMAP, 2-AP, and OM (Meijer 1995, Kim *et al.* 1996). Apart from the association of the $p34^{cdc2}$ kinase with specific cyclin, another process must take place to allow for CDK activation. The conserved ATP-binding motif (GXGXXG) contains two amino acids, Thr14 and Tyr15, which become phosphorylated by the products of *wee1/mik1* genes. Activation of CDK/cyclin B complex requires dephosphorylation of both residues of the $p34^{cdc2}$ kinase, which is mediated by a highly specific protein phosphatase, cdc25 (Solomon *et al.* 1992, Draetta 1994).

The mechanisms by which vanadate can mediate cellular reactions comprise stimulation of tyrosine kinase (Brown and Gordon 1984), inhibition of ATPase and, consequently, either deformation of $p34^{cdc2}$/cyclin B complex, or disruption of the ubiquitin pathway indispensable for cyclin B degradation (see Ajiro *et al.* 1996). Considering the above data it seems clear that, by whatever means, using either the CDK inhibitors (6-DMAP, 2-AP, and OM), or $NaVO_3$, the applied

treatments converge to produce a common final effect. Consistently with this hypothesis, we speculate that cellular commitment to DNA endoreduplication in *Pisum* (and probably in other plant species) may appear not only as a consequence of suppression imposed directly upon CDK activity, but also as an indirect output connected with the decreased activity of cdc25 protein phosphatase, an enzyme necessary to turn the switch on for appropriate conformation of the CDK/cyclin B complex.

Our results conform, thus, to the model of Grafi and Larkins (1995) in showing that inhibition of CDK-like proteins and, consequently, the decrease in CDK-dependent phosphorylation is capable of inducing extra rounds of DNA synthesis in differentiating cells of *Pisum*. Furthermore, the similarity of effects induced by 6-DMAP, 2-AP, OM, and $NaVO_3$ extend the number of inhibitors capable of inducing endoreduplication, as well as the range of possible regulatory pathways leading to the transition from cell division cycles to the endo-reduplication cycles. However, due to morphogenetic constraints and cytophysiological conditions to which the cells are subjected within the root cortex of intact seedlings, the degree of induced endoreduplication is considerably lower than that evidenced earlier by Nagl (1993) in suspension cell cultures of *Phaseolus coccineus* treated with the staurosporine analogue, K-252a.

One of the challenging questions is: how does an inhibition of the key cell cycle-related enzymes, CDKs, get translated into stimulation of DNA endo-reduplication? The best we can do is to adopt the concept of Grafi (1998, see also references therein), based on a view that not just S and M, but the entire cell cycle, provides a mechanism to assure a correct and adequate genetic inheritance to each recipient daughter cell produced by mitosis. Thus, a variety of factors acting at different molecular control levels may disrupt these mechanisms and induce the phenomenon of endocycles. The activity of S and M phase kinases (SPF and MPF, respectively) seem to negatively regulate DNA re-replication by inhibiting the transition of replication origins to a competent state licensing a new round of DNA synthesis (Dahmann *et al.* 1995). In concert with the general idea presented by Grafi (1998), our studies on root cells of *Pisum* provide direct evidence that decreased catalytic activity of CDK is a prerequisite of, or a signal for endoreduplication.

The increased condensation of chromatin in endopolyploid cells from pea roots treated with CDK inhibitors, and vanadate-induced decondensation of chromatin appear to be in agreement with numerous effects evidenced in experiments on animal cells. 6-DMAP has been found to affect the first step of chromatin decondensation during formation of sea urchin male pronucleus (Cothren and Poccia 1993). On the other hand, Chinese hamster mitotic mutant tsTM13 cells exposed to sodium vanadate reversed all kinds of mitosis-specific defects, with concommitant inactivation of $p34^{cdc2}$/H1 kinase and decondesation of chromatin (Ajiro *et al.* 1996).

Among a number of factors determining nuclear condensation, the mechanism controlled by the balance of protein kinase and protein phosphatase activities is generally regarded as one of the most important (Lewin 1990). Despite correlation between the $p34^{cdc2}$/H1 kinase activation and the initiation of mitosis, it is still unknown whether phosphorylation of histones determines chromatin condensation, or the condensed state of chromatin is subsequent to phosphorylation-dependent activation of downstream regulatory factors directly involved in remodelling of chromatin. Our microspectro-photometric studies of Feulgen-stained endopolyploid cell nuclei suggested that the degree of chromatin condensation, and the extent of observed changes varies depending on the duration of treatment and the nature of the inhibitor. A series of OD screenings indicates that incubation with 6-DMAP, 2-AP and OM brings about significant compaction of large areas of 8C cell nuclei, whereas incubation with $NaVO_3$ results in considerable decondensation of chromatin. However, the initial condensation appeared to be a reversible step in endopolyploid root cells treated with 2-AP, and nuclear decondensation was shown to be transient in endo-polyploid cells treated with $NaVO_3$. In the former case, decondensation of chromatin (following 7 days of incubation with 2-AP) brings about no net increase in the fraction of endopolyploid cells. On the other hand, the appearance of condensed chromatin after prolonged incubation with $NaVO_3$ is correlated with a marked increase in cells approximating the 8C nuclear DNA level. It may be, than, that acquizition of a certain critical level of chromatin condensation constitutes a prerequisite for additional rounds of DNA synthesis.

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