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Effects of water soluble oncostatic fraction from *Rheum officinale* Baill. rhizomes on *Allium cepa* root meristem

I. The mitotic activity.

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Abstract

The effects of the oncostatic extracts from *Rheum officinale* rhizomes on the activity of meristematic cells from *Allium cepa* roots were investigated. A statistically significant decrease of the I_M value was noted as well as of the total number of mitoses during incubation. The disturbances in the course of mitosis and cytokinesis are described and discussed. The kind of disturbances during postincubation points to damage of the S and G_2 phases of the interphase nuclei. Cytochemical and autoradiographic studies demonstrated a diminished intensity of staining of DNA and RNA and inhibition of DNA synthesis during incubation, this leading in turn to a lower intensity of protein staining in postincubation. Disturbances in mitosis and cytokinesis after treatment wth 2,6-dihydroxyantraquinone, supposed to be the antimitotically active compound of the extract, are the same as those produced by the total water soluble fraction.

INTRODUCTION

The search among natural substances and plant extracts for compounds with an antimitotic activity has a tradition of many years (for review see Hartwell, 1967; Harrod, 1969). This problem is full of present interest as may be seen not only from numerous individual investigations (e.g. Boratyńska and Borkowski, 1957; Szuleta, 1961; Farnsworth and Svoboda, 1966; Farnsworth, 1968; Tarkowska, 1971; Tarkowska and Matuszewska, 1975), but above all from research work on a wide scale initiated by CCNSC (Cances Chemotherapy National Service Center) in 1960. The results were published partially in 1962. The main purpose of these studies was the

isolation and demonstration of the structure of the oncostatically active components. A list of the most important oncostatic compounds of natural origin was compiled by Borkowski (1966a, b). None of the so far elaborated substances was found to be of decisive significance, nevertheless the partial effects observed encourage to further research. Among the substances tested such antimitotics as *Vinca rosea* alkaloids or podophyllotoxin derivatives from *Podophyllum peltatum* have found official application in therapy (Borkowski, 1966a, b).

The present study was undertaken to investigate the effect of an aqueous extract of *Rheum officinale* rhizomes on the activity of apical meristem in adventitious roots of *Allium*. The water and alcohol extracts of *Rh. officinale* inhibit growth of spontaneous and transplanted neoplastic tissues (Belkin and Fitzgerald, 1952; McKenna et al., 1960; Farnsworth, 1961).

The chemical composition of the aqueous extract of *Rheum officinale* rhizomes is given by Hegnauer (1969). The genus *Rheum* is characterized by the general occurrence of anthraquinones. In *Rheum palmatum* they constitute a considerable (3—7.5% dry weight) proportion of the rhizomes (Bukowiecki and Furmanowa, 1972). The literature supplies no corresponding data for *R. officinale*. Besides anthraquinones and their derivatives the rhizomes contain from among flavonoids — rutine, from stilbenes — raponticine, from tannins — catechin (Hegnauer, 1969). The essential component as regards quantity and chemotaxonomic value are anthraquinones. Their oxidative properties, unsaturated character and the presence of carbonyl groups explain their high reactivity and bactericidal, fungicidal and antimitotic properties (Kersten, 1971). On the basis of the foregoing data is was also decided to verify the supposition concerning the dominating role of anthraquinones in the activity of complex extract.

MATERIAL AND METHODS

The experimental material consisted of adventitious roots of *Allium cepa* bulbs growing at room temperature in dark glass vessels (0.5l) in tap water changed every 24 h. The experiment was started when roots reached a length of 3 cm. The extract was prepared according to Tarkowska (1971). Root incubation lasted 6, 12 and 24 h in the aqueous extract solutions within the dilution range of the outset extract 1—25 per cent. In the separate experiment shorter incubation times of 1, 2.5, 4 h were also applied. For establishing the degree of reversibility of the changes the same bulbs were transferred to tap water. During post-incubation material was collected at 24-h intervals up to 72 h. Each experimental combination included 5 bulbs and 2 control ones. The roots

for squash preparations were fixed in aceto-alcohol (1:3 vol/vol) and then stained with aceto-orcein. Roots for specific cytochemical staining on microtome sections were fixed in aceto-formalin (0.5—20). DNA was stained by Schiff's reagent (the Feulgen reaction), both NA were stained with toluidine blue in citrate-phosphate buffer, pH 3.4. Proteins were stained with toluidine blue pH 7.4 and with fast green, pH 8 after hydrolitic removal of NA with 5 per cent TCA at 90°C. The NA and protein levels were estimated on visual evaluation of color intensity in the cells. The roots for autoradiography were fixed in uranyl-nitrate with formalin (0.2—20). ^{3}H-thymidine (Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia) with specific activity 19.69 Ci/mM was used. The roots were incubated in ^{3}H-thymidine of 3 μc/ml concentration for 1 h. The microtome preparations were covered with stripping film (Kodak AR 10).

On squash preparations the total number of mitoses in the roots and the mitotic index were stated. I_M was calculated as the mean per cent of dividing cells in a total of 6000 meristematic cells (1000 such cells from each of 6 roots). The significance of the difference between the experimental and control data was statistically verified by Student's test. Estimation of the total number of mitoses were based on mean data from 3—5 preparations of 3-mm root apexes.

The presence of anthraquinones in the aqueous extract was confirmed by Borntrager's reaction (T u r o w s k a et al., 1970). In the experiment 2,6-dihydroxyanthraquinone of 0.15 mg/ml concentration (6×10^{-4}M) was used.

RESULTS AND DISCUSSION

Total number of mitoses and mitotic index during incubation in extract and postincubation in water

The growth of roots in the extract is strongly inhibited and obvious even on macroscopic observations. The growth increment of control roots occurs regularly in each time interval examined, whereas incubation in 5 and 12.5 per cent solutions completely inhibits growth (Fig. 1). It was found that 6-h and 12-h incubation in 25 and 12.5 per cent solution, respectively, is the limiting treatment. Stronger action is lethal. The further results presented here concern only the effect of a 5 per cent solution which is completely nontoxic and after which in the postincubation period reversion of the changes readily occurs.

It was supposed that one of the causes of root growth inhibition is the decrease in mitotic activity in the meristem. Calculation of the I_M indicates that incubation in 5 per cent solution produces a considerable fall in the number of mitoses (Fig. 2). Statistical analysis of the results

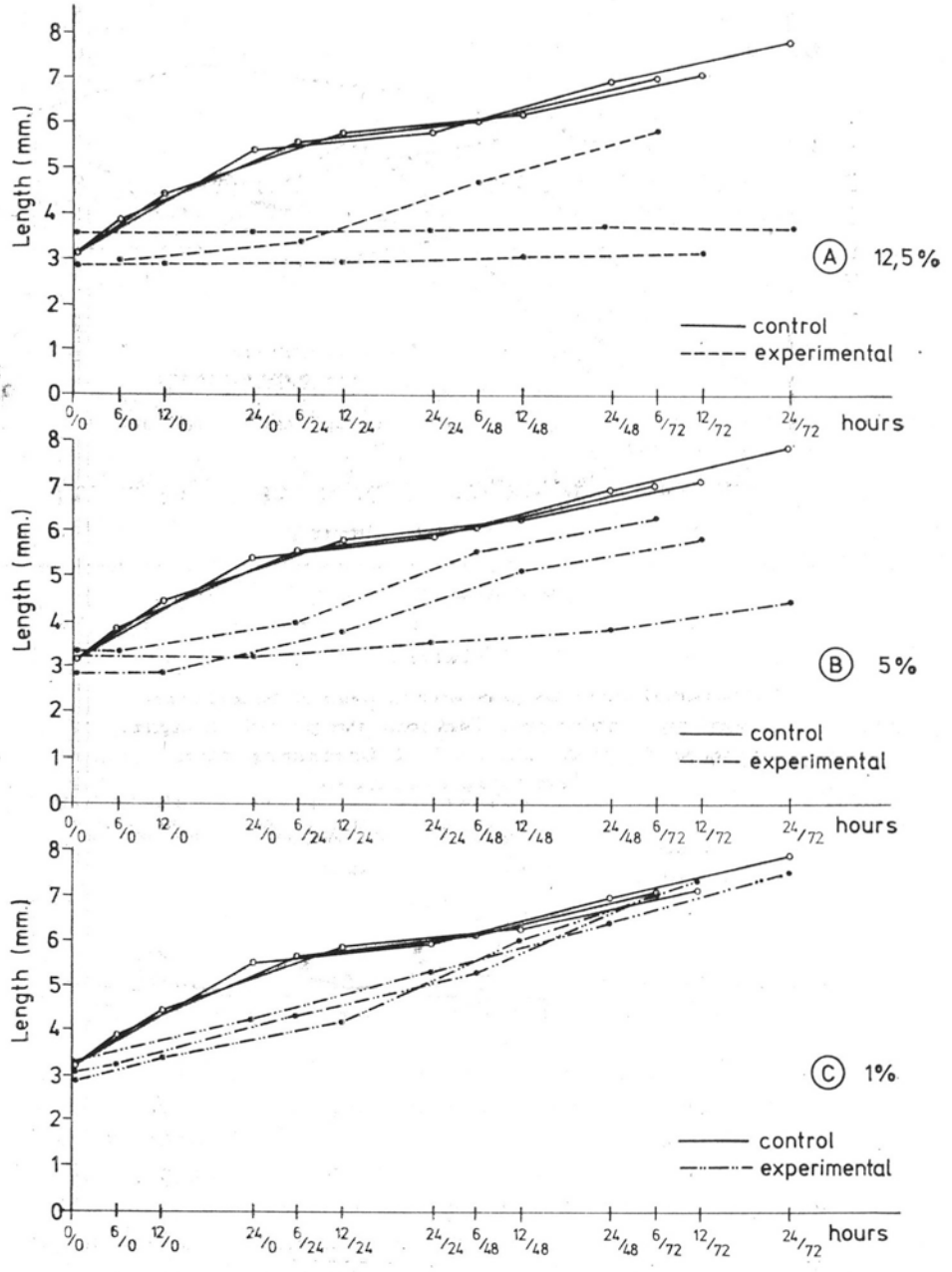

Fig. 1. Elongation growth of roots during incubation in aqueous extract from *Rheum officinale* rhizomes and postincubation in water. A. B. C — different concentrations of the extract

confirms the significance of the difference between experimental and control data (Table 1). A rise of the I_M value to the control level or even higher was noted during postincubation. This is best visible in Fig. 3

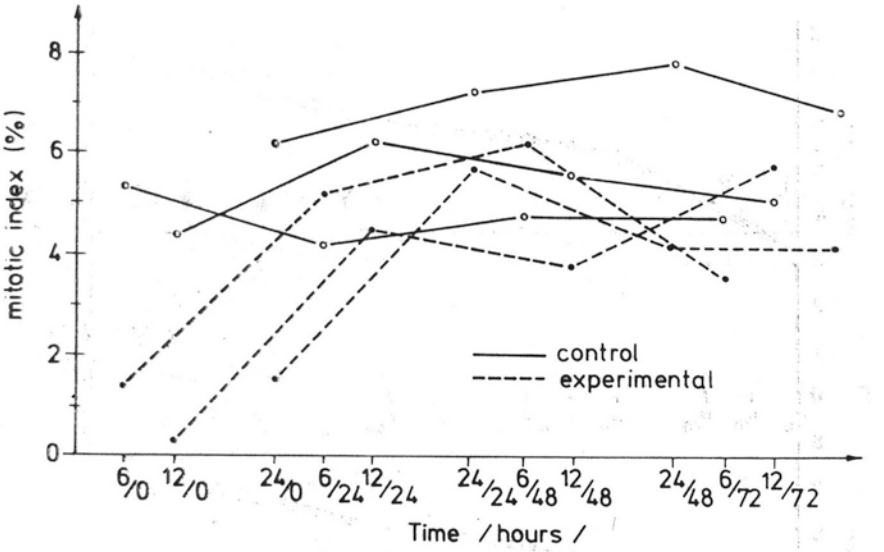

Fig. 2. Changes in mitotic index (I_M) during incubation in 5% extract and post-incubation in water

Table 1

Significant differences (t) between I_M values of the control and experimental combinations. Confidence level p = 0.95, 10 degrees of freedom, theoretical value $t_a = 2.228$. Difference significant if $t_a > t$ and not significant if $t_a < t$

Extract concentration	Incubation	Postincubation	t
	hours		
5%	24	0	3.790
	24	24	1.112
	24	48	2.181
	24	72	1.931
5%	12	0	5.732
	12	24	1.874
	12	48	1.833
	12	72	1.386
5%	6	0	9.074
	6	24	0.584
	6	48	1.574
	6	72	0.946

which shows data concerning the change in the total number of mitoses in the meristem. The transient increase in the number of mitoses following 24 h of postincubation (Fig. 3) may be the consequence of prolonged duration of mitosis as well as of mass reasumption of mitosis. In another

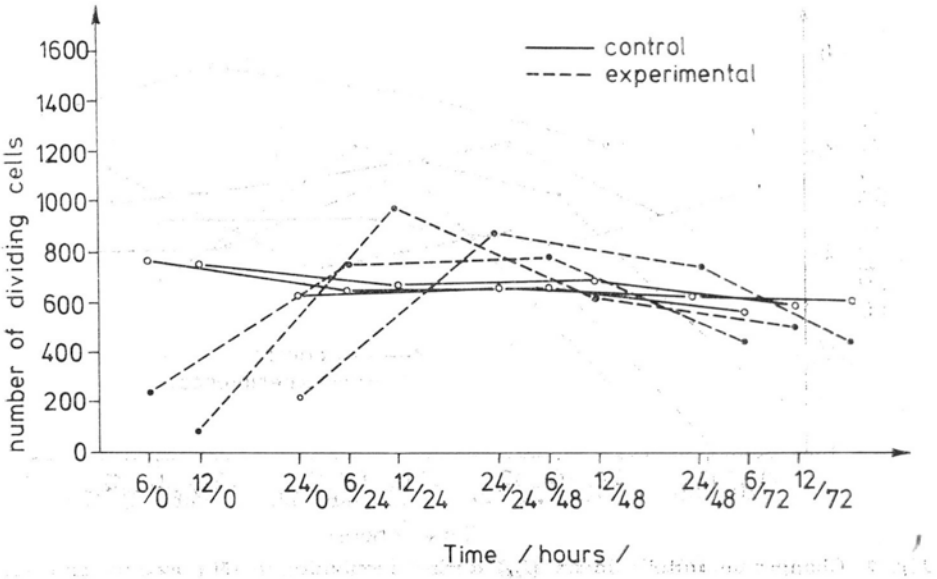

Fig. 3. Total number of dividing cells during incubation in 5% extract and post-incubation in water

experiment changes in I_M during 6-h incubation in 12.5 per cent solution were investigated. A sudden fall of the I_M (Fig. 4) was noted as early as after 1 h of incubation, this indicating a drastic inhibition of the $G_2 \rightarrow M$ transition. The transient increase of I_M after 4 h of incubation suggests accumulation of the slowly running mitoses and puts in doubt the total blockade of $G_2 \rightarrow M$. The low I_M value after 6 h of incubation seems to point to a highly restricted $G_2 \rightarrow M$ cell transition. A similar

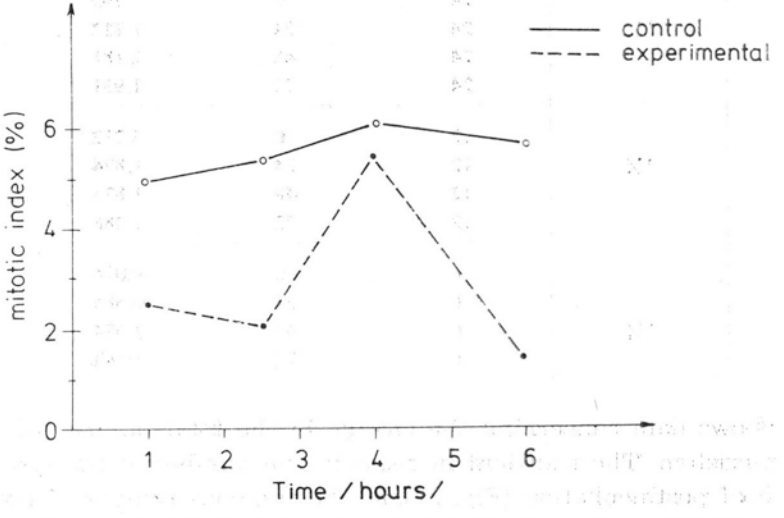

Fig. 4. Changes in mitotic index (I_M) during 6-h incubation in 12.5% extract

argumentation and interpretation is given by Mäkinen (1958) when analysing accumulation of mitoses during 5-h exposure of *Allium cepa* roots to 10^{-3} and 10^{-4}M CH_3COOH and 10^{-4} and 10^{-5}M H_2O_2.

The fragmentary data supplied by analysis of variance of the I_M are confirmed and supplemented by the calculation of all mitoses in the 3-mm root apex (Fig. 3). The strong effect of mitosis depression and the readiness with which reversion occurs in the postincubation period were confirmed. From the last data phase indices were calculated (Table 2). Comparison between experimental and control phase indices allows to determine eventual disturbances in the duration of particular phases (Mäkinen, 1963).

In the control the ratio of prophases to metaphases (P/M) does not exceed 2.7 value, whereas the ratio of metaphases to the sum of anaphases and telophases (M/A+T) is 1.01. In the experiment after 6 h and 12 h incubation the per centual contribution of prophases greatly increases (P/M 9.0 and 10.9, respectively). The more reduced is the number of dividing cells the more pronounced is the domination of prophases within scarce mitoses (e.g. after 12 h). The M/A+T value is practically unchanged in relation to the control, therefore the results point to a prolongation of prophase. Reversion of the metaphases and anaphases also contributes to the rise of the prophase index. During postinbucation the phase indices returns to normal.

Effect of rhizome extract and 2,6-dihydroxyantraquinone on the course of mitosis and cytokinesis

Penetration of the *Rh. officinale* extract into root meristematics cell causes various disturbances in the course of mitosis and cytokinesis and changes in the chromosome structure. As early as after 1.5 h of incubation the chromosomes contract, they become shorter and thicker than normal ones (Plate I, Photos 1, 2). The increase in chromosome diameter is due to their despiralization. It results in gradual reversion of prophases and restitution of interphase nuclei from the reverting metaphases and anaphases (Plate I, Photos 3, 4, 5). A similar interpretation of chromosome shortening may be found in the paper of Gimenez-Martin (1973). Regression of the initiated meta — and anaphases points to damage of the mitotic spindle. The observed disturbances in anaphase movements also indicate impairment of the mitotic spindle (Plate II, Photo 1). The presence of acentric chromatid fragments and chromatid bridges were observed (Plate II, Photos 2, 3). The fragments and bridges observed were always chromatid, thus they must have formed in the S or G_2 periods of the mitotic cycle. The maximal number of micronuclei (measure of chromatid fragmentation) was observed with a striking

Table 2

Numerical characteristic of mitotic activity in meristem during incubation in 5 per cent extract and postincubation in water (mean data from 3—5 roots)

Incubation / Post-incub. (hours)	Total number of mitoses		Content, %								P/M		M/A+T	
			Prophases		Metaphases		Anaphases		Telophases					
	Expt.	Contr.	Expt.	Contr.	Expt.	Contr.	Expt.	Contr.	Expt.	Contr.	Expt.	Contr.	Expt.	Contr.
6 / 0	241.6	751.0	78.5	56.9	8.6	19.2	3.8	9.8	8.3	9.9	9.0	2.8	0.73	0.83
6 / 24	737.6	622.0	56 5	55.0	22.4	22.3	12.7	12.8	13.04	9.9	2.5	2.4	0.8	0.9
6 / 48	792.4	668.0	53.4	51.09	22.3	19.2	13.1	10.9	11.2	17.1	2.3	2.7	0.9	0.68
12 / 0	66.0 948.0	764.0	82.7	52.6	7.6	22 3	2.4	11.07	6.6	10.7	10.9	2.3	0.8	1.05
12 / 24	622.0	668.0	49.6	51.2	21.2	19.2	12.08	10.9	13.5	17.1	2.3	2.7	0.8	0.7
12 / 48		688.0	49.5	52.6	3.3	23.9	12.1	11.3	15.0	12.9	2.1	2.5	0.8	1.0

regularity during postincubation (Table 3) as a result of disturbances during semiconservative DNA replication in S or morphological formation of chromatids in G_2 period. The course of cytokinesis is also disturbed. Binucleate cells were observed, most frequently in the dermatogen and external periblem layers, as the result of disorders during formation of the cell plate. After disappearance of the phragmoplast the two daughter nuclei come close together and become flattened along the contact surface (Plate II, Photo 4).

The effect of 6×10^{-4}M 2,6-dihydroxyanthraquinone is strikingly similar to that of the total extract. Contraction of chromosomes, restitution nuclei formation from reverting mitotic nuclei, chromatid type of

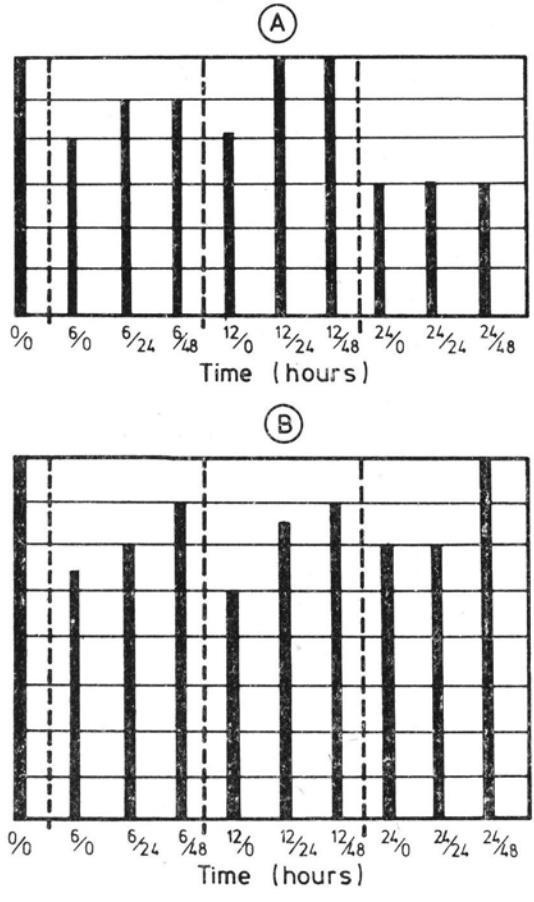

Fig. 5. Visual estimation of DNA staining in interphase nuclei with 0.25% toluidin blue, pH 3.4 (A) and by the Feulgen method (B) during incubation in 5% extract and postincubation in water

Table 3

Total number of micronuclei in 3-mm root apex during incubation in 5 per cent extract and postincubation in water (mean data from 3—5 roots in each sample)

Incubation (hours)	Postincub. (hours)	Number of micronuclei Expt.	Number of micronuclei Contr.	Incubation (hours)	Postincub. (hours)	Number of micronuclei Expt.	Number of micronuclei Contr.	Incubation (hours)	Postincub. (hours)	Number of micronuclei Expt.	Number of micronuclei Contr.
6	0	5.5	6.0	12	0	6.6	4.0	24	0	3.6	6.3
6	24	14.6	3.4	12	24	25.6	3.5	24	24	17.0	6.3
6	48	15.0	2.0	12	48	16.6	0.0	24	48	59.3	0.33
6	72	11.33	1.66	12	72	16.0	1.0	24	72	23.6	1.66

fragmentation, manifest during postincubation, and the same pattern of disturbances in the course of cytokinesis were observed.

Such facts as inhibition of the $G_2 \rightarrow M$ transition and appearance during postincubation of chromatid type aberrations suggest that they are the result of the antimitotic action on the interphase. From among the many possible causes of metabolism disorders in interphase cells, estimation of the influence of the extract on the DNA synthesis level (^{3}H-thymidine incorporation) and the NA and protein content was attempted (after specific cytochemical staining).

The DNA content in interphase nuclei after incubation was lower than in the control as indicated by the differences in the intensity of the Feulgen reaction and staining with toluidine blue. The intensity of RNA staining in the nucleoli and cytoplasm changes similarly. Preliminary autoradiographic investigations confirm the above mentioned observations. After 3 and 6 h of incubation ^{3}H-thymidine incorporation is low and after 12 h complete absence of labeled nuclei was noted. In the postincubation period DNA synthesis is reassumed after 24 h, but the control level is reached only as late as after 48 h. Changes observed in the protein content are less marked than in NA. It seems that a slight decrease in the acid and basic protein amounts occurs during postincubation as the result of previous inhibition of DNA and RNA synthesis (Fig. 6).

On the basis of presented data it seems that the component of the extract deciding of its cytostatic properties are anthraquinone compounds.

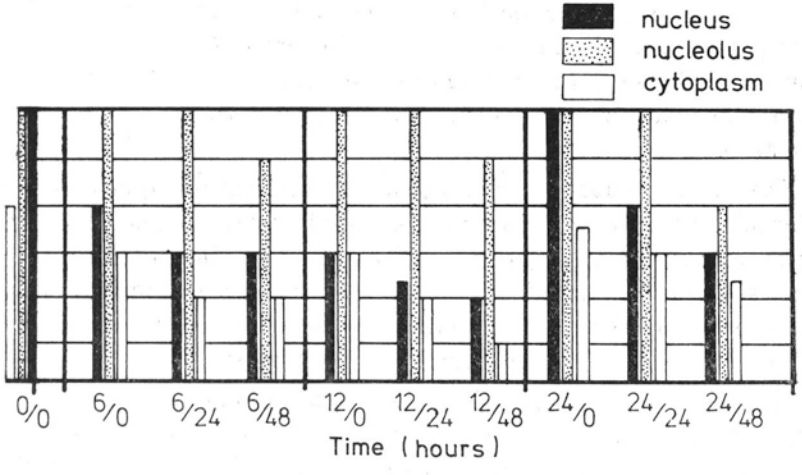

Fig. 6. Visual estimation of intensity of protein staining with 0.25% toluidin blue, pH 7.4 in the nucleus, nucleolus and cytoplasm during incubation in 5% extract and postincubation in water

The biochemical mechanism of quinone inhibition is attributed to the "attack" on the -SH protein groups (Hoffmann and Ostehof, 1963). A confirmation of this mechanism is the partial reversion of inhibition after addition of cystein or glutathione (Hoffmann--Ostenhof, 1963) protecting the enzymes. This author gives a list (incomplete) of the enzymes inhibited by quinones: alkaline and acid phosphatase, urease, glutaminase, proteinase, deoxyribonuclease, pyruvic acid carboxylase (the latter particularly susceptible to the action of anthraquinones). Thus, the inhibition involves various metabolic pathways, among them also DNA, RNA and protein synthesis.

The results here obtained are mostly comparable with those observed after acting with maleic acid hydrazide (MH) on meristematic plant cells (Hughes and Spragg, 1958; Evans and Scott, 1964 and Kihlman, 1969). It was found that the action of MH has the same chemical background as that characteristic for quinones, that is formation of irreversible bonds between MH molecules and -SH protein groups (Hughes and Spragg, 1958).

CONCLUSIONS

Comprehensive analysis of the results allows the following characteristic of the antimitotic activity of the extract from *Rheum officinale* rhizomes:

1. The inhibition of root growth is mainly due to the decrease of the mitotic activity manifested in a statistically significant fall of the I_M value and the total number of mitoses. This depression of mitosis is reversible in the postincubation period. The rapid decrease of mitotic activity points to a considerable fall of the phase $G_2 \rightarrow M$ cell transition. Incubation in the extract prolongates the duration of mitosis. The highest absolute and relative frequency, thus longest duration, is characteristic of prophase.

2. The extract causes chromosome despiralization appearing in their considerable contraction. Premature despiralization leads to prophase reversion and interphase nuclei restitution from the initiated meta- and anaphases. Impairment of the mitotic spindle and phragmoplast was noted. The consequence of cytokinesis disorders is the presence of binucleate cells, mainly in the peripheral root layers.

3. Chromosome fragmentation of chromatid type was found. The type of fragmentation and its manifestation in the postincubation period indicate that the damage may have occurred in the periods S or G_2 of the mitotic cycle. Differences in the intensity of specific cytochemical stainings as compared with the control suggest a lower NA content in the interphase nuclei during incubation. According to preliminary

autoradiographic investigations, it seems that during incubation in the extract DNA synthesis is inhibited. Owing to previous inhibition of NA synthesis a reduced intensity of protein staining was observed.

4. It is probable that the active antimitotic agent in the extract consists of anthraquinone compounds. Treatment with 6×10^{-4} M 2,6-dihydroxyanthraquinone gives a similar picture of cytological deformations as does the total extract. It seems that these effects appear as the result of disturbance of various metabolic pathways, among them of NA and protein synthesis. Probably the biochemical mechanism of inhibition consists above all in the formation of irreversible bonds between anthraquinone compounds and the -SH groups of proteins.

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Wpływ rozpuszczalnej w wodzie onkostatycznej frakcji z kłączy Rheum officinale Baill. na merystem wierzchołkowy korzeni Allium cepa L.

I. *Zmiany aktywności mitotycznej w merystemie*

Streszczenie

Zbadano wpływ onkostatycznego ekstraktu z kłączy *Rheum officinale* na aktywność mitotyczną korzeni przybyszowych cebul *Allium cepa* L. oraz podjęto próbę zdefiniowania czynnego antymitotycznie składnika. Stwierdzono statystycznie istotny spadek wartości I_M oraz całkowitej liczby mitoz w okresie inkubacji, czego następstwem było hamowanie przyrostu korzeni. Wyciąg wywołuje różnorodne zaburzenia toku mitozy i cytokinezy: kontrakcję i despiralizację chromosomów, zaburzenia ruchów chromatyd w anafazie, zaburzenia wrzeciona mitotycznego i fragmoplastu, rewersję profaz, powstawanie jąder restytucyjnych z metafaz i anafaz oraz powstawanie komórek dwujądrowych. Obecność acentrycznych fragmentów chromatyd podczas postinkubacji wskazuje na uszkodzenia faz S i G_2

jąder interfazowych. Badania cytochemiczne sugerują, że podczas inkubacji w ekstrakcie obniża się zawartość DNA i RNA, na co wskazuje zmniejszenie intensywności zabarwienia NA w porównaniu z kontrolą. Wstępne badania autoradiograficzne wykazały, że podczas inkubacji jest hamowana synteza DNA. Podczas postinkubacji, w następstwie hamowania syntezy DNA, obserwowano zmniejszoną intensywność barwienia się białek. Składnikiem decydującym o antymitotycznych właściwościach ekstraktu są prawdopodobnie związki antrachinonowe. Zakłócenia w przebiegu mitozy i cytokinezy pod wpływem 2,6-dwuhydroksyantrachinonu są takie same, jak po działaniu ekstraktu całościowego. Opisane efekty są prawdopodobnie rezultatem inhibicji, jaką powodują antrachinony, tworząc nieodwracalne połączenia z grupami –SH białek.

Plate I

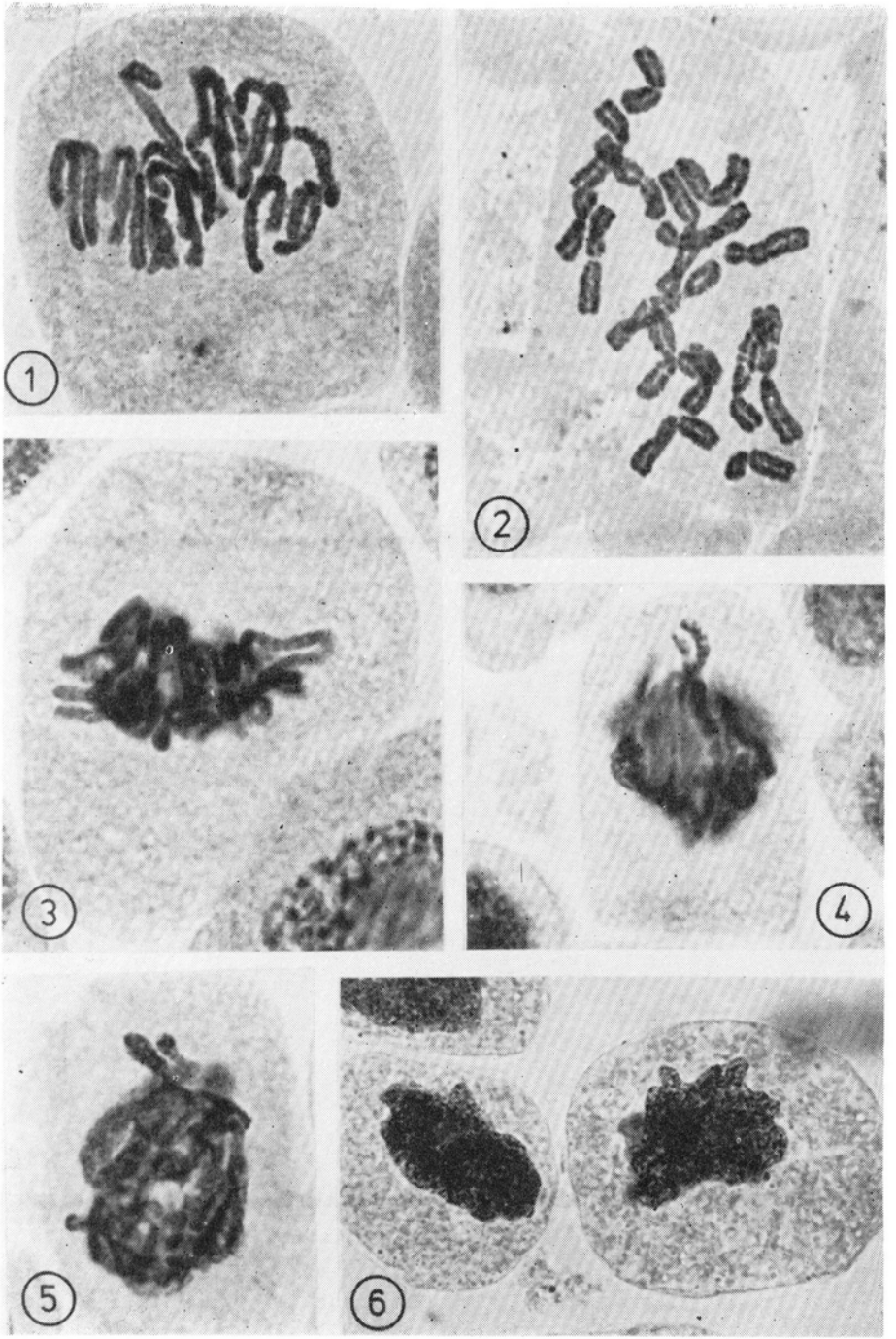

Photo 1. Late prometaphase in control root. Photo 2. Shortened and thickened metaphase chromosomes after 6 h incubation in 5% extract. Photos 3, 4, 5. Restisution of interphase nuclei. Photo 6. Restitution nuclei formed from reverting mitoses

Squash fixed in AA, aceto-orcein, × ca. 1300

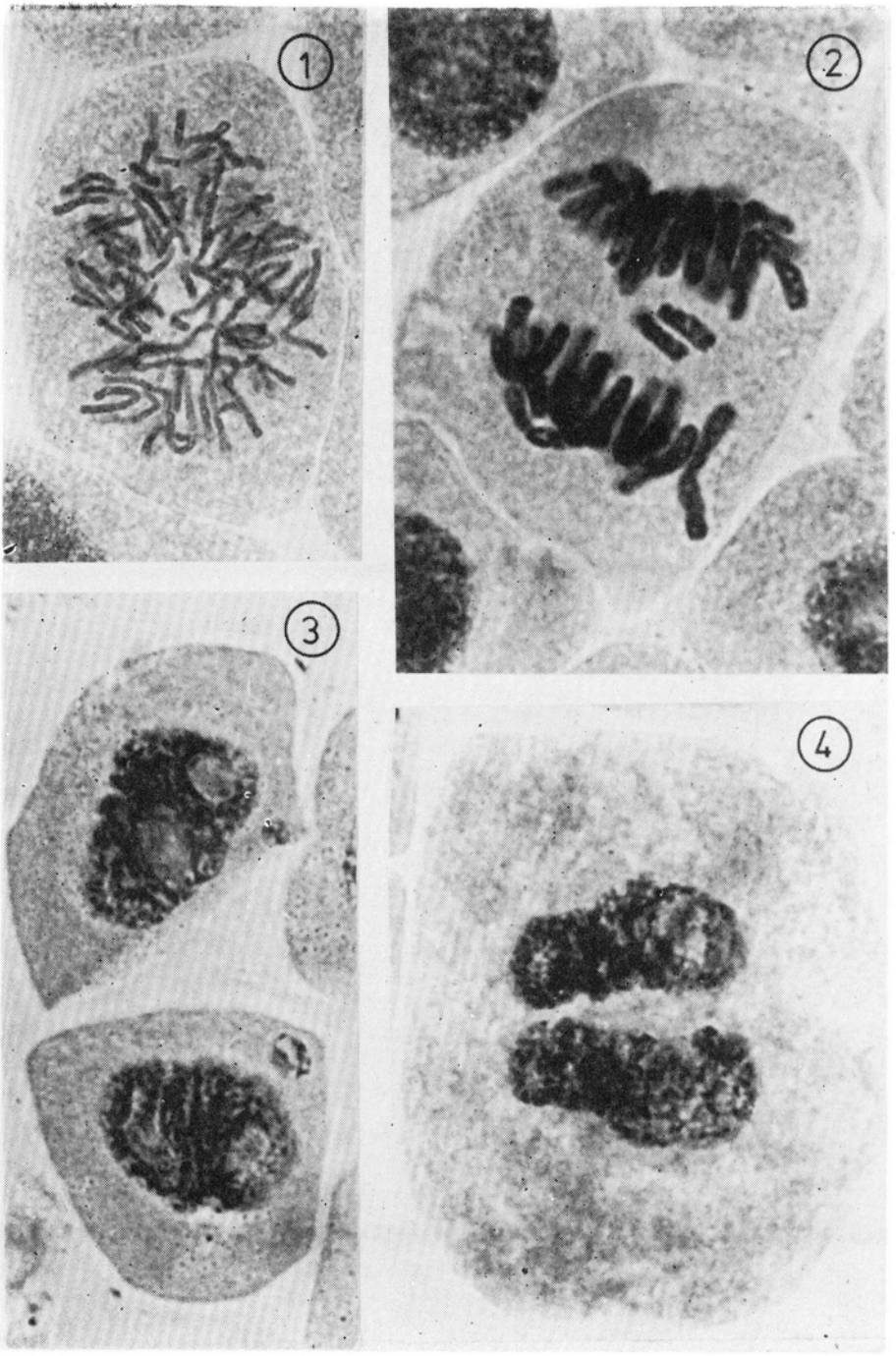

Photo 1. Disturbances in chromatid translocation under the influence of 6-h incubation in 5% extract. Photo 2. Chromatid fragment in late anaphase, 24-h postincubation after 6 h of exposure to 5% extract. Photo 3. Micronuclei formed from excentric chromatid fragments. Postincubation 48 h after 6 h of exposure to 5% extract. Photo 4. Binucleate cell formed as the result of cytokinesis disturbances after 6 h incubation in 5% extract

Squash fixed in AA, aceto-orcein; Photo 1 × ca 1300; Photos 2, 3, 4 × ca 1820