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CELL PROLIFERATION IN *ALLIUM CEPA* L. MERISTEMS UNDER 8-HYDROXYQUINOLINE, A CHELATING AGENT THAT AFFECTS DNA AND RNA POLYMERASES

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SUMMARY

8-Hydroxyquinoline (HQ) chelates Mg^{2+} and Mn^{2+} and, secondarily, affects the activities of DNA and RNA polymerases. The *in vivo* effect of HQ has been estimated in *Allium cepa* L. meristems growing under new growth kinetics in the presence of this agent. HQ (at both 5×10^{-5} M and 10^{-4} M) depressed incorporation of [^{3}H]uridine much more effectively than that of [^{3}H]-thymidine. Cycle kinetics in meristems behaved as if they were independent of the rates of synthesis or accumulation of RNA since, under HQ, cycle time was only moderately modified and the new cycle kinetics achieved could be explained by the new rates of [^{3}H]thymidine incorporation. Lengthened *S* periods were partially compensated for by shortened G_2 phases, suggesting that, in these cells, both the growth cycle and its coupling with the DNA-division cycle were not disturbed by a decreased amount of RNA.

Finally, the nucleolar cycle during mitosis, but not the interphase nucleolus, was modified under the new rates of RNA synthesis.

INTRODUCTION

Proliferating cells are mostly characterized by the continuous growth of all of their components so that after each cycle time the cell can be divided into two cells identical to the initial one. These general processes of growth of cellular mass take place in the so-called growth cycle (Mitchison, 1974). Replication of DNA is one of the processes that occurs in a parallel sequence of events, the DNA-division cycle (Mitchison, 1971), in a discrete segment of the whole interphase, the *S* period. Involved in the control of the replication and distribution of the replicated chromosomes, i.e. mitosis, there are a number of points at which synthesis or assembly of specific molecules conditions further progression of the cell towards the completion of its cycle. Such regulatory steps are located in the DNA-division cycle, and they are the final consequence of qualitative cycle-specific gene functions, as mutational analysis has proved (Hartwell, 1971; Howell & Naliboff, 1973; Liskay & Prescott, 1978).

Gene activity, both replication and transcription, depends on cations for activities of the different DNA and RNA polymerases, which rely on supply of Mg^{2+} and

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Mn^{2+} to different extents (Kornberg, 1980; Onishi & Muramatsu, 1976; Marzluff, 1976).

Previous work on *Schizosaccharomyces pombe* has shown the rapid inhibition of the synthesis of ribosomal and messenger RNA, but not of 5 S and transfer RNA, produced by HQ (Fraser & Creanor, 1974), and that the *in vitro* 8-hydroxyquinoline (HQ)-mediated RNA synthesis inhibition was brought about by the chelation of Mg^{2+} and Mn^{2+} (Fraser & Creanor, 1975).

The aim of the present work was to study how a chelating agent, such as HQ, affects the process of DNA and RNA synthesis in *Allium cepa* L. meristems *in vivo* and how these modifications affect the proliferative kinetics.

MATERIALS AND METHODS

The material used was the root meristem of *A. cepa* L. bulbs. They were grown in the dark at a temperature of 15 ± 0·5 deg. C, in cylindrical glass receptacles of about 80 ml capacity, in tap water renewed every 24 h with constant aeration. The bulbs were placed so that only their bases remained immersed in the water. After 2–3 days most of the roots ranged from 2–3 cm in length.

Treatments

Roots of 20–30 mm, still attached to the bulbs, were transferred to the different HQ solutions (10^{-4} M, 5×10^{-5} M), under the same conditions as usual for growing.

Measurement of root growth

When the roots attained a length of approximately 20–30 mm they were all removed, except 10–15. Elimination of new roots was continued throughout the time during which measurements were made, so that only the initially selected roots were allowed to grow. This procedure enabled each root to be identified individually. The length of each root was measured daily to ±0·25 mm with a transparent paper ruler. The first reading was taken to coincide with the selection of roots, and the increases found were recorded every 24 h.

Cytological procedures

Labelling with [^{3}H]thymidine. [*methyl*-^{3}H]thymidine ([^{3}H]dThd) (from the Radiochemical Centre, Amersham, U.K.) with a specific activity of 9·25 TBq mmol^{-1} and at a concentration of 0·37 MBq ml^{-1} was used. In the experiments applying Mak's (1965) method, a 30 min pulse of [*methyl*-^{3}H]thymidine was used. The roots were fixed immediately at the end of the thymidine pulses in 3:1 (v/v) ethanol/acetic acid. They were then stained by the Feulgen reaction and the appropriate meristem squashes were prepared on subbed slides. Autoradiography was done with Kodak AR-10 stripping film. After exposure for 7 days they were developed with a Kodak D19 developer and fixed with a Kodak ultrarapid acid fixer.

Staining techniques. For cytological analysis roots were fixed in a 3:1 (v/v) ethanol/acetic acid mixture. Some meristems were stained with acetic orcein according to Tjio & Levan (1950). Other meristems were fixed in a mixture of 10 % (v/v) formaldehyde and 1 % (v/v) hydroquinone for 2 h, and the silver impregnation technique was used for staining nucleoli.

Analysis of cell frequencies. The mitotic and phase indices were determined. Frequency distribution of cells in G_1, S and G_2 was obtained by using Mak's (1965) method, which combined autoradiography for detecting cells in the S period, and Feulgen spectrophotometry of the unlabelled interphase cells to distinguish between G_1 and G_2 cells ($2C$ and $4C$ content of DNA, respectively).

Synchronous binucleate cell population

Control roots and roots incubated for 48 h in HQ (10^{-4} M and 5×10^{-5} M) were immersed, still attached to the bulbs, in 5 mM-caffeine plus treatment solution for 1 h, and then returned to treatment solution until the binucleate cells reached mitosis.

Kinetics of incorporation in segments of the root meristems

Roots after 4 days of treatment (10^{-4} M and 5×10^{-5} M-HQ) were immersed in the same solution containing the radioactive precursor, and incubated for the required period.

[^{3}H]uridine incorporation

[5,6-^{3}H]uridine incorporation ([^{3}H]dUrd) was used at a final concentration of 1·5 MBq ml^{-1} (with a sp. act. of 1·75 TBq mmol^{-1}).

At the times indicated (30, 60, 90, 120 min) the apical 30–40 mm of 10 roots were collected and thoroughly washed with 1 % calcium hypochlorite in order to eliminate contaminating bacteria (Sarrouy-Balat, Delseny & Julien, 1973). Afterwards, the roots were washed with distilled water (at 4 °C). The first 0·7 mm from the root apex was discarded, while the next 1 mm of the root was taken as representative of the portion of meristems with the highest growth fraction, i.e. most of its cells are cycling. These 1-mm segments were harvested at −20 °C. Later on, these meristematic segments were homogenized in a Potter grinder in the presence of 0·1 M-glycine–NaOH buffer, pH 9, containing 0·01M-EDTA and 0·1M-NaCl. Three 0·1-ml samples of the homogenate were immediately taken and transferred onto GF/A Whatman glass fibre filters, dried and counted in order to determine radioactive uptake. After that, 0·3 ml from the homogenate was precipitated with 20 % trichloroacetic acid in order to estimate the incorporation into the acid-insoluble fraction. Precipitates were recovered by filtration through GF/A filters, washed, dried and counted. Each experiment included a control in the absence of HQ and such control incorporation curves were normalized to a single control.

RESULTS

Effect of HQ on root growth

The first aim of the work was to find two concentrations of HQ that moderately and severely, respectively, affected growth in *A. cepa* L. roots without blocking it. Root growth is the result of both the continuous supply of new cells by the meristem and the elongation of these cells to the final size achieved by differentiated cells. Concentrations above 10^{-4} M-HQ produced a blocking of root growth from the first day of treatment; moreover, 2×10^{-3} M-HQ stopped cells at metaphase. Concentrations of HQ below 5×10^{-5} M did not produce any substantial change in the rate of root growth.

Cycle kinetics under HQ

From the concentrations tested: 5×10^{-5} M-HQ inhibited (by about 46 %) root growth (Fig. 1) but allowed the maintenance of new growth kinetics; HQ at 10^{-4} M apparently blocked growth after 4 days of continuous treatment (Fig. 1), allowing from the second day to the fourth for similar root growth, which was 30 times lower than in the control (0·35 mm day^{-1} *versus* 12 mm day^{-1} in control).

The rate of incorporation of [^{3}H]dThd was measured in root meristems on the fourth day of growth in HQ. Fig. 2 shows that after such long treatment (even at 10^{-4} M) there is still incorporation, though 1·6 times lower than in the control cells.

HQ at 5×10^{-5} M gives a rate of incorporation 1·25 times lower than control. The ratio between [^{3}H]dThd incorporation into DNA and its cellular uptake was fairly constant (0·37) at all the times and conditions studied.

The cellular distribution among the different cycle compartments was analysed by giving short treatments with [^{3}H]dThd and by recording the frequency of labelled cells on autoradiograms, as well as the frequency of interphase cells with both 2*C* and 4*C* DNA contents. The frequency of mitosis was also recorded. Values presented in Table 1 represent cell distribution in control conditions, as well as after 4 days of continuous treatment with HQ. It can be observed that the relative frequency of both G_1, *S* and G_2 changed under HQ. *S* increased while G_2 decreased. The maximum *S* period took place in 10^{-4} M-HQ, at which not only G_2 but also G_1 was reduced. Apparently, mitosis was not affected.

In order to measure the absolute duration of interphase, we labelled as binucleate those cells traversing telophase, by using a 1-h treatment with 5 mM-caffeine, which inhibits the formation of the cytokinetic plate (López-Sáez, Risueño & Giménez-Martín, 1966). We followed that population throughout their cycle by fixing the meristems at different times after the end of caffeine. Table 2 presents the frequencies of binucleate cells in mitosis at different times after their formation.

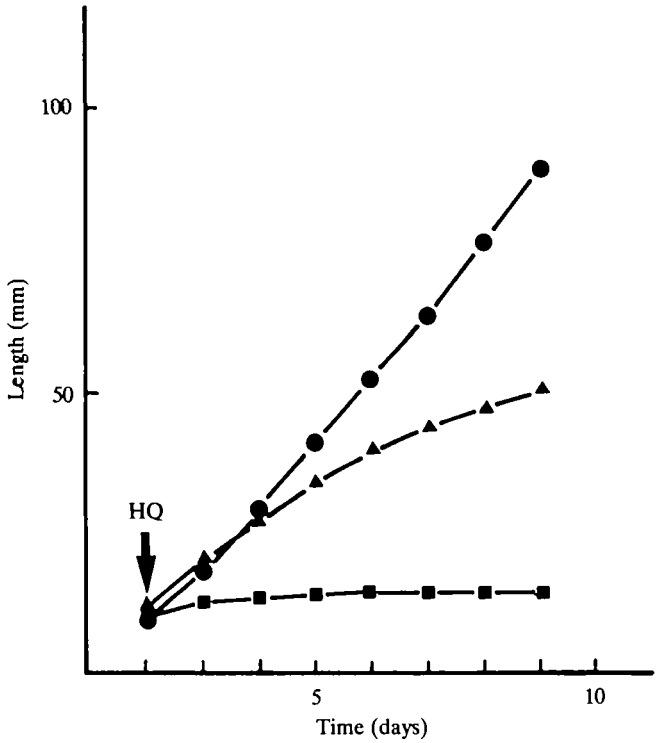

Fig. 1. Mean root length at different times after sprouting. Treatments with HQ were initiated on the 2nd day. (●——●) Control; (▲——▲) 5×10^{-5} M; (■——■) 10^{-4} M.

From these data it could be estimated that the fastest 20 % of the control population reached mitosis at 25·2 h of interphase. Under continuous treatment with 5×10^{-5} M-HQ, entry into mitotis was displaced only 2·1 h (8 %) in relation to the control, while

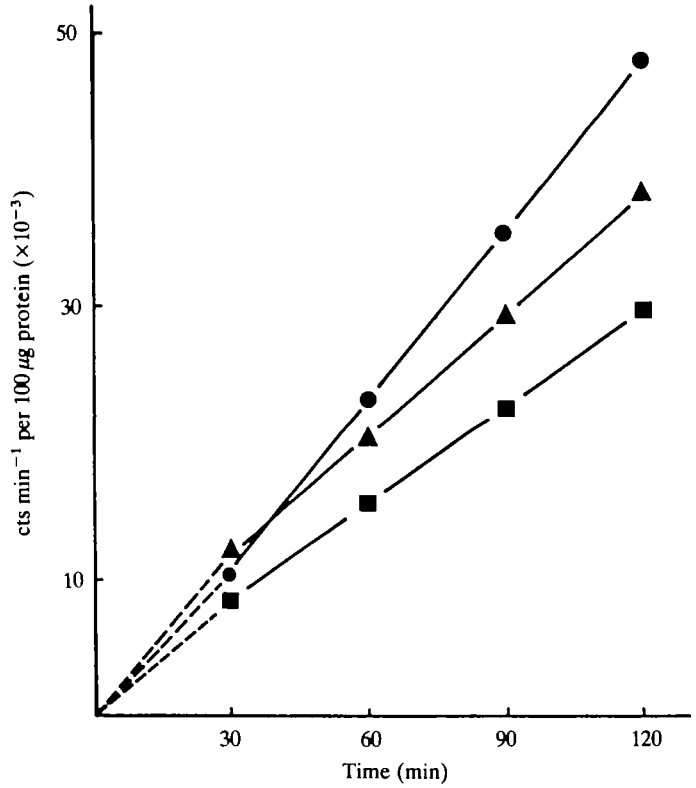

Fig. 2. [^{3}H]dThd incorporation in roots collected on the 6th day after sprouting, i.e. after 4 days of continuous growth in HQ in the case of treated roots. The incorporation was measured in the presence of HQ at different times of [^{3}H]dThd treatment *in vivo* (30–120 min), and is expressed in cts min^{-1} per 100 μg of protein content, measured in a sample of the same root segments as used for estimating DNA precursor incorporation. Symbols as for Fig. 1.

Table 1. *Recorded frequencies of cellular distribution under HQ and in control conditions*

	G_1	S	G_2	M	Cycle time (h)*
Control	0·28 (8·1)	0·42 (12·2)	0·17 (4·9)	0·13 (3·8)	29·0
5×10^{-5} M	0·29 (9·0)	0·49 (15·2)	0·10 (3·1)	0·12 (3·7)	31·0
10^{-4} M	0·25 (8·7)	0·54 (18·8)	0·10 (3·5)	0·11 (3·8)	34·8

Values in parentheses represent the estimated durations (in h) of the different cycle compartments.

* Estimated from the interphase durations (Table 2), taking into account the recorded mitotic indices (column *M*).

Table 2. *Frequency of binucleate cells in mitosis, at different times after caffeine (%)*

Time (h)	Control	5×10^{-5} M-HQ	10^{-4} M-HQ
23	3·6 (250)	—	—
25	10·0 (200)	—	—
27	40·4 (210)	15·2 (200)	—
29	75·7 (203)	43·5 (350)	12·1 (150)
31	57·9 (200)	60·1 (150)	23·5 (125)
33	32·3 (205)	50·9 (250)	24·6 (150)
Interphase duration (h)*	25·2	27·3	31·0

Values in parentheses represent the size of the population studied.

* Estimated from the time when 20% of each binucleate cell population had reached mitosis, after obtaining the corresponding regression lines from the ascending portion of the curve.

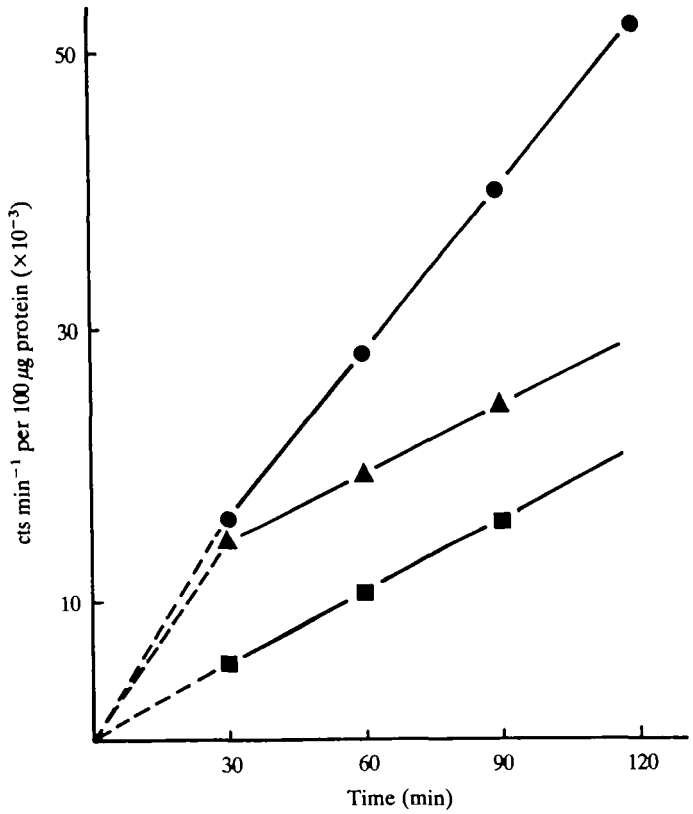

Fig. 3. [^{3}H]dUrd incorporation estimated in segments of roots on the 6th day after sprouting. At this time, treated roots had had 4 days of continuous growth in HQ. [^{3}H]dUrd was supplied simultaneously with HQ, from 30–120 min and the incorporation was expressed in cts min^{-1} per 100 μg of protein. Symbols as for Fig. 1.

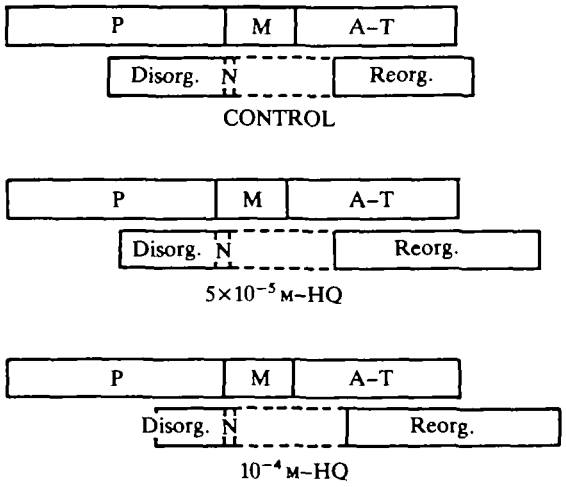

Fig. 4. Diagram showing the fitting of nucleolar and mitotic cycles in control (top) and under 5×10^{-5} M and 10^{-4} M-HQ, respectively. P, M and A–T represent prophase, metaphase and ana–telophase in upper bars. Disorg. represents the disorganization phase of the nucleolus, which is characterized by the appearance of nucleolar segregation and the progressive loss of nucleolar portions. N in lower bars represents the persistent nucleoli that are sometimes observed after nuclear envelope breaking. Reorg. represents the stage characterized by the presence of either scattered prenucleolar material only or this material plus the new growing nucleoli on NORs.

for the population growing at 10^{-4} M-HQ there was a displacement of 5·8 h in relation to the control (23 % of the interphase timing). These data were also used to estimate the duration of the different cycle phases, taking into account the relative frequencies previously recorded in Table 1. As we can deduce from such estimations of timings (values in parentheses in Table 1), the *S* period was lengthened 1·25 and 1·6 times in 5×10^{-5} M and 10^{-4} M-HQ, respectively. Such lengthenings are proportional to the rates of [^{3}H]thymidine incorporation under HQ (estimated from data in Fig. 2).

Effect of HQ on transcription and the nucleolar cycle

We also analysed the rate of incorporation of [^{3}H]uridine in 1-mm segments of the root apex. As shown in Fig. 3, the rate of incorporation was depressed under HQ. That rate was 1·8 and 2·5 times lower, under 5×10^{-5} M and 10^{-4} M-HQ, respectively, than in the control. Moreover, the ratio between [^{3}H]dUrd incorporated and the total cellular uptake varied in the three experimental conditions.

Finally, the fitting of the nucleolar cycle to mitosis was accomplished by studying the frequency of the different mitotic phases, as well as the frequencies of the different phases of the nucleolar cycle: its disorganization, and the reappearance of prenucleolar bodies in ana–telophasic cells.

Fig. 4 represents the fitting of both sets of results, taking into account that the end of the disorganization stage occurs precisely when the nuclear envelope breaks down (prophase to metaphase border). The main features we can discern under HQ are a

shortened disorganization phase and a longer reorganization period, while the length of mitosis is similar at both HQ concentrations. The initiation of the reorganization of nucleoli under HQ occurred slightly later than in the control.

No segregation was observed in interphasic nucleoli even under such long treatments with HQ.

DISCUSSION

HQ stops cycle progression at metaphase when used at high concentrations (2×10^{-3} M) in plant cells, so it is used for chromosomal analysis (Tjio & Levan, 1950; Terasaka & Tanaka, 1974). We have confirmed such an effect on *A. cepa* L. meristems. However, the severe depression of root growth observed at 10^{-4} M-HQ is mostly unrelated to any cycle blocking either in metaphase or in any other mitotic or interphase stage. Probably, the elongation that characterizes the early stages of the cells approaching the differentiating zone of the root should be severely depressed under HQ.

Both DNA and RNA syntheses are negatively affected by HQ, as expected of such an agent chelating Mn^{2+} and Mg^{2+}, which are involved in the activity of both RNA polymerase (Fraser & Creanor, 1975) and DNA polymerase (Fraser & Creanor, 1974; Cannon & Jiménez, 1974). *In vivo*, synthesis of RNA was found to be more sensitive than synthesis of DNA to both concentrations of the chelating agent in these plant cells (compare Figs 2 and 5).

Synthesis of DNA

The lower rates of DNA synthesis under steady-state kinetics in HQ-treated meristems were paralleled by S periods that were larger than those recorded in the control. Not only were their relative durations in the cycle lengthened, but also their absolute durations.

Coupling of the growth and DNA-division cycles in meristems

The lengthening of the S period was accompanied by a shortening of the subsequent G_2 phase. This observation suggests that the two sequences of events, which run in parallel (the DNA-division cycle and the growth cycle), are related or interconnected in late interphase, as well as in the initiation of the S period, as proposed for such meristematic cells by Navarrete, Cuadrado & Cánovas (1983) and Cuadrado, Navarrette & Cánovas (1985). Furthermore, there must be some G_2-specific functions in the plant DNA-division cycle, so that G_2 cannot be completely dispensed with, for G_2 was shortened up to 70 % of its control duration, but no more. The indispensability of a G_2 phase has been suggested for animal (Rao Satya-Prakash & Wang, 1984) and plant cells (Navarrete *et al.* 1983).

Finally, when shortening of G_2 was not enough to compensate for the lengthening of S, G_1 was also shortened, a phenomenon observed when using low concentrations of different inhibitors of DNA replication (Navarrete *et al.* 1983).

Synthesis of RNA and the growth cycle in these proliferating cells

The completion of the cell cycle in a time 1·2 times longer than the control, when the rate of [^{3}H]dUrd incorporation is 2·5 times lower at 10^{-4} M-HQ than in the untreated meristems, makes it evident that, in these plant cells, the rate of cycle progression is not strictly linked to the total rate of synthesis or accumulation of bulk RNA. Moreover, the new cycle kinetics achieved under HQ are fully explainable by the depressed rates of replication.

This lack of correlation between cycle kinetics and rate of transcription appears to be in simple contradiction to the results obtained in animal cells (Darzynkiewicz *et al.* 1979), though another approach makes it evident that such a relationship may not be so obvious, or applicable to all animal cells (Mercer *et al.* 1983).

Our data suggest that the accumulation of RNA per cycle either is not one of the controlling elements of the growth cycle or, if it is, it remains cryptic due to RNA being far in excess in these plant cells. This observation in onion agrees with the apparent independence of G_1 length on the rate of rRNA production in root meristems of maize (De la Torre, Moreno & Clowes, 1986).

Nucleolus

Though the inhibition of the synthesis of RNA is known to bring about the segregation of fibrillar and granular components (Bernhard, 1971), its mere depression under new growth kinetics does not seem to affect the distribution of the nucleolar components in interphase since no segregation was observed under HQ. The completion of the nucleolar reorganization depends on the re-starting of RNA transcription in late mitosis (De la Torre & Giménez-Martín, 1982), and probably on the rate at which it is initiated, since depressing the rate of RNA synthesis slightly lengthened the time of nucleologenesis (nucleolar reorganization).

Finally, the disassembly of nucleoli during prophase must not be considered a passive process, since depression of RNA synthesis delays its onset, as was shown in maize under severe inhibition of RNA synthesis (Clowes & De la Torre, 1974).

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