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Participation of the Intracellular Enzymes in the Control of the Mutation Process

III. Influence of the Inhibition of Repair and Replication on γ-Ray Induced Chromosomal Aberrations in Barley

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Summary. The influence of repair and replication on the frequency of spontaneous chromosome aberrations and of those induced by gamma-irradiation is reported.

Using the technique of labelling DNA with radioactive ^{3}H-thymidine and measuring the radioactivity of DNA isolated from embryos, the time of initiation and the duration of DNA synthesis in barley seeds was studied after the soaking of the seeds had begun. The average duration of each phase of the first DNA synthesis cycle in soaking barley seeds was found to be as follows: pre-DNA synthesis stage, 10-11 hrs; DNA synthesis stage, 8 hrs. After gamma-irradiation, the intensity of DNA synthesis decreased and the beginning of DNA synthesis was delayed.

It was found that the inhibition of repair by caffeine led to an increase in the frequency of both spontaneous and induced chromosome aberrations. Caffeine enhanced several times the frequency of chromosome and chromatid aberrations at the time of the maximal activity of repair enzymes. During DNA replication, caffeine had a lower effect on the realization of premutational lesions.

An inhibitor of DNA replication – hydroxyurea – had no influence on the frequency of spontaneous chromosome aberrations during the replication period, whereas after gamma-irradiation, hydroxyurea enhanced the frequency of aberrations mainly at the stage of DNA replication.

The relatively small mutagenic action of both agents (caffeine and hydroxyurea) was observed during all stages of the cell cycle of germinating barley seeds.

Key words: Intracellular enzymes – Mutagens – Mutation process Caffeine – Hydroxyurea – Barley.

Introduction

It has been supposed that intracellular enzymes interacting with DNA during repair, replication and recombination are involved in the process of stabilization of premutational damages induced by various mutagens (Borstel 1969; Soyfer 1969). On the model of prokaryotes, evidence was obtained which suggests that in vivo such influence of repair enzymes (e.g. UV-specific endonucleases) on mutagenesis is reflected both on the total rate of induced mutagenesis (Soyfer 1971; Soyfer and Titov 1976) and on the interrelation of complete and mosaic mutations (Soyfer et al. 1977b).

There are data that the inhibition of semiconservative replication in bacteria also led to a change of the mutation frequency and the ration of complete to mosaic mutations (Soyfer and Titov 1976). Thus, all these observations support the general idea, formulated very long ago, that mutagenesis is connected with 'copy-error mistakes' of enzymes operating with DNA (Dobzhansky 1963; Glass and Novick 1959; Kubitschek 1960). This idea was transferred into the study of aging: Burnet (1974) proposed that a major source of cellular deterioration during aging may be an accumulation of somatic mutations that are the products of 'error-prone' DNA repair and replication.

The interest in the study of the role played by repair and replication in mutation production, not only in bacteria and viruses but also in animals, became especially great after the discovery of different repair systems and the number of DNA-polymerases in various representatives of animals.

At the same time in plants, similar efforts gave more modest results. After the evidence of photoreactivation in higher plants had been presented (Trosco and Mansour 1968), many attempts to find dark repair in plants (Painter and Wolff 1973; Trosco and Mansour 1968, 1969) failed. Using the modified techniques of incorporation of labelled thymidine in the seedlings of higher plants and improved chromatographic analysis of the hydrolysis products of irradiated DNA, Soyfer and Cieminis (1974) obtained evidence for excision of thymine dimers from the DNA of plant seedlings. Similar observations in relation to excision of dimers from plant DNA were made with the DNA of cultured carrot cells (Howland 1975).

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Accumulation of thymine dimers in the acid-soluble fraction of plant cells, repair of single-strand breaks in DNA after dimer excision and repair replication in higher plants were studied by Soyfer and Cieminis (1976) and the possibility of the repair of damages induced in plant DNA by γ-rays was demonstrated by Howland et al. (1975). The repair of single-strand breaks induced in plant DNA by alkylating agents (Veleminsky et al. 1972) and the influence of repair of these breaks induced by propyl methanesulfonate on the frequency of chromosome and chromatid aberrations in barley (Soyfer et al. 1977a) have been studied.

However, the question of influence of repair and replication on the total rate of mutagenesis and on the frequency of chromosome and chromatid aberrations, especially in higher plants, are still rather obscure.

Having studied the incorporation of radioactive precursors in DNA, we obtained data on the duration of DNA synthesis stage in barley and then had the possibility for investigating the administration of repair and replication inhibitors in different points during cycle of DNA replication and their influence on the frequency of chromosome and chromatid aberrations in plants irradiated by gamma-rays.

Materials and Methods

Irradiation of Barley Seeds with Gamma-rays. Barley var. Moskovsky 121 (reproduction of 1972 and 1973 year) was used. Dry seeds (humidity about 10%) were irradiated on laboratory γ-apparatus LMB-γ-IM (radioactivity source ^{137}Cs).

Caffeine and Hydroxyurea Treatment. Control and irradiated seeds were released from scales and soaked in sterile tap water. Part of the seeds were treated with caffeine (Calbiochem, USA, A grade, Lot 1007) and hydroxyurea (Calbiochem, A grade, Lot 802031) for the following time intervals: 0-5, 10-15, 15-20 and 19-24 hours. The final caffeine concentration was 0.1% and the final concentration of hydroxyurea was 10^{-3}M and 5×10^{-2}M. Seeds were transferred to Petri dishes with solutions of appropriate drugs and were placed into thermostat at 25°C and after treatment were washed in tap running water at 25°C.

Study of DNA Synthesis in Control Germinating Barley Seeds and after Irradiation. Seed scales were removed from unirradiated and irradiated seeds. The seeds were then put on Petri dishes and flooded with an aqueous ^{3}H-thymidine solution (10 μ Curie/ml). Every 2 h after the beginning of germination the samples (12 seeds per each point with 3 repeats) were transferred into a freezing chamber (−20°C). Germs were detached from the seeds and put into 0.2 ml of a buffer solution (0.15 M NaCl + 0.1 M EDTA). Then the germs were ground in an ice mortar to a homogenous state; 1.8 ml of the same solution + sodium dodecylsulfate (Koch-Light, England) (a final concentration of 1%) + pronase (Calbiochem, USA, B grade, Lot 53702) (a final concentration of 2 mg/ml) were added and the mixture was incubated for 2.5 h at 37°. The samples were poured into a double volume of chilled ethanol; polymeric molecules were then wound onto glass rods and dissolved in 0.1 SSC (0.015 M NaCl + 0.0015 M sodium citrate, pH 7.0). Crystalline RNAse (Calbiochem, A grade, lot 100688) (concentration 50 μg/ml) was added and after another hour of incubation at 37°C, pronase (2 mg/ml) was added. After a 2 h incubation DNA was isolated by re-sedimentation in chilled ethanol. The material wound on the rod was dissolved in 0.1 SSC and analyzed for the DNA content in a recording spectrophotometer SP 8000 of Pye Unicam (England). Radioactivity was counted in a dioxane scintillator (dioxan 400 ml, sublimed naphtalene 30 g, methanole 100 ml, ethyleneglycol 10 ml, PPO 2 g, POPOP 0.2 g) in a liquid scintillation spectrometer Mark II Nuclear Chicago. The DNA thus recovered yielded a distinct peak at 254-256 nm and had $E_{255}/E_{230} = 1.4 - 1.6$ and $E_{255}/E_{280} = 1.6$. Radioactivity (D.P.M.) was measured per microgramm of DNA in the sample. There were three independent determinations for each point.

Study of the frequency of chromosome and chromatid aberrations. The control experiments and experiments with caffeine treatment were performed with barley seeds of a 1972 reproduction. The seeds of the 1973 reproduction were used for experiments with hydroxyurea. The germs of control and irradiated seeds, treated by caffeine and hydroxyurea (10^{-3} M), as was shown before, were fixed in the mixture of glacial acetic acid and ethanol (1:3) at 21.5 h after the beginning of soaking and fixations were made after every 2 h (5-6 fixations). The germs of seeds treated by 5×10^{-2} M of hydroxyurea (except the variant of 0-5 hrs) were fixed at 32-34 hr after soaking because in these conditions mitosis was delayed. Rootlets were macerated by their incubation in the mixture: glacial acetic acid: I N HCl (15:1) at 60°C. They were then transferred into I N HCl for 15 min, then into Shiff's reagent for 20 min. Rootlet tips (about 1 mm) were cut off and mashed in a drop of 22% acetic acid. Anaphases and early telophases were taken into account. The total number of cells at preparation, the number of cells with aberrations, the number of single and double fragments, single and double bridges, bridges with fragments or without them and rings were measured.

Results

a) A Study of DNA Synthesis in the Germinating Barley Seeds in the Normal Conditions and Following Gamma-Irradiation

Since there are no plants defective for the synthesis of repair enzymes available, we could not modify the enzymic activity by a genetic block of the enzymic synthesis, but had to inhibit metabolism of the intracellular reactions.

Prior to these experiments we had to investigate the parameters of the cell cycle in normally germinating barley seeds and in seeds after irradiation. To this end we studied the kinetics of DNA synthesis by a rigorous direct biochemical analysis of the incorporation of radioactive precursors into plant cell DNA by analogy with prokaryotes. This method enabled the usual difficulties of autoradiographic studies in plants to be avoided.

The results of the study of the kinetics of DNA synthesis in barley seedlings are given at Fig. 1. It can be seen that DNA synthesis both in normal conditions and following gamma-irradiation begins at the 10th hour after the dry seeds are soaked and continues to the 16-17th hour.

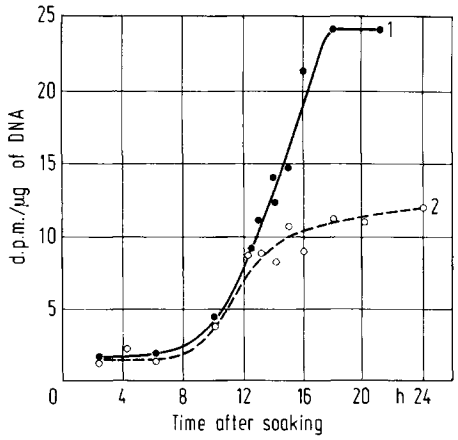

Fig. 1. Kinetics of DNA synthesis in developing barley embryos. Each point is a mean of three independent determinations 1 – control; 2 – γ-irradiation

It should be noted that the initiation of DNA synthesis in the seeds was highly synchronous. This confirms once more the idea that dry seeds of barley are a naturally synchronized system with respect to the first division, making them useful subjects for various cytogenetical and biochemical studies.

DNA synthesis in plants, quite similarly to what was observed in the experiments with animal and prokaryotic cells, was heavily inhibited by gamma-irradiation. The rate of DNA synthesis in the irradiated seeds was two times lower than in the unirradiated ones. In addition a short delay in the beginning of replication of DNA was observed in these experiments.

b) The Frequency of Chromosome and Chromatid Aberrations in the Seedlings Grown from the Unirradiated Seeds

A study of the frequency of spontaneous chromosome and chromatid aberrations in the seedlings of the control seeds has shown (Table 1) that the frequency of chromatid aberrations is, on the average, higher than that of chromosomal ones by a factor 3. Bridges were in the majority among chromatid aberrations (about 82%) but amounted to only 20% of chromosomal aberrations. As a result of the caffeine administration during the initial five hours of soaking the seeds when replication had not yet started, the aberration frequency increased 9.8 times (from 0.0046 in the control to 0.045 after caffeine treatment), and the mutation spectrum changed. During DNA replication (from 10th to 15th h) caffeine had a lower effect on the aberration frequency and the mutation spectrum did not change.

Thus, this series of experiments has shown that the action of caffeine on the aberration frequency is maximal outside the phase of DNA replication. In relation to hydroxyurea such regularity is not revealed. During all the cell cycles, with the exception of the time of replication, the frequency of aberrations increased, ranging from 6.5 to 10.5 times higher compared to the level of aberration frequencies in the control. At the time of DNA replication (from 15 h to 20 h after the beginning of soaking) the aberration frequency slightly decreased.

c) The Influence of Caffeine on the Frequency and Spectrum of Chromosome and Chromatid Aberrations Induced by Gamma-irradiation

As a result of exposure of the dry seeds to gamma-irradiation, the aberration frequency increased sharply. The summary frequency of aberrations of both types increased 100-fold as compared to spontaneous background (Table 2). The proportion of the two types of aberrations also changed abruptly. Gamma-irradiation is known to induce a large number of double-stranded breaks in chromosomal DNA. Conforming to this, the proportion of chromosome aberrations increased from 20.8% in the control group to 62.1% in the experimental group. The frequency of chromatid aberrations dropped as abruptly (from 70.8% to 33%).

In the series studying the effect of caffeine, as in the experiments with the unirradiated seeds, caffeine had an effect only in the time period prior to DNA synthesis (during the initial 5 h after soaking the seeds). At this time the frequency of aberrations of both types was higher by a factor of 2.

In the other periods of the cell cycle caffeine had no significant effect on the frequency of aberrations of either type. Nor did it have any effect on the proportion of chromosome to chromatid aberrations.

d) The Influence of Hydroxyurea on the Frequency of Chromosome Aberrations

If in the control, the treatment by hydroxyurea led to a roughly similar increase of mutation frequency during the first 24 h after soaking (except the phase of DNA synthesis), then in the case of sharp increase in the number of premutational damages in DNA under the action of gamma-irradiation, the influence of hydroxyurea on mutation frequency became quite noticeable (Table 3).

In complete agreement with the molecular mechanism of action of this agent as a DNA replication inhibitor, hydroxyurea in irradiated seeds exerts maximal influence upon aberration frequency during DNA replication (from 15 to 20 h). During this period, the application of hydrox-

Table 1. The frequency of chromatid and chromosome aberrations in the seedlings grown from unirradiated barley seeds

Character of treatment	Total of examined cells	No. of cells with aberrations	Aberrations						% of a given aberration type to the total		Aberration frequency per cell	The factor by which aberration frequencies increased as compared to control ones
			Chromatid		Chromosome		Other total		Chromatid	Chromosome		
			Fragments	bridges	Fragments	bridges						
Control	5231	24	3	14	4	1	2	24	70.8	20.8	0.0048	1
Caffeine treatment												
0- 5 h	2115	95	15	48	19	10	3	95	66.2	30.5	0.045	9.8
10-15 h	2231	40	17	19	6	2	1	45	79.9	17.8	0.018	3.9
15-20 h	1007	24	3	11	5	5	1	25	56.0	40.4	0.024	5.2
19-24 h	2255	37	3	20	6	6	2	37	62.2	32.4	0.016	3.5
Hydroxyurea treatment												
10^{-3} M												
0- 5 h	1823	49	19	23	7	8	–	57	74.0	26.0	0.030	6.5
5×10^{-2} M												
0- 5 h	733	32	18	3	12	2	–	35	60.0	40.0	0.048	10.5
15-20 h	1465	29	17	6	7	3	1	34	67.6	29.4	0.023	5.0
19-24 h	1417	49	36	2	20	3	1	62	61.2	38.6	0.044	9.6

Table 2. The frequency of chromosome and chromatid aberrations upon Gamma-irradiation of the dry seeds and subsequent treatment of the seedlings with caffeine

Character of treatment	Total of examined cells	No. of cells with aberrations	Aberrations						% of a given aberration type to the total		Aberration frequency per cell	The factor by which aberration frequencies increased as compared to control ones
			Chromatid		Chromosome		Other total		Chromatid	Chromosome		
			Fragments	bridges	Fragments	bridges						
Gamma-irradiation without caffeine	1747	629	119	138	306	178	38	779	33.0	62.1	0.45	1
Administration of caffeine after irradiation:												
from 0 to 5th h	1833	1149	210	348	819	276	42	1695	32.9	64.6	0.92	2.04
from 10 to 15th h	2104	855	168	196	544	174	29	1111	32.8	64.6	0.53	1.17
from 15 to 20th h	1260	499	140	146	286	102	16	690	41.4	56.3	0.54	1.20

Table 3. The frequency of chromosome and chromatid aberrations upon Gamma-irradiation of the dry seeds and subsequent treatment of the seedlings with hydroxyurea (5×10^{-3} M)

Character of treatment	Total of examined cells	No. of cells with aberrations	Aberrations					% of a given aberration type to the total		Aberration frequency per cell	The factor by which aberration frequencies increased as compared to control ones
			Chromatid		Chromosome		Other total	Chromatid	Chromosome		
			Fragments	bridges	Fragments	bridges					
Gamma-irradiation without hydroxyurea	2065	172	26	59	52	42	2	179	47.5	51.4	0.09	1
Administration of hydroxyurea after irradiation:											
from 0 to 5th h	2342	270	32	72	104	129	8	288	36.1	61.1	0.12	1.34
from 10 to 15th h	1327	153	32	34	55	48	6	175	37.7	58.8	0.13	1.45
from 15 to 20th h	1478	397	171	22	232	71	1	497	38.8	60.9	0.34	3.77
from 19 to 24th h	1432	200	108	26	94	32	4	464	50.7	47.7	0.18	2.00

yurea results in a 3.8 fold increase in mutation frequency. Before DNA replication the action of hydroxyurea was negligible, whereas from 19 to 24 h its influence was nearly 2 times weaker than in the preceding five hours.

Discussion

The present work studies the duration of the presynthetic phase and the phase of the first DNA synthesis after the beginning of soaking of barley seeds. These experiments were performed using the technique of studying the incorporation of radioactive precursors into newly synthesized DNA, isolation of the DNA preparations from embryos each 20-30 min and measurement of the radioactivity of these preparations. Such determinations were made both in relation to control (unirradiated) seeds and in seeds irradiated in a dry state by gamma-rays.

Having determined the time of the pre-DNA-synthetic phase, we obtained data that this period is equal to 10 hours. As was demonstrated earlier, the excision of UV-dimerized thymines from plant seedlings' DNA is completed during a 6-hour period after UV-irradiation (Soyfer and Cieminis 1974). Thus we can say with confidence that the first repair cycle of DNA, after soaking the seeds, must be performed during the first 6-10 hours.

The exact determination of the time of the first DNA synthesis in germinating seeds gave us the possibility of using inhibitors of repair and replication exactly at the time when both these processes occur and of determining the influence of these inhibitors on the frequency of spontaneous and induced chromosome and chromatid aberrations.

It was found that caffeine has its maximal mutagenic activity before DNA replication; i.e., at the time of DNA repair. Hydroxyurea was more active during the process of DNA replication.

During this study the mutants of plants defective in repair or replication were not available, we had to use only inhibitors of these processes in our study of the influence of repair and replication on mutagenesis. An indication of using caffeine as an inhibitor of repair in plants has been described in the literature (Ahnstrom and Natarajan 1971; Swietlinska and Zuk 1974; Yamamoto and Yamaguchi 1969). In addition, we demonstrated (Soyfer et al. 1977b) that caffeine was most effective at the phase of repair of plant DNA and that caffeine selectively inhibited the repair processes in plants. Although many authors have come to the conclusion (especially on the model of animal cells) that caffeine is the inhibitor of post-replicative repair, in our experiments where caffeine was used during exactly the G_1, S and G_2 phases, we found that the maximal influence of caffeine on the increase of the frequency of chromosomal aberrations was

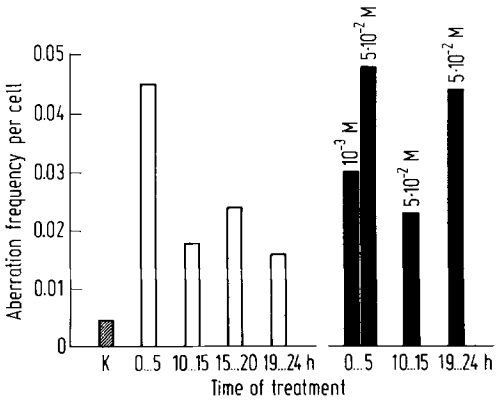

Fig. 2. The relationship between the frequency of chromosome aberrations per cell and the time of administration of caffeine and hydroxyurea in unirradiated barley seeds. 0-5, 10-15, 15-20, 19-24 is the time (hours) of treatment of seeds with drugs □ 0.1% caffeine; ■ hydroxyurea; □ untreated seeds

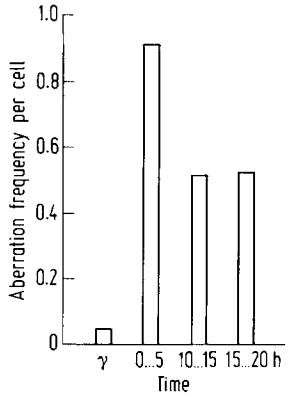

Fig. 3. The relationship between the frequency of chromosome aberrations per cell and the time of treatment of gamma-irradiated barley seeds by caffeine γ-aberration frequency in gamma-irradiated seeds; 0-5, 10-15, 19-24 hours – the time of treatment of irradiated seeds by caffeine

observed during the time of pre-DNA-synthetic stage of cellular cycle, that is during stage of excision repair. Our experiments with seedlings have also shown that hydroxyurea is an effective inhibitor of DNA replication in higher plants (Soyfer et al. 1973 and our unpublished data).

The results presented here completely support the hypothesis proposed earlier about the role of repair and replication in the mutation process (Soyfer 1969, 1972, 1975). It was supposed that inhibition of these processes would lead to an increase of mutations including chromosomal and chromatid aberrations. As shown by the present data, the administration of caffeine was followed by a sharp increase of the mutation frequency at the same time when repair occured. Such results were observed both in the case of spontaneous background (Fig. 2) and in the case of aberrations induced by gamma-irradiation of seeds (Fig. 3).

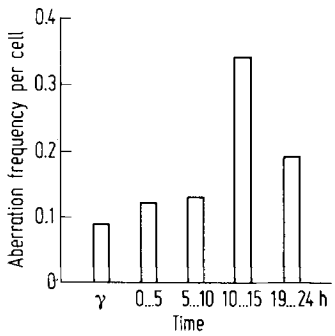

Fig. 4. The relationship between the frequency of chromosome aberrations per cell and the time of treatment of gamma-irradiated barley seeds by $5 \cdot 10^{-2}$ M hydroxyurea

The use of hydroxyurea led to a roughly similar increase of chromosome aberration frequencies in unirradiated seeds during all stages of the first cell cycle, except at the time of the first DNA replication (Fig. 2). These data about the influence of caffeine and hydroxyurea indicate, perhaps, that the mistakes of replication make the greatest contribution to spontaneous mutagenesis.

At the same time the treatment of gamma-irradiated seeds by hydroxyurea revealed that premutational lesions of chromosomal DNA are diminished both with repair enzymes and with DNA-polymerases and the other enzymes participating in the replication process since an inhibition of replication in gamma-irradiated seeds by hydroxyurea sharply enhanced the mutation frequency (Fig. 4).

Besides this specific action on repair and replication processes, both caffeine and hydroxyurea have a small nonspecific mutagenic action at each point when a mutation was checked.

Thus, just as it was demonstrated in the case of genic mutations of prokaryotes (Soyfer 1972; Soyfer and Titov 1976), the present experiments demonstrated the influence of repair and replication on the frequency of chromosome aberrations. Together with data about the influence of repair of DNA single-strand breaks on the frequency of chromosomal aberrations induced by chemical mutagen propyl methanesulfonate (Soyfer et al. 1977a). and data received with bacteria (Soyfer and Titov 1976), these results have clearly shown the complex interaction of different intracellular enzymes in the control of the mutation process, both at the level of prokaryotes and eukaryotes.

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