Hydroxylamine Induced Chromosome Aberrations in *Lens esculenta* and Combined Effects with Ionizing Radiation

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Introduction

Hydroxylamine hydrochloride has been tested in *Vicia faba* primary root-tips where it induced chromatid inter- and intra-changes of a delayed type (Nicoloff *et al.* 1971). Besides an increase in these effects with rising temperature and at higher pH, a larger proportion of incompleteness has also been observed compared to other chemicals. Chemically, hydroxylamine is known to react with cytosine moiety in nucleic acid and to induce main cleavage in DNA (Borenfreund *et al.* 1964). This reaction with cytosine has been investigated in detail in TMV (Fraenkel-Conrat *et al.* 1972) where the formation of N4-hydroxycytosine has been demonstrated under definite experimental conditions depending on pH among other factors. At pH 6.0 there is predominance of the N4-substitution over the C-5,6 addition reaction. Some results obtained in phage (Soyfer 1969) support the conclusion that the primary DNA structure can be influenced by HA, but that the lesions which change the secondary configuration of DNA are reparable.

Neurospora studies (Malling *et al.* 1971a and 1971b) consistently agree with the hypothesis that point mutations are predominantly produced by GC-AT base-pair transitions. These results are in contrast with those obtained with mammalian cells cultivated *in vitro* in which HA used at lower concentrations is more readily oxidized. It can be inferred that these oxidative products are the substances responsible for the higher number of chromosome aberrations observed in mammals *in vitro* (Somers *et al.* 1962) or *in vivo* (Ramayia 1968, 1969).

However, other chemical data suggest that hydroxylamine mutagenesis involves more complex processes than base-pair transitions. Some other factors like *e.g.* the specificity of replicating enzymes can also be at work (Janion *et al.* 1969). Cytological studies dealing with chromosome breakage also indicated that reactions in the cytosine moiety of DNA are not responsible for the chromosome damage but that other more complex processes are involved (Nicoloff *et al.*, *loc. cit.*).

The mechanisms by which HA affects the recovery of broken chromosomes is however not fully understood. From the literature review, it can be seen that few studies are dealing with combination of mutagens, which may help to get a new insight in these mechanisms. We can mention the experiments in *Vicia faba* where hydroxylamine has been combined with X-rays (Cohn 1964a and 1964b) and with maleic hydrazide (Nicoloff *et al.*, *loc. cit.*) and in *E. coli* combining hydroxylamine

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with acridine (Soyfer 1970).

The present investigation aims to recognize the nature of aberrations produced by X-rays and hydroxylamine singly and in combination, on dry and presoaked seeds of lentil (*Lens esculenta* Moench) which is grown mainly for its protein rich edible seeds and sometimes as a forage crop.

Material and methods

Dry seeds of lentil (microsperma) variety B-18 obtained from the pure line genetic stock maintained at the experimental station, were irradiated with 1, 5, 10 and 15 kR X-rays (9 mA and 125 kV, target distance 10 cm from the source) with a dose-rate 30 R/sec. The initial seed water content was 12 per cent. For presoaking experiments, seeds were initially immersed in water for 6-12 hr (in Erlenmeyer flask with sufficient water to cover the seeds and shaken intermittently) removed and spread uniformly on moist filter paper in Petri dishes before irradiation in the same conditions as dry seeds. Both dry and presoaked seeds were treated with hydroxylamine solutions (British Drug House) prepared extemporaneously by dissolving 0.5 and 0.1 gm of pure hydroxylamine hydrochloride crystals in 100 ml distilled water. The aqueous solutions were neutralised with 1 N NaOH solution to maintain pH 7.0. Pure NaCl crystals ($\approx$ 1N) were added to the solution in equal proportion of HA (Freese *et al.* 1961). All treatments were performed at 22°C for 2hr. After the treatment, the seeds were washed carefully in distilled water and allowed to germinate in Petri dishes. For combined treatment 6 hr presoaked seeds were irradiated with X-rays and then immediately treated by HA. Slides were prepared from aceto-orceine or from usual Feulgen squashes.

Results

In general, the irradiated seeds germinated slowly and the seedling growth was considerably reduced. Mitotic delay or inhibition, stickiness of chromosome (Fig. 1) secondary association of chromosomes (Fig. 3) and polyploid cells (although not frequent) were observed after irradiation. The major types of structural changes produced by X-rays included both chromosome and chromatid breaks, exchanges and microfragments, at metaphase. At anaphase, bridges and fragments were most common with occasional occurrence of rings.

Effects of X-rays on dry and presoaked seeds

When dry seeds were irradiated with 5-15 kR, the yield of aberrations increased linearly with the dose (Table 1). At 15 kR, the increase of abnormal cells was only 2.8 per cent more than at 10 kR whereas the frequency of aberrations per cell increased to 2.12 compared to 1.04 at 10 kR indicating a greater damage per cell at the higher dose. The dose-response relationship showed that the proportion of fragments was directly proportional to the dose (Graph 1, Fig. 1), whereas the production of bridges was almost the same at 5 and 10 kR, but increased twofold at 15 kR suggesting a two-hit kinetics. The frequencies of metaphase aberrations (10 kR) on dry seeds are shown in Table 2.

Figs. 1-8. 1, stickiness of metaphase chromosome after X-irradiation (10 kR). 2, rejoining of fragments of chromosome VI with chromosome III (15 kR—dry seed). 3, secondary association of somatic chromosomes (10 kR—dry seed). 4, chromatid break and break near the centromeric region (0.1% HA—2 hr). 5, chromatid break (5 kR X-rays—6 hr presoaked seeds). 6, multiple gaps and breaks (0.1% HA—2 hr). 7, isochromatid breaks and microfragments (5 kR+0.1% HA—2 hr). 8, breaks in chromosome V involving centromere and secondary constriction (10 kR—dry seed).

Graph 1a-b. a, yield of anaphase aberrations after X-irradiations of dry seeds. b, yield of abnormal cells and anaphase aberrations after 5 kR X-rays on presoaked seeds.

Table 1. Frequency of anaphase aberrations after X-irradiation of dry seeds (500 cells analysed for each treatment)

Irradiation kR	Abnormal cells %	% aberrations		
		Bridges	Fragments	Total
5	12.8	15.4	5.4	20.8
10	38.0	16.4	23.2	39.6
15	40.8	35.2	51.4	86.6
Control	0.4	0.8	0.0	0.8

Table 2. Proportion of different kinds of metaphase aberrations after 10 kR X-irradiation of dry seeds (300 cells analysed)

Irradiation kR	Abnormal cells %	% aberrations				
		B''	B'	Interchanges	Micro-fragments	Total
10	42.0	44.6	5.3	2.0	3.3	55.2
Control	0.2	0.2	0.0	0.2	0.0	0.4

Table 3. Effects of X-rays (5 kR) on presoaked seeds
(500 cells analysed for each treatment)

Treatment	Abnormal cells %	% aberrations		
		Bridges	Fragments	Total
Dry seed	12.4	15.4	5.4	20.8
6 hr soaking	93.0	129.4	148.2	277.6
12 hr soaking	100.0	207.4	278.0	485.4

Figs. 9-13. 9, bridges and fragments at anaphase after HA treatment of presoaked seeds (0.2%—2 hr). 10, laggards (10 kR—dry seeds). 11, single bridge (0.2% HA—2 hr). 12, multiple bridges and fragments (5 kR—presoaked seeds). 13, bridges, large fragments and ring (10 kR—presoaked seed).

Table 4. Frequency of anaphase aberrations resulting from HA treatments
(0.1%—2 hr)—500 cells analysed

Treatment	Abnormal cells %	% aberrations		
		Bridges	Fragments	Total
Dry seed	1.4	2.0	0.6	2.6
6 hr soaking	5.4	3.4	3.4	6.8
Control	0.4	0.6	0.2	0.8

The hydration of seeds for 6 and 12 hr in water before irradiation with 5 kR enhanced considerably the radiosensitivity, and almost all cells appeared to be abnormal (Table 3). The number of aberrations per cell increased with the time of presoaking, the highest yield being at 12 hr/presoaking. Varying proportions of rings were observed at anaphase after presoaking but not in case of dry seeds.

Effects of HA on dry and presoaked seeds

Treatments of dry and presoaked seeds with 0.1 per cent HA for 2 hr at pH 7.0

Table 5. Effect of different pH during HA treatments (0.1%—2 hr)
Frequency of anaphase aberrations (500 cells analysed)

Treatment	% aberrations (Total)			
	6.5 pH	7.0 pH	7.5 pH	8.0 pH
Dry seed	3.4	2.6	2.4	3.0
6 hr soaking	8.4	7.0	6.8	8.0
Control	0.8	0.8	0.6	0.6

Table 6. Frequency of anaphase aberrations after combined treatment
of X-rays and HA (0.1%—2 hr)—500 cells analyzed.

Treatment	Abnormal cells %	% aberrations		
		Bridges	Fragments	Total
1 kR	29.2	22.0	30.2	52.2
5 kR	93.0	129.4	148.2	277.6
HA	4.4	2.8	4.8	7.6
1 kR + HA	35.8	27.4	36.2	63.6
5 kR + HA	100.0	158.2	259.2	417.4

Table 7. Proportion of different kinds of metaphase aberrations after combined
treatments of X-rays (1 kR) and HA (0.1%—2 hr) (300 cells analysed per treatment)

Treatment	Abnormal cells	% aberrations					
		B''	B'	Intra-changes	Intra-changes	Micro-fragments	Total
X-rays	35.0	33.0	8.0	9.0	3.0	15.3	67.6
HA	5.3	6.6	0.6	1.6	0.0	0.3	9.1
X-rays + HA	42.3	46.6	5.0	14.6	15.0	0.3	81.5

caused chromosome aberrations to a lesser extent in case of dry seeds, (Table 4). HA reduced the mitotic index. This reduction was very high with 0.5 per cent/2 hr and hence the dose was limited to 0.1 per cent only in the present experiments. Multiple gaps and breaks throughout the entire chromosome length comparable to a banded pattern (Fig. 6) were observed in several cells. The breaks were not restricted to centromeric regions. In dry seeds, most of the aberrations were of the chromosome type and chromatid exchanges were few.

When dry and presoaked seeds were treated with 0.1 per cent at HA different pH, viz. 6.5, 7.0, 7.5 and 8.0 for 2 hr and allowed to germinate and grow for 7 days, there was reduction in seedling height at all pH levels compared to the control. The maximum reduction in height was at 6.5 pH. This trend was also noticed in the induced chromosome aberrations (Table 5) with no conspicuous change in the proportion of bridges and fragments.

Effects of combined treatments of X-rays and hydroxylamine

It was observed that when 6 hr presoaked seeds were X-irradiated followed immediately by 0.1 per cent HA treatment for 2 hr varying results were obtained depending upon the doses of X-rays. The total aberration frequency in combined treatment of 1 kR and HA was close to the additive effect of two single treatments, but there was a significant increase in total aberrations after combined treatment of 5 kR and HA over the additive yield.

Different types of aberrations were observed at metaphase and their proportions were investigated (Table 7). After combined treatment, there was a greater increase in B'' than interchanges. At 5 kR, anaphase aberrations per cell was 2.98 which increased to 4.17 after the combined treatment of HA.

Discussion

The effects of X-rays on dry and presoaked seeds of lentil are in general comparable with those obtained by Sinha and Godward (1968) on *Lens culinaris*. It can be seen that the dose-response relationship is linear in both cases. As expected, the seed water content enhanced the radiosensitivity. Even after 6 hr soaking, the number of abnormal cells increased to almost hundred per cent.

The effects of treatments of dry seeds of *Lens* are similar to those obtained with other materials (Jana *et al.* 1966) yielding only aberrations of the chromosome class e.g. *Nigella damascena* (Moutschen *et al.*, 1968, Moutschen *et al.* 1969) and *Allium* (Moutschen, unpublished data). On the other hand, chromatid aberrations are scored after treatments of 6 hr presoaked seeds. This proves that the result obtained in *Vicia faba* where HA induced chromatid aberrations (Cohn, 1964 a,b and Nicoloff *et al.* 1971) can also be extended to chromosome aberrations.

On the specific effects of HA, Cohn (*loc.cit.*) reported that it prevents rejoining of X-ray broken chromosomes and Nicoloff *et al.* (*loc. cit.*) observed a similar inhibition with chromosome broken with maleic hydrazide. A relatively large number of interchanges have continuously been scored in the present investigation confirming the results of Somers and Hsu (1962). At this stage, it is difficult to conclude that HA completely prevents repair as hypothesised before (*loc. cit.*). The present data also suggest that the action of HA can involve different mechanisms. On the other hand, the cytosine substitution can not be considered as the main process leading to chromosome breakage whereas the inhibition of recovery processes can, but partly explain the distribution of induced chromosome aberrations.

From the present investigation, as well as from the data of Somers and Hsu (*loc. cit.*) it can be seen that HA can cause gross changes in the morphology of chromosomes and that its effect is not restricted to some heterochromatic regions as

indicated by Natarajan and Upadhyaya (1964). Combined treatments with X-rays and hydroxylamine show a synergistic effect as observed in *Vicia faba* by Cohn (*loc. cit.*). The interesting point that emerges from the present experiment is the dependence of the effect from the doses of X-rays. It is clearly seen that, when the exposures were low, the combined effect was not far from additive, but when the exposure was increased, the synergistic effect became conspicuous. It would be interesting to know if the synergistic effects obtained in other experiments (*e.g.* Rieger 1965) would vary with the relative changes in the doses of the mutagens as observed in the present experiment.

Summary

Dry and presoaked seeds (6 and 12 hr) of *Lens esculenta* were irradiated with 1, 5, 10 and 15 kR X-rays and the effects of radiation were measured from the yield of induced chromosome aberrations observed in roots. The yield of X-ray induced aberrations increased linearly with the exposure. When seeds were presoaked before irradiation there was a remarkable increase in the yield of total aberrations still increasing at the longer presoaking time.

Treatments of dry and 6 hr presoaked seeds with HA (0.1%/2 hr) resulted in chromosome breakage not restricted to the heterochromatic regions and influenced by the pH.

Post-treatments of 1 and 5 kR X-irradiated seeds (presoaked) with HA (0.1%/2 hr) showed that the yield of aberrations in combined treatment was more than additive, the synergistic effect was depending on the dose of X-rays.

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