SOME ASPECTS OF THE CHEMICAL PROTECTION AGAINST RADIATION DAMAGE TO VICIA FABA CHROMOSOMES [1]

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SINCE 1933 when CRABTREE and CRAMER first reported that anaerobiosis decreased the effects of radium treatment on cancerous tumors, there have been numerous reports on the recovery of living cells, both animal and plant, from the effects of ionizing radiations. LEA (1945) and GRAY (1952) have reviewed the literature concerning these effects.

An excellent area for the study of these effects has been the production of chromosomal aberrations in plant tissues. THODAY and READ (1947) discovered that low oxygen tensions reduced the amount of chromosomal aberrations in actively growing *Vicia faba* roots; GILES and RILEY (1950) found a reduction in the chromosomal aberrations of Tradescantia microspores if the microspores were irradiated in nitrogen; and HAYDEN and SMITH (1949) showed a reduction of chromosomal aberrations in barley seed irradiated *in vacuo*.

The above work, however, is of theoretical interest only, as it seems impractical to keep aerobic organisms in oxygen-free atmospheres for lengthy periods of time. Thus much of the recent work has centered on the method of reducing the internal cellular oxygen tension by chemical means. Chief among the chemicals that do this are: sulfhydryl compounds, alcohols, glycols, and sodium hydrosulphite (BURNETT et al. 1951). In light of this, MICHAELSON (1952) was able to decrease the amount of chromosomal aberrations caused by X-rays in Tradescantia root tips by pretreatment with glutathione solutions. And RILEY (1952) was able to decrease the number of aberrations in onion roots by treating with sodium hydrosulphite.

Ostensibly, these chemicals work by reducing the number of active radicals formed in irradiated water as postulated by WEISS (1944, 1947) and thus they can protect against the "indirect effect" of ionizing radiations as first described by DALE (1943).

This present paper is concerned with the protective effects of some of the chemical reducing agents against chromosome damage in *Vicia faba*. The study is divided into three parts ascertaining 1) whether or not the agents can reduce damage if given to resting cells in the seed; 2) whether or not there is an additional effect of the chemicals beyond that of simple oxygen removal;

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3) whether or not it is the original breakage of the chromosomes or the restitution of ends already broken that the chemicals affect.

MATERIALS AND METHODS

Seeds of Argentine *Vicia faba* were used in all experiments. This particular organism is well suited for studies in radiation cytology for several reasons. Chief among these are 1) that there are but twelve chromosomes in a diploid cell, 2) the chromosomes are very large, 3) the root tips respond to the squash technique very well, and 4) by using seed, one is certain of irradiating all cells at resting stage and thus of avoiding the complications that arise due to the differential sensitivity of the various stages of mitosis to radiation.

The method of handling the seed was basically that of GRAY and SCHOLES (1951) with several major modifications. To decrease the amount of fungal infection, only unmarred seeds were used and these were first soaked in a 5% solution of calcium hypochlorite for thirty minutes. The seeds were then washed several times in distilled water to get rid of all traces of the oxidizer used as a disinfectant. The seeds were then soaked for twenty-three hours in glass-distilled water. The water was changed frequently during this period of time and to further decrease fungal infection the seed coat was removed as soon as it had softened.

The seeds were then transferred for exactly one hour, to solutions of the reducing agent to be tested. The solutions were all 2×10^{-3} M and made up with double-distilled water, the second distillation being done in an entirely glass system. This was necessary since some of the chemicals are rapidly oxidized by minute quantities of copper ions. The seeds were then transferred to sterile Petri dishes, the bottoms of which were lined with filter paper. The paper was moistened with the solutions and the seeds then immediately irradiated in this condition.

TheX-ray tube was operated at 160 KVP and 10 MA. All dosages were delivered at 200 r/min. as measured by a dosimeter in the radiation chamber at the actual time of irradiation. The only filtration utilized was that inherent in the tube itself and the Pyrex glass covers of the Petri dishes.

The seeds were then allowed to germinate on boards similar to those described by GRAY and SCHOLES. After a period of three days the roots were 2–3 centimeters long and the first mitotic divisions were occurring. One centimeter of each root was then cut off and placed for exactly seven hours into a 0.1% solution of colchicine to shorten the chromosomes and to collect metaphases. The root tips were then fixed in a solution of six parts ethyl alcohol, three parts glacial acetic acid, and one part chloroform. The latter was added to dissolve any oil in the cells that might obscure the chromosomes.

After at least three days in the fixative, aceto-carmine squash preparations were made of individual root tips. A detailed chromosome analysis was made of every scorable metaphase plate on the slide. Usually 3–8 slides were made of each treatment in order to get a large enough number of cells for statistical significance. The aberrations scored were rings and dicentric chromosomes

TABLE 1

Effect of chemical reducing agents on radiation-induced chromosome aberrations in Vicia faba. 500 r total dose at rate of 200 r/min. All chemical concentrations 2×10^{-3}M.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (no radiation)	200	199	1	....	....	....
Water	316	202	64	48	20.2 ± 2.6	15.2 ± 2.0
BAL	530	406	60	46	11.3 ± 1.4	8.7 ± 1.5
Cysteine	406	288	66	56	16.3 ± 1.8	13.8 ± 1.7
$Na_2S_2O_4$	354	256	64	37	18.0 ± 1.8	10.4 ± 1.6
Glutathione	236	162	48	38	20.3 ± 2.6	16.1 ± 2.5
Ascorbate	261	126	56	40	21.4 ± 1.5	15.3 ± 2.2

which are two-hit aberrations (SAX 1941). Since other workers have scored fragments in anaphase figures as an indication of chromosomal damage, the number of rod and dot deletions have been included to enable comparison of this experiment with others. However, as RICK (1940) has indicated, a proportion of the dot deletions are two-hit aberrations and so the number of rods and dots cannot be considered an accurate measure of one hit aberrations. The data were then computed on the basis of the percentage of cells containing the various types of aberrations. The " t " test of statistical significance was used both as a test of homogeneity for all slides receiving the same treatment and as a test of significance for different treatments. Fiducial limits were set up at the five percent level of significance.

RESULTS

In order to determine whether or not the chemical reducing agents were capable of decreasing the amount of radiation damage to seeds, the seeds of *Vicia faba* were soaked for one hour previous to irradiation in solutions of BAL, cysteine, sodium hydrosulphite, glutathione, or sodium ascorbate. They were then exposed to 500 r of X-rays delivered in two and one-half minutes. The results are presented in table 1.

TABLE 2

Effect of chemical reducing agents on radiation-induced chromosome aberrations of Vicia faba. In vacuo. 500 r total dose at rate of 200 r/min. All chemical concentrations 2×10^{-3}M.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (no radiation)	115	114	1	....	....	....
Water	215	169	31	17	14.4 ± 2.4	7.9 ± 1.8
BAL	392	314	58	25	14.6 ± 1.8	6.4 ± 1.2
Cysteine	268	208	41	20	15.3 ± 2.2	7.5 ± 1.6
$Na_2S_2O_4$	211	167	29	15	13.7 ± 2.4	7.1 ± 1.8
Glutathione	208	174–	28	11	13.4 ± 2.4	5.3 ± 1.6
Ascorbate	216	172	36	12	16.6 ± 2.5	5.6 ± 1.6

It will be seen that the BAL and the sodium hydrosulphite both afforded significant protection against the formation of two-hit chromosome aberrations It is interesting to note that these are the two chemicals that were found to be the most protective in bacteria (BURNETT et al. 1951).

Since these protective agents are reducing agents, they can decrease the internal oxygen tension of the cell and thus lower the amount of radiation damage. However, since some of these chemicals have other physiological properties, it was decided to test whether or not there was any additional effect of the chemicals after all the oxygen was removed. The seeds were, therefore irradiated *in vacuo*. The treatment was the same as in the previous experiment with the following exception. One-half hour before irradiation, the seeds were placed in the lucite tanks first described by KING, SCHNEIDERMAN and SAX (1952). They were then evacuated and the seeds were irradiated while in the vacuum. The results are presented in table 2.

TABLE 3

Effect of BAL (2×10^{-3}M) plus dosage fractionation on the production of radiation-induced chromosome aberrations in Vicia faba. 200 r/min. 3 doses with 1-hr. intervals.

Treatment	No. cells	Normal	Rods & dots	Rings & dic. (2-hit)	% Rods & dots	% Rings & dic.
Water (constant dosage)	549	278	173	109	31.6 ± 2.0	19.9 ± 1.7
Water (fractionated dosage)	500	253	164	92	32.8 ± 2.1	18.4 ± 1.7
BAL (constant dosage)	584	449	94	50	16.1 ± 1.5	8.6 ± 1.2
BAL (fractionated dosage)	658	381	102	36	15.5 ± 1.4	5.4 ± 0.9

None of the chemicals was able to decrease significantly the amount of chromosome aberrations obtained by irradiating in a vacuum. This indicates that the chemicals are only able to exert their protective effect when there is oxygen present in the cell. There is no additive effect noticed beyond that afforded by the direct removal of intracellular oxygen.

In view of the fact that some of the reducing agents are able to decrease the amount of aberrations induced by irradiation, the question arises as to the manner in which these chemicals work. Do they decrease the original breakage of the chromosomes or do they facilitate the restitution of ends already broken?

The classical method for the study of restitution is that which SAX (1941) proposed. That is to fractionate the radiation dosage, in which case the two-hit aberrations are decreased due to the restitution of broken ends.

Vicia faba seed seems to be an ideal organism on which to test the response of the chromosomes to fractionated dosages, since SAX and BRUMFIELD (1943) have hypothesized that the breaks remain open for a long period of time. The experiments were carried out as previously with the following changes. 600 r were given at the rate of 200 r/min. Those seeds that received

fractional dosage received three 200 r doses with one-hour intervals between treatments. The only chemical tested was the BAL which had proved to be the most effective in the earlier experiments.

The results are presented in table 3 and in figure 1.

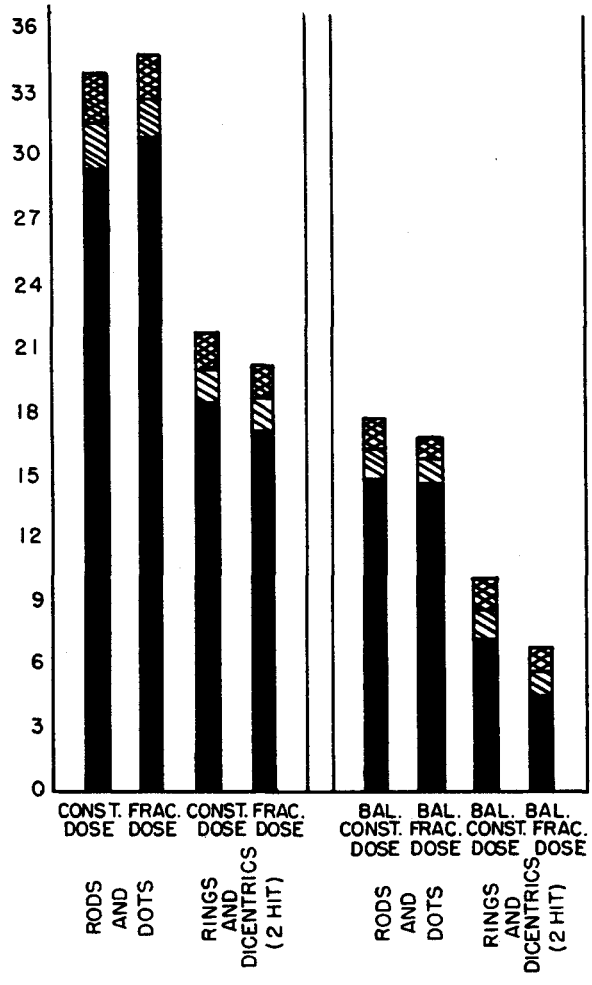

FIGURE 1.—Effect of BAL (2×10^{-3} M) plus dosage fractionation on the production of radiation induced chromosome aberrations in *Vicia faba*. All figures are $\pm$ 1 σ.

It will be seen that the fractionation of the dosage resulted in a significant decrease in the number of two-hit aberrations only in the presence of BAL.

DISCUSSION

As was pointed out previously both BAL and sodium hydrosulphite were able to protect against aberration production. However, it will be noted that the sodium ascorbate, another powerful reducing agent, gave no protection at all. This is in complete accord with studies made in other systems with this

particular chemical (PATT et al. 1950). It is therefore assumed that the ascorbate does not exert any action in those areas that are sensitive to the irradiation.

In light of the work done with bacterial (BURNETT et al. 1951) and mammalian systems (PATT et al. 1950), an anomalous result occurs when the seed is treated with cysteine. Significant protection is not attained. Since cysteine is in many respects a similar molecule to BAL one would certainly expect it to penetrate the plant cells if the BAL does. However, as was pointed out (BURNETT et al. 1951), cysteine has only one-half the protective value of BAL. This is what would be expected since the BAL has two SH groups whereas cysteine has only one. And indeed upon checking the rods and dots, one finds that although there is a statistically insignificant amount of protection, it is approximately one-half the amount of protection given by BAL. Even in the two-hit data if one varies the figure recorded by as little as one standard deviation, one gets one-half the protection of the BAL.

MICHAELSON (1952) in the only other attempt to test glutathione on plant chromosomes, found he could get significant protection only for fragments and not for anaphase bridges when he treated Tradescantia root tips. However he soaked the roots in glutathione for twenty-four hours, and it is quite probable that in the present experiments enough glutathione, a tripeptide, did not diffuse into the seed during the one-hour soaking.

It is quite evident none of the chemicals is able to give complete protection against the effects of the radiation. This is consistent with the results of all the other experiments ever performed on the protective effect of these chemicals.

It may be noticed that the placing of the seeds *in vacuo* caused no breakage to the chromosomes *per se*. Furthermore, as would be expected from the results of THODAY and READ (1947) and of HAYDEN and SMITH (1949), placing the seed in a vacuum resulted in protection from much of the damage.

According to the equations of WEISS (1944, 1947) the presence of the oxygen in the cells causes the formation of two compounds that would not be found in the absence of oxygen. These are the highly reactive hyperoxyl radical and hydrogen peroxide.

$$H + O_2 \rightarrow HO_2$$
$$2HO_2 \rightarrow H_2O_2 + O_2$$

It is quite logical, since these are the only two products that are excluded when water, which is omnipresent in cells, is irradiated anaerobically, that the chemicals protect by preventing the formation of these two substances. The other active radicals are present whenever the water is exposed to X-rays, anaerobically or not.

$$H^+ + OH^- \rightarrow H + OH$$

Since this is so and since the protective chemicals have no additive effect beyond oxygen removal as these experiments indicate, then it must be assumed that the chemicals are only able to protect against that portion of the " in-

direct effect" that is mediated through the hyperoxyl radical and hydrogen peroxide. The remaining chromosomal aberrations came therefore from two sources; 1) from that portion of the "indirect effect" mediated by H and OH radicals, and 2) from the direct ionization of the chromosome threads by the irradiation.

The question remains as to where the active radicals exert their influence. Do they break the chromosomes or do they inhibit restitution? Other groups of workers have already made contributions toward the solution of this problem (GILES and RILEY 1950; SCHWARTZ 1952; RILEY, GILES and BEATTY 1952; BAKER and VON HALLE 1953). However, they disagree as to the mechanism of lowering the oxygen tension. GILES and RILEY interpret their experiments on Tradescantia as indicating that oxygen affected the original breakage of the chromosomes. However, the possibility that the oxygen effect was due to a reaction on broken ends at the time of radiation was not excluded.

In contrast, both SCHWARTZ' work with endosperm mosaics in maize and BAKER and VON HALLE's work with recessive lethals in Drosophila indicate that it is the restitution process that is being affected.

The results of the present dosage fractionation experiments on *Vicia faba* indicate that the number of two-hit aberrations is dependent on total dosage and not on the method of administration. The two-hit aberrations are not decreased by fractionating the dosage as is the case in the classical Tradescantia experiments. This is consistent with the hypothesis of SAX and BRUMFIELD (1943) and is probably due to the fact that the ends remain open for periods longer than three hours.

Upon the addition of the BAL, however, fractionation results in a significant decrease of the two-hit aberrations. This is explainable if the addition of the chemical sped up the restitution time of the broken ends so that it was now less than the three hours in which the dosage was administered. This would then allow some of the breaks to become healed before other breaks occurred, and consequently would mean that there would be fewer breaks in the system at any given time that could form interchanges.

It therefore appears that the chemicals that reduce the oxygen tension within the cells increase the restitution rate of broken chromosomes. If one were to generalize from this hypothesis, then it would seem that the initial breakage hypothesis of GILES and RILEY is improbable, and that in the presence of oxygen the chromosomes probably react at the time of irradiation in some manner so as to decrease their ability to restitute.

SUMMARY

1) The seeds of *Vicia faba* were treated with 2×10^{-3} M solutions of BAL, sodium hydrosulphite, cysteine, glutathione, or sodium ascorbate, and then were given 500 r X-rays. The BAL gave good protection against radiation-induced chromosomal aberrations. The sodium hydrosulphite gave some protection but not as much as the BAL. The other chemicals did not enhance radioresistance.

2) The same chemicals were given and the seeds irradiated *in vacuo*. The vacuum gave partial protection against the production of aberrations. The chemicals had no additive effect over that of the vacuum.

3) The seeds were given 600 r of X-rays both in a constant dose and an intermittent one. There was no change in the number of aberrations when the dosage was fractionated. The experiment was repeated with the additional treatment of BAL. The BAL-treated seeds showed a decrease in the number of two-hit aberrations when the dosage was fractionated. This is interpreted as indicating that a reduction of the oxygen tension within the cells at the time of radiation increases the subsequent restitution rate of broken chromosome ends.

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