The Effect of Respiratory Inhibitors and Chelating Agents on the Frequencies of Chromosomal Aberrations Produced by X-Rays in *Vicia*

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ABSTRACT

Nitrosophenylhydroxylamine-ammonium (cupferron), potassium cyanide, sodium azide, ethylenediaminetetraacetate (EDTA), α,α'-dipyridyl, and *o*-phenanthroline were tested (1) for their ability to enhance the frequencies of chromosomal aberrations produced by x-rays in the root tip cells of the broad bean, *Vicia faba*, and (2) for their ability to inhibit oxygen consumption of excised roots of the same plant. In all cases a close correlation was found between the inhibitory effect on respiration and the enhancement of the sensitivity to x-rays at low oxygen pressures. EDTA, dipyridyl, and *o*-phenanthroline did not affect respiration to any greater extent, and they were without influence on the radiosensitivity. Cyanide, azide, and cupferron, which strongly inhibited respiration, also increased the frequencies of chromosome aberrations produced by x-rays at low oxygen pressures.

The relation between oxygen concentration and radiosensitivity was determined both in the presence and the absence of the respiratory inhibitor cupferron. When cupferron was present, the radiosensitivity was influenced by oxygen concentrations 30 times lower than those effective in the absence of the inhibitor.

In an atmosphere of pure oxygen, an increase of radiosensitivity of about 20 per cent was obtained with cupferron, EDTA, and potassium cyanide.

INTRODUCTION

The fact that the radiosensitivity of bean root tips may be strongly influenced by potassium cyanide (KCN) was established by Lilly (19, 18). She observed that in an atmosphere of tank nitrogen the growth inhibition and chromosome damage produced by x-rays was much greater in the presence than in the absence of KCN. These results were confirmed by Kihlman, Merz, and Swanson (14), who also found that the chelating agent cupferron had a similar effect on the sensitivity of *Vicia* chromosomes to x-rays. Previously, another chelating agent, ethylenediamine tetraacetate (EDTA), was found by Wolff and Luippold (36) to enhance the frequencies of aberrations produced by x-rays in *Vicia*. Wolff and Luippold suggested that the effect of EDTA was a result of a complex formation between the chelating agent and calcium and/or magnesium ions within the chromosomes. They based their explanation on the Mazia-Steffensen model of the chromosome. According to Mazia (21) and Steffensen (32), the chromosome consists of small units linked together by calcium and/or magnesium ions. The cyanide-cupferron effect was explained by Kihlman *et al.* in much the same way as Wolff and Luippold explained the EDTA effect. Kihlman *et al.* believed, however, that the metal involved was more likely to be iron or some other heavy metal than calcium or magnesium. Since their results were obtained under apparently anaerobic conditions and since cupferron was not known to be a respiratory inhibitor, they did not consider the possibility that the effect of KCN and cupferron on x-ray sensitivity was related to the effect of these agents on respiration. The finding of Alper and Howard-Flanders (1) that even such low concentrations of oxygen as those present in ordinary tank nitro-

gen may influence radiosensitivity of micro-organisms, suggested, however, that the effect of cyanide and cupferron on radiosensitivity might be the result of an inhibition of respiration by these agents. The fact that oxygen strongly increases the chromosome damage produced by x-rays in *Vicia* was known previously (35, 11), but the oxygen effect became, as a rule, not evident before the oxygen concentration in the gas phase exceeded 3 per cent. However, Alper and Howard-Flanders showed that radiosensitivity actually may be influenced by oxygen concentrations as low as 0.07 per cent. According to these authors, the reason why higher concentrations of oxygen are needed when organized tissues such as bean roots are used is that, in these cases, oxygen has to diffuse through actively respiring cells in order to reach the central part of the root. When the oxygen concentration in the irradiation chamber is low, completely anaerobic conditions are likely to prevail in the greater part of the meristem because of the respiratory activity of the outer cells of the root. In the presence of a respiratory inhibitor, however, even low concentrations of oxygen may be able to diffuse into the cells of the whole root meristem.

In the light of the results of Alper and Howard-Flanders, it was, therefore, necessary to reexamine the cyanide-cupferron effect. In the new experiments, great care was taken to remove oxygen from the system. The tank nitrogen used was made oxygen-free by passing it over active copper in a Meyer-Ronge column (23) and the gas was introduced into the system through a fritted glass filter instead of through the glass capillary used in previous experiments. The result of these experiments was that cupferron did not produce any significant increase of radiosensitivity in the absence of oxygen (13). The effect obtained in the presence of 1 per cent oxygen and cupferron was as high as that obtained in air. In the absence of cupferron, 1 per cent oxygen did not influence the sensitivity of the root tip chromosome to x-rays. In preliminary experiments, cupferron was found to produce a marked inhibition of respiration of bean roots. These results strongly suggested that the observed effect of cupferron was due to the ability of cupferron to inhibit oxygen consumption. More evidence in favour of this explanation of the cupferron effect is presented below.

Method

Material:

The experimental material consisted of lateral roots of the broad bean, *Vicia faba* (Carter's "Seville Long-pod"). The seedlings were grown essentially as described by McLeish (22). After the beans had been soaked for 24 hours at 25°C., the seed coats were removed and a celluloid strip passed through the cotyledons. By the aid of this strip, the beans were suspended over the openings of glass tubes containing about 40 ml. of tapwater in such a way that the roots, but not the cotyledons, were immersed in water. The seedlings were grown in the dark at 19°C., and every 24 hours the water in the tubes was changed. After 4 days, when the main root was about 4 cm. long, the tips were removed. After another 4 days at 19°C., the seedlings had developed 1 to 2 cm.-long lateral roots and were ready for treatment.

Chemicals and Gases:

The respiratory inhibitors and chelating agents used were the ammonium salt of nitrosophenylhydroxylamine (cupferron), sodium azide (NaN_3), potassium cyanide (KCN), *ortho*-phenanthroline, α,α'-dipyridyl, and ethylenediaminetetraacetic acid (EDTA). The structural formula of cupferron, the inhibitor used in most of the experiments, is shown in Fig. 1. The chemicals were dissolved in phosphate or phosphate-citrate buffer solutions, the concentrations of these buffers being M/60 in the respiration experiments and M/150 in the irradiation experiments.

Analysed oxygen-nitrogen gas mixtures containing 0.06, 0.1, 1.0, 2.8, 5.4, 10.1, and 99.7 per cent oxygen, were supplied by the Swedish Gasaccumulator Company. The other oxygen concentrations used in the experiments were obtained by mixing gases with known concentrations of oxygen in desired proportions by means of flowmeters.

Oxygen-free nitrogen was obtained by passing tank nitrogen over active copper in a Meyer-Ronge column (23).

Radiation:

The x-rays were generated at 180 kv, 5mA in a Müller therapy tube, type 71780. The dose rates used were 20.6 r/minute (Figs. 2 to 4, Table IV), and 54 r/minute (Figs. 5 and 6, Tables III, V, and VI). The radiation was filtered through 1 mm. Al. The dose was measured by a Philips ionization chamber, type 37480.

Irradiation Vessel:

The sides of the vessel were made of 5 mm. thick lucite. The gases were introduced into the vessel through a glass tube provided with a fritted glass filter, and they escaped through a narrow glass tube, the

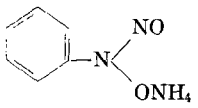

FIG. 1. Cupferron.

outer end of which was kept below water in order to prevent back-diffusion of air. The vessel also contained tubes through which solutions could be introduced and removed. Two bean seedlings were always irradiated simultaneously. The seedlings were placed into a perforated lucite holder which was attached to the removable front side of the vessel. Since the distance between the front and the back side of the perforated holder was not more than 5 mm., the laterals were kept on approximately the same level. Immediately behind the holder was a lucite tube which contained the ionization chamber. Between the roots and the ionization chamber was 5.5 mm. of water and lucite. Careful measurements have shown that the measured dose had to be multiplied by a factor of 1.08 in order to get the dose the root tips actually received (16). The entry and exit tubes, as well as the lucite tube containing the ionization chamber, were fitted into holes in the top of the vessel by means of rubber stoppers. When filled up to the cotyledons of the seedlings, the vessel contained 330 ml. of solution.

Procedure in the Irradiation Experiments:

The two bean seedlings to be irradiated were introduced into the irradiation vessel, and the vessel was filled with tap water or buffer solution up to the level of the cotyledons. When the irradiation was to be performed at other oxygen concentrations than that of air, the air was removed from the vessel by bubbling oxygen-free nitrogen through the vessel for 5 minutes. If the seedlings were to be treated with some chemical before and during irradiation, a solution of the chemical in question was made oxygen-free in a separate vessel by bubbling oxygen-free nitrogen through it. The vessel was connected with the irradiation vessel through a rubber tubing. After about 5 minutes of bubbling, the water was removed from the irradiation vessel. This was accomplished by closing the exit tube for the gas and opening the exit tube for the solution. When the water had been completely removed, the exit tube for the gas was again opened and the exit tube for the solution closed. The tubing connecting the irradiation vessel and the vessel containing the solution was opened and the solution was allowed to flow into the irradiation vessel until the surface of the solution was level with the cotyledons. The connecting tube was closed. The desired oxygen-nitrogen mixture was now bubbled through the solution for 30 minutes. To prevent diffusion of air into the system, copper tubings were used instead of rubber tubings whenever possible. When this could not be done, vacuum rubber tubings were used. The bubbling was continued during the irradiation which concluded the ½-hour treatment period. The temperature during treatment and irradiation was 22°C. When the irradiation was finished, the vessel was opened and the seedlings removed and thoroughly washed with running tap water. The seedlings were then transferred to tubes containing tap water. During the recovery period, the seedlings were kept in the dark at 19°C.

Eighteen hours after the irradiation, the root tips were fixed in alcohol-acetic acid 3:1. Before fixation the roots were immersed for 4 hours in 0.05 per cent colchicine. The slides were prepared as Feulgen-squashes and made permanent according to the dry ice method (4).

In most of the experiments, the effect was expressed as percentages of abnormal metaphases and as isolocus breaks (34) and exchanges per 100 cells. Most of the isolocus breaks were of the isochromatid type, but some chromosome breaks were also present. Similarly, the exchanges were mostly of chromatid type, but a few isolocus-chromatid exchanges (triradials) and isolocus-isolocus (chromosome) exchanges were included in this group. Metaphases containing structural chromosome changes were scored as abnormal. The structural chromosome changes observed were in addition to isolocus breaks and exchanges, "true" chromatid breaks (as distinct from achromatic lesions), and minute fragments. For each treatment 100 to 200 cells from the root tips of two seedlings were analysed. The standard deviation in per cent was calculated according to the formula $100\sqrt{a}/n$, in which a is the number of aberrations or abnormal cells counted and n the number of cells analysed.

Procedure in the Respiration Experiments:

The oxygen consumption was determined by means of a Warburg constant volume respirometer. The experiments were made at 20°C. with 25 about 1-cm.-long excised bean root tips in each flask. In most of the experiments, the flasks contained 3 ml. of the M/60 phosphate or phosphate-citrate buffer in question, the center well 0.2 ml. of 15 per cent sodium hydroxide solution, and the side arm 0.5 ml. buffer or inhibitor solution. When the inhibitors were volatile (azide, cyanide) the standard method had to be modified. In the azide experiments at low pH, the side arm contained a solution of azide in a neutral M/120 M phosphate buffer. In the cyanide experiments, the methods of Krebs (15) were used in order to counteract the HCN distillation. When a gas mixture other than air was used, the desired gas mixture was run through the system for about 20 minutes before the manometer stopcock and the gas vent in the sidearm-stopper were closed.

Table I shows how the experiments were performed

TABLE I

Determination in a Warburg Apparatus of the Inhibitory Action of 400 μM/liter Cupferron on the Respiration of Excised Bean Root Tips

Gas phase: air. Flasks Nos. 1 and 2: inhibitor added; flasks Nos. 3, 4, and 5: no inhibitor added.

Flask No.	Time, *min.*							
	0–15	15–30	30–60 ↓	65–80	80–95	95–110	110–140	140–170
1	14	13	24	7	7	3	9	8
2	13	12	25	7	6	2	8	6
3	15	13	28	13	15	10	25	22
4	14	13	28	13	15	9	25	21
5	13	12	25	12	13	9	24	21
$\frac{\text{Mean of 1 and 2}}{\text{Mean of 3, 4, and 5}}$	0.96	0.98	0.91	0.55	0.45	0.27	0.34	0.33
Mean	0.95			0.39				
Inhibition, *per cent*	$\frac{100\ (0.95-0.39)}{0.95} = 59$							

and the inhibition calculated. The method is essentially the same as that used by Stenlid (33). The oxygen consumption is given in arbitrary units (change in mm. of the manometer readings, corrected for the thermobarometer reading). At ↓ the inhibitor was added from sidearms of flask 1–2, to give a concentration of 400 μM/liter. From the sidearms of flasks 3–5, an equivalent quantity of buffer was added. The initial pH in the experiment was 5.7, the final 5.8.

RESULTS

Cupferron as a Respiratory Inhibitor:

Cupferron proved to be a strong inhibitor of respiration of excised bean root tips. As appears from Table II, the inhibition is dependent on both the pH of the solution and on the pressure of oxygen. The inhibition increases strongly when the pH is lowered from 7.3 to 5.5. The study of the influence of oxygen pressure on the inhibitory action is complicated by the fact that at very low oxygen pressures most of the cells in the root do not take part in the oxygen consumption at all, since all the oxygen is consumed by the outer cells of the root. When respiration is slowed, oxygen penetrates further and the number of respiring cells increases. Since the amount of respiring tissue increases with increasing degree of inhibition at oxygen concentrations below 5 per cent, the figures obtained for the inhibitory effect of cupferron at 2.8 per cent oxygen are too low. Therefore, the results indicate that the inhibitory action increases when the oxygen concentration decreases.

TABLE II

The Effect of Cupferron on Respiration of Excised Bean Root Tips at Different Concentrations of Oxygen and at Different pH Values

Experiment No.	pH	Concentration of cupferron	Concentration of oxygen in the gas phase	Inhibition of oxygen consumption
		μM/liter	*per cent*	*per cent*
I	5.5	400	99.7	47
I	5.5	400	*Circa* 21 (air)	59
II	5.5	400	*Circa* 21 (")	59
II	5.5	400	2.8	67
III	5.5	200	2.8	83
IV	5.9	400	2.8	100
V	7.3	200	2.8	11

The Relation between Sensitivity to X-rays at Low Oxygen Tensions and Respiratory Activity:

Table III shows that there is strong correlation between the effect on oxygen consumption of the agents tested, and their effect on radiosensitivity at 1 per cent oxygen. EDTA, α,α′-dipyridyl, and *o*-phenanthroline did not markedly suppress oxygen consumption during an experimental period of 2 hours. In the x-ray experiments, these compounds were found to be either without effect (EDTA, α,α′-dipyridyl) or to have only a slight effect on

TABLE III

The Relation between Sensitivity to X-rays at 1 Per cent Oxygen and Respiratory Activity of Bean Root Tips

Inhibitor	Concentration of inhibitor	pH of solution	X-ray experiments						Respiration experiment	
			Concentration of oxygen in the gas phase	Dose of x-rays	No. of metaphases analysed	Abnormal metaphases	Aberrations/100 cells		Concentration of oxygen in the gas phase	Oxygen consumption per cent of control
							Isolocus breaks	Exchanges		
	μM/liter		*per cent*	*r*		*per cent*			*per cent*	
—	—	5.8	1.0	81	100	13.0	10.0	3.0	—	—
Potassium cyanide	400	7.2	1.0	81	100	51.0	32.0	39.0	*Circa* 21 (air)	26
EDTA	1000	7.0	1.0	81	200	9.5	8.0	1.5	"	100
α,α'-Dipyridyl	1000	7.0	1.0	81	100	16.0	14.0	3.0	"	89
o-Phenanthroline	1000	7.0	1.0	81	100	21.0	11.0	12.0	"	79
—	—	7.1	99.7	81	200	48.5	43.0	21.0	—	—
—	—	5.3	1.0	108	100	15.0	13.0	3.0	—	—
—	—	7.3	1.0	108	100	14.0	11.0	4.0	—	—
Cupferron	200	5.5	1.0	108	100	68.0	52.0	50.0	2.8	17
"	200	7.3	1.0	108	100	22.0	9.0	15.0	2.8	89
Sodium azide	400	4.7	1.0	108	100	63.0	61.0	34.0	*Circa* 21 (air)	27
" "	400	7.3	1.0	108	100	20.0	10.0	9.0	"	63
—	—	5.8	99.7	108	200	59.0	48.0	35.0	—	—

radiosensitivity (*o*-phenanthroline). Potassium cyanide, on the other hand, is an effective inhibitor of respiration, and, in its presence, the effect produced by 81 r in an atmosphere of 1 per cent oxygen, is of the same magnitude as the effect of 81 r in pure oxygen. At low pH values, when the oxygen consumption is strongly suppressed by both cupferron and sodium azide, there is also a strong enhancement of the frequencies of x-ray–produced chromosome aberrations by these compounds. At pH 7.3 the oxygen consumption is but little affected by azide and cupferron and so is the x-ray–induced chromosome damage. As shown by the control experiments in Table III, variations in pH between 5.3 and 7.3 have no effect per se on radiosensitivity.

The Effect of Cupferron at Low Oxygen Tensions:

Table IV shows how the effect of cupferron on the frequencies of x-ray–produced chromosome aberrations is influenced by pH. At pH 4.8 the toxic effect of cupferron becomes so strong that the mitotic activity is completely suppressed even 18 hours after the treatment. When the pH is increased, the toxic effect of cupferron decreases, being still considerable at pH 5.4, however. At pH 5.9 the mitotic inhibition produced by cupferron and x-rays together (see Fig. 6) is not stronger than that produced by x-rays alone.

TABLE IV

The Effect of 400 μM/liter Cupferron and 7.1 μM/liter (0.55 Per cent) Oxygen on the Production of Chromosome Aberrations by 108 r of X-rays at Different pH Values

pH	No. of metaphases analysed	Abnormal metaphases	Aberrations/100 cells	
			Isolocus breaks	Exchanges
		per cent		
4.8	No mitoses	—	—	—
5.4	100	67	44	43
5.9	100	65	44	41
6.7	100	45	28	24

Since the effect of cupferron on radiosensitivity is the same at pH 5.9 as at pH 5.4 (Table IV), the pH in all subsequent cupferron experiments was kept at pH 5.9. At pH 6.7 the x-ray effect is much less influenced by cupferron.

Fig. 2 shows how the percentage of abnormal metaphases produced by 86 r of x-rays at an oxygen concentration of 1 per cent is influenced by various concentrations of cupferron. Above 300 μM/liter any further increase of the concentration of cupferron is without influence on the x-ray effect. In subsequent experiments, the concentration of cupferron used was 400 μM/liter.

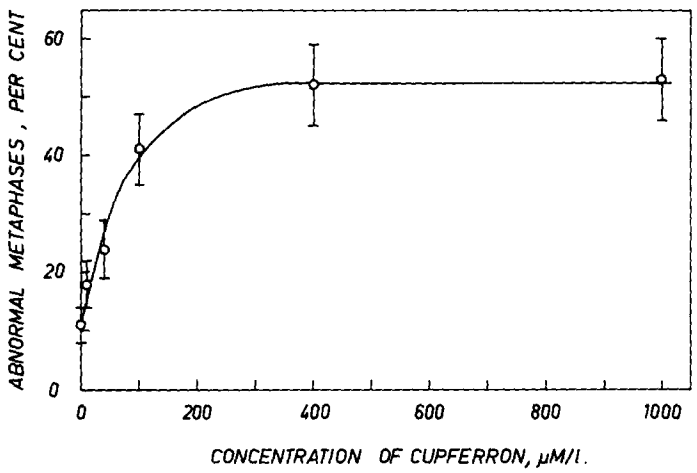

FIG. 2. The relation between the concentration of cupferron and the percentages of abnormal metaphases induced by 86 r of x-rays in the presence of 1 per cent oxygen (in the gas phase).

The Effect of Oxygen on X-ray Sensitivity in the Presence and Absence of Cupferron:

Figs. 3 and 4 show how the percentage of abnormal metaphases produced by 108 r of x-rays is influenced by the oxygen tension in the presence and in the absence of cupferron (400 μM/liter). When the inhibitor is absent, there is no significant increase of radiosensitivity until the oxygen concentration exceeds 60 μM/liter (5 per cent in the gas phase). Above 60 μM/liter the frequencies of structural chromosome changes increases with increasing oxygen concentration until a maximum is obtained at about 500 μM/liter of oxygen (*circa* 35 per cent in the gas phase). Similar curves for the relation between oxygen concentration and x-ray-induced chromosome damage have previously been obtained in *Tradescantia* by Giles and Beatty (6), and Riley, Giles, and Beatty (28) and in *Vicia* by Kihlman (11).

In the presence of cupferron, the slope of the curve is much steeper. The percentage of abnormal metaphases is influenced by oxygen concentrations as low as 1.3 μM/liter (0.1 per cent in the gas phase), and the maximum effect is obtained at an oxygen concentration of about 26 μM/liter (2 per cent in the gas phase). Fig. 4 also shows that the maximum effect in the presence of cupferron is about 20 per cent greater than that obtained in the absence of cupferron. In order to study this phenomenon more in detail, a number of experiments was performed, the results of which will be described below under a separate heading.

Attempts have also been made to determine the

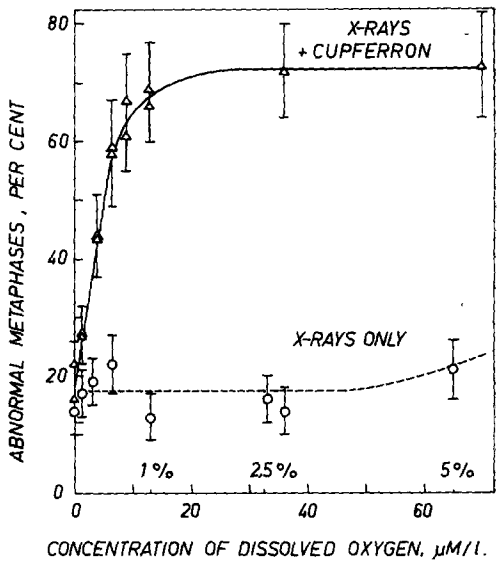

FIG. 3. The influence of low oxygen concentrations on the percentages of abnormal metaphases induced by 108 r of x-rays in the presence and absence of cupferron (400 μM/liter).

constants K and m of Howard-Flanders and Alper (9). K is defined by these authors as "that oxygen concentration which will give $(m + 1)/2$ times the nitrogen radiosensitivity" and m represents "the maximum enhancement ratio." The method used in the present study was less laborious but also less accurate than that used by Howard-Flanders and Alper.

The factor m, which had to be determined first,

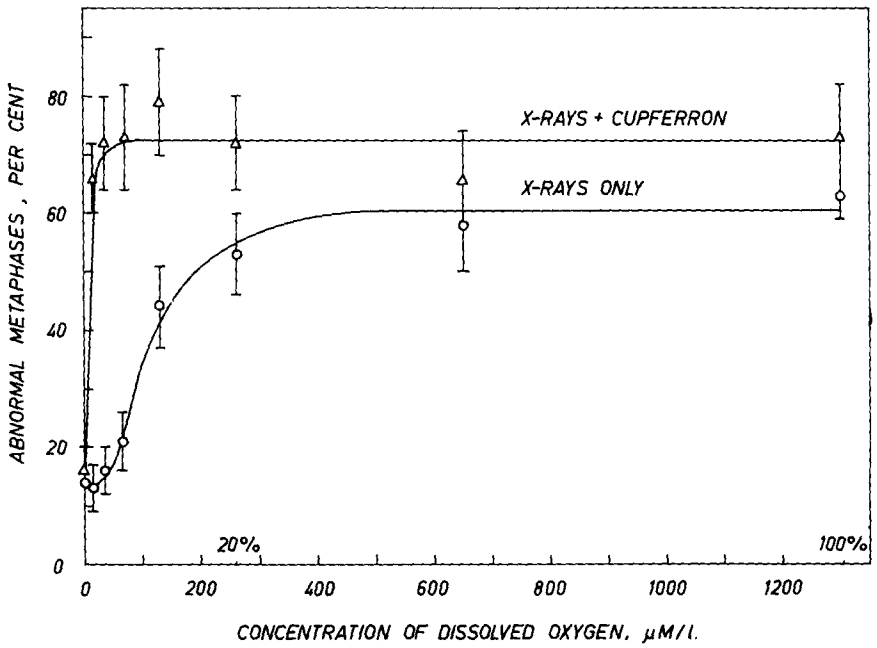

FIG. 4. The relation between the concentration of oxygen and the percentages of abnormal metaphases induced by 108 r of x-rays in the presence and absence of cupferron (400 μM/liter).

was obtained by determining the dose which at 100 per cent oxygen gave the same number of isolocus breaks and exchanges as a given dose in the absence of oxygen (oxygen-free nitrogen). The ratio, m, between the oxygen sensitivity and the nitrogen sensitivity was found to be 3.0 in the presence of cupferron and 2.7 in its absence. The dose which at oxygen concentration K would give the same number of aberrations as the dosages used in nitrogen and oxygen was calculated from the formula $S_K/S_N = (m + 1)/2$ (9) in which S_K is the sensitivity at oxygen concentration K and S_N the nitrogen sensitivity. With a dose D, the sensitivity S is equal to $1/D$. When the dosage at K had been calculated, the oxygen concentration K was determined experimentally by exposing root tips to this dose at a number of different oxygen concentrations. The results appear from Table V. In the presence of cupferron K was found to be higher than 3.3 μM/liter but lower than 5.2 μM/liter. In the absence of cupferron K appears to be about 132 μM/liter.

The Enhancement of Radiosensitivity at High Oxygen Pressures:

The results described above show that the maximum enhancement ratio, m, is greater in the presence than in the absence of cupferron. It appears from Fig. 4 that the maximum effect in the presence of cupferron is about 20 per cent higher than in its absence. Fig. 5 shows the effect of cupferron on the frequencies of isolocus breaks + exchanges obtained by various doses of x-rays at 0.0, 1.0, and 99.7 per cent oxygen. It appears from Fig. 5 that there are two main levels of sensitivity to x-rays, the higher sensitivity level being about 3 times higher than the lower one. A high sensitivity is obtained at 99.7 and 1.0 per cent oxygen in the presence of cupferron and at 99.7 per cent oxygen in the absence of the inhibitor. A low sensitivity is obtained at 0.0 per cent oxygen both in the presence and the absence of cupferron and at 1.0 per cent oxygen in the absence of cupferron. At each of the two levels, however, there appear differences between the effects of the different treatments. This is particularly true at the higher level, where the effect obtained at 99.7 per cent oxygen in the presence of cupferron is significantly greater than the effect obtained at the same oxygen tension in the absence of cupferron. At the lower sensitivity level, the differences are not so pronounced, but here, too, a somewhat greater effect is obtained in the presence of cupferron.

A number of other compounds were also tested

TABLE V

Determination of Constants m and K (9) in the Presence and in the Absence of Cupferron

Concentration of oxygen		Concentration of cupferron	pH of solution	Dose of x-rays	No. of metaphases analysed	Abnormal metaphases	Aberrations per 100 cells			Constants m and K
Gas phase	Solution						I Isolocus breaks	II Exchanges	I + II	
per cent	*μM/liter*	*μM/liter*		*r*		*per cent*				
99.7	1299	400	5.8–6.0	72	100	48.0	30.0	21.0	51.0	
99.7	1299	400	5.8–6.0	72	100	53.0	31.0	30.0	61.0	
					Mean	50.5	30.5	25.5	56.0	
0.0	0.0	400	5.8–6.0	216	100	47.0	34.0	19.0	53.0	
0.0	0.0	400	5.8–6.0	216	100	58.0	43.0	24.0	67.0	
					Mean	52.5	38.5	21.5	60.0	$m = 3.0$
0.25	3.3	400	5.8–6.0	108	100	35.0	29.0	15.0	44.0	
0.25	3.3	400	5.8–6.0	108	200	38.5	31.0	11.5	42.5	
					Mean	36.8	30.0	13.3	43.3	
0.30	3.9	400	5.8–6.0	108	200	38.5	29.5	16.5	46.0	
0.30	3.9	400	5.8–6.0	108	100	44.0	42.0	15.0	57.0	
0.30	3.9	400	5.8–6.0	108	100	44.0	39.0	16.0	55.0	
0.30	3.9	400	5.8–6.0	108	200	53.0	41.5	19.5	61.0	
					Mean	44.9	38.0	16.8	54.8	
0.35	4.6	400	5.8–6.0	108	100	54.0	41.0	24.0	65.0	
0.35	4.6	400	5.8–6.0	108	100	48.0	32.0	15.0	47.0	
0.35	4.6	400	5.8–6.0	108	200	44.0	32.5	24.5	57.0	
					Mean	48.7	35.2	21.2	56.4	
0.40	5.2	400	5.8–6.0	108	200	57.5	49.5	27.0	76.5	$3.3 < K < 5.2$
0.40	5.2	400	5.8–6.0	108	200	57.5	46.5	22.5	69.0	or
					Mean	57.5	48.0	24.8	72.8	$K = 4.2 \pm 0.9$ μM/l.
99.7	1299	—	*Circa* 6.5	108	100	58.0	49.0	29.0	78.0	
99.7	1299	—	*Circa* 6.5	108	200	63.0	52.5	27.5	80.0	
					Mean	60.5	50.8	28.2	79.0	
0.0	0.0	—	*Circa* 6.5	292	100	57.0	50.0	26.0	76.0	
0.0	0.0	—	*Circa* 6.5	292	100	61.0	48.0	34.0	82.0	
					Mean	59.0	49.0	30.0	79.0	$m = 2.7$
10.1	132	—	*Circa* 6.5	158	200	54.0	47.0	25.0	72.0	K = *circa* 132 μM/liter

for their ability to increase the sensitivity to x-rays in oxygen and nitrogen atmosphere. The results of these experiments appear from Table VI. In oxygen, a greater effect than in the control was obtained with KCN and EDTA, whereas α,α′-dipyridyl and *o*-phenanthroline seemed to decrease the effect. The figures indicate that the frequencies of chromatid exchanges particularly are increased at high oxygen pressures in the presence of cupferron, EDTA, and KCN. The increase obtained with KCN is much greater than that obtained in the presence of cupferron and EDTA. This may be explained by the fact that, in the absence of radiation, only the KCN treatment produces a marked radiomimetic effect. In nitrogen atmosphere, cupferron and KCN produce a slight but hardly significant increase of the x-ray effect whereas EDTA is quite ineffective. The finding that KCN enhances the x-ray effect even in an atmosphere of pure oxygen does not agree with the

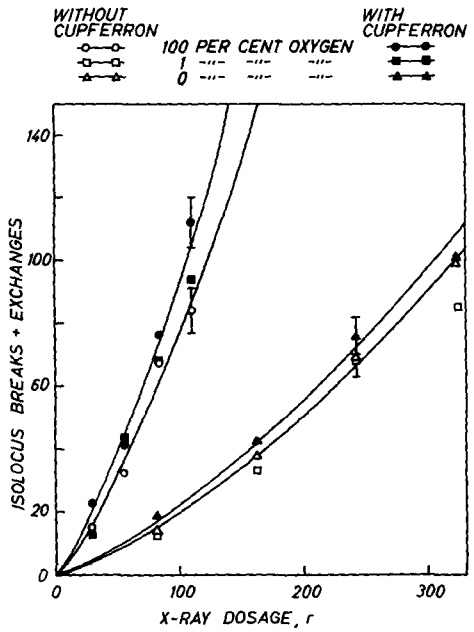

FIG. 5. The relation between the x-ray dosage and the frequencies of isolocus breaks + exchanges induced in the presence and absence of cupferron (400 μM/liter) and with 0, 1, and 100 per cent oxygen in the gas.

results of Lilly (18). She did not observe any enhancement of the x-ray sensitivity at high oxygen pressures.

Since the effect obtained in *Vicia* by a given dose of x-rays is greatest 4 to 6 hours after irradiation (27) and then slowly decreases when the period between irradiation and fixation increases, it was conceivable that the increase obtained in the presence of cupferron, KCN, and EDTA was due to the mitotic inhibition produced by these agents. If the combined chemical-radiation treatments delayed cell division more than the x-ray control, the maximum effect would appear later after the combined treatments. That this is not the correct explanation of the increased radiosensitivity obtained appears from Fig. 6, which shows the effect of cupferron on the percentage of abnormal anaphases and on the inhibition of mitosis produced by 65 r of x-rays. There is no significant effect of cupferron on the frequency of cells in division, although the percentage of abnormal anaphases is considerably higher in the presence than in the absence of cupferron.

DISCUSSION

Very little has been known previously about cupferron as a respiratory inhibitor. According to Lerner (17), cupferron inhibits polyphenoloxidase *in vitro*. In Lerner's experiments, the respiration of *Hedera*, *Aucuba*, and *Euonymus* leaves was nonspecifically inhibited by cupferron, provided that the leaves were injected with the inhibitor. When administered through the petiole, cupferron increased respiration.

The results described in the present paper have shown that cupferron is an effective inhibitor of respiration of excised bean root tips, provided that the pH of the solution is below 7. The inhibitory action of sodium azide on root tip respiration is similarly dependent on the pH of the solution (33). In the case of azide, the greater inhibitory action is believed to be due to the fact that the undissociated hydrazoic acid penetrates more easily. It seems very likely that the greater inhibitory effect of cupferron at low pH values has a similar explanation, *i.e.* that nitrosophenylhydroxylamine penetrates more easily into the root in an undissociated condition. Since the inhibition of bean root respiration produced by cupferron does not differ quantitatively from that produced by KCN and NaN_3, and since the terminal oxidase in plant root tips usually seems to be cytochrome oxidase (10), the present study indicates that cupferron is an inhibitor of cytochrome oxidase.

Under the same conditions in which they are effective as respiratory inhibitors, both cupferron and azide enhance the effect of x-rays at low oxygen pressures (Table III). It should be mentioned that in a previous study, azide was found to be without influence on radiosensitivity (14). The explanation is that in the experiments of Kihlman *et al.*, the treatments were performed with neutral NaN_3 solutions. The present study has shown that such solutions do not effectively suppress respiration.

The reason why acid solutions were not tested was that neutral NaN_3 solutions had been found to inhibit effectively the radiomimetic effect of 8-ethoxycaffeine (11, 26), which indicated that NaN_3 did penetrate into the root under these conditions. The influence of NaN_3 on the radiomimetic effect of ethoxycaffeine was interpreted in the same way as the similar effect of KCN; *i.e.* it was believed to be due to its inhibitory effect on respiration. In the light of the results of the present experiments, it seems more likely that NaN_3 inhibited the radiomimetic effect of ethoxycaffeine because of its ability to uncouple phosphorylation from respiration, an effect which is obtained with lower azide concentration than the effect on oxygen consumption (31). In the same ethoxycaffeine experiments, the uncoupling agent 2,4-dinitrophenol effectively suppressed the radiomimetic effect of ethoxycaffeine (11, 26).

TABLE VI

The Influence of Some Respiratory Inhibitors and Chelating Agents on Radiosensitivity at 99.7 and 0.0 Per cent Oxygen

Inhibitor or chelating agent	Concentration of agent	Duration of treatment	Concentration of oxygen in the gas phase	Dose of x-rays	Number of metaphases analysed	Abnormal metaphases	Aberrations/100 cells		
							I Isolocus breaks	II Exchanges	I + II
	μM/liter	hr.	per cent	r		per cent			
—	—	—	99.7	54	200	26.5	18.5	11.5	30.0
—	—	—	99.7	54	200	29.0	18.0	14.5	32.5
Cupferron	400	½	99.7	54	200	32.5	23.5	18.0	41.5
EDTA	1000	½	99.7	54	200	40.5	26.5	17.5	44.0
—	—	—	99.7	81	200	46.5	46.5	16.5	63.0
—	—	—	99.7	81	200	48.5	43.0	21.0	64.0
Cupferron	400	½	99.7	81	200	60.0	47.5	28.0	75.5
"	400	½	99.7	81	200	54.5	43.5	33.5	77.0
EDTA	1000	½	99.7	81	200	54.0	43.5	28.5	72.0
"	1000	½	99.7	81	200	56.0	47.0	27.0	74.0
Potassium cyanide	400	½	99.7	81	100	73.0	64.0	36.0	100.0
" "	400	½	99.7	81	100	66.0	40.0	52.0	92.0
α,α′-Dipyridyl	1000	½	99.7	81	100	48.0	39.0	12.0	51.0
o-Phenanthroline	1000	½	99.7	81	100	47.0	34.0	18.0	52.0
Cupferron	400	½	99.7	—	100	2.0	2.0	0.0	2.0
EDTA	1000	½	99.7	—	100	3.0	2.0	1.0	3.0
Potassium cyanide	400	½	99.7	—	100	20.0	20.0	4.0	24.0
—	—	—	0.0	81	200	13.5	8.0	6.0	14.0
Cupferron	400	½	0.0	81	200	16.0	16.0	3.0	19.0
EDTA	1000	½	0.0	81	200	12.5	11.0	2.5	13.5
Potassium cyanide	400	½	0.0	81	100	20.0	16.0	8.0	24.0
—	—	—	0.0	243	200	55.0	45.5	23.5	69.0
Cupferron	400	½	0.0	243	200	59.0	49.0	27.0	76.0
EDTA	1000	½	0.0	243	200	51.5	43.5	22.0	65.5

The striking correlation which, in the case of heavy metal complexing agents, exists between ability to inhibit respiration and ability to enhance the x-ray sensitivity at low oxygen pressures (Table III) clearly indicates that the two phenomena are related. Apparently oxygen is not consumed by the outer cells of the root in the presence of respiratory inhibitors, but is able to diffuse into the cells of the whole meristem even in cases when the outside oxygen concentration is very low. Consequently, the relation observed between oxygen concentrations and x-ray effect is very similar to that observed when free cell suspensions such as bacteria and ascites tumour cells are used. By two different methods, Howard-Flanders and Alper (9) found the factor K (see page 484) to be 4.0 ± 0.4 and 4.7 ± 0.3 μM/liter, respectively, in experiments with the bacterium *Shigella flexneri*. In the present *Vicia* experiments, which were performed at 22°C., K was found to be 4.2 ± 0.9 μM/liter in the presence of cupferron, compared to *circa* 132 μM/liter in its absence. The K value obtained in non-respiring root tip cells is, within experimental limits of error, the same as for *Shig. flexneri*. That this K value might represent the "true" relation between oxygen concentration and x-ray sensitivity is further indicated by the fact that Deschner and Gray (5) have found K to be 5 ± 2 μM/liter at 18°C. for ascites tumour cells of the mouse. It must be borne in mind, however, that the mean inhibition obtained with 400 μM/liter cupferron at 2.8 per cent oxygen and at pH 5.8 was not stronger than 85 per cent. Although at lower concentrations of oxygen, a higher degree of inhibition probably is obtained, the remaining oxygen consumption might be strong enough to

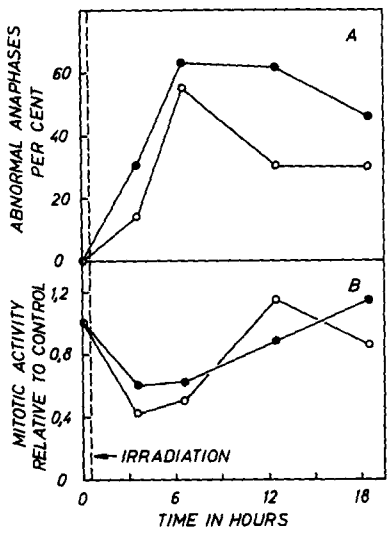

FIG. 6. The percentages of abnormal anaphases (*A*) and the inhibition of mitosis (*B*) produced by 65 r of x-rays in the presence (●—●) and absence (○—○) of cupferron (400 μM/liter). The cupferron treatment was given between 0 and 0.5 hour. The irradiations performed with air as gas.

influence the oxygen tension inside the root. Thus, there remains the possibility that the "true" K value is lower than that obtained in the present study.

The experiments reported in the present paper have shown that the oxygen present in ordinary tank nitrogen is sufficient to increase the radiosensitivity of bean root tip chromosomes. The rather weak increase observed in an atmosphere of tank nitrogen cannot explain the substantial enhancement observed by Kihlman *et al.* (14) in their study of the cyanide-cupferron effect, however. Apparently this enhancement was due mainly to the fact that oxygen was not effectively removed by the method used, *i.e.* bubbling nitrogen into the solution through a glass capillary. An effective removal of the oxygen dissolved seems to be obtained, however, by introducing the gas into the system through a fritted glass filter, as was done in the present study.

The idea that respiratory inhibitors may increase radiosensitivity in organized tissues by decreasing the oxygen gradient is not new. Hall, Hamilton, and Brues (8) interpreted in this way their finding that cyanide somewhat increases the sensitivity of tumour fragments irradiated in the presence of oxygen, and such an effect of respiratory inhibitors has also been discussed by other authors, *e.g.*, Patt (24), Gray (7), Scott (29), and Lilly (18).

It remains to consider the effect of KCN, cupferron, and EDTA on radiosensitivity at high oxygen pressures (Table VI).

The effect of cyanide can partly be accounted for by the radiomimetic effect of this compound, but the cupferron and EDTA treatments applied do not have any marked radiomimetic effects.

All the compounds in question are metal complexing agents, but EDTA differs from the other two by not being a respiratory inhibitor. This difference might be important. Unfortunately, the enhancement produced by EDTA is not quite as well documented as that produced by KCN and cupferron. Some support for the significance of the EDTA effect may, however, be obtained by the facts that EDTA was found by Wolff and Luippold (36) to increase the chromosome damage produced by x-rays in *Vicia* and by Bair and Hungate (2) to increase the growth inhibition produced by alpha and beta radiation in yeast.

If the EDTA effect is not taken into account and the enhancement produced by KCN and cupferron at high oxygen pressures is ascribed to their effect on respiration, it cannot be the oxygen concentration as such which is of significance. Since the oxygen consumption has already reached a maximum at 21 per cent oxygen, the concentration of excess oxygen must be much higher in the absence of an inhibitor at an oxygen concentration of 100 per cent than in the presence of an inhibitor at 2 per cent oxygen. As shown on Fig. 4, the enhancement, nevertheless, is greater in the latter case.

The facts are more consistent with the view that the effect is produced by organic peroxides. The peroxide hypothesis has been advocated by Sobels (30) as an explanation of his findings that pretreatments with cyanide and azide significantly enhance the rate of x-ray–induced mutations in *Drosophila*. According to the peroxide hypothesis, the enhancement of x-ray sensitivity obtained after treatments with cyanide and azide is produced by peroxides which are believed to accumulate in the cell as a result of the inhibitory action of cyanide and azide on cytochrome oxidase and catalase enzymes. Like cyanide and azide, cupferron inhibits catalase (12) and its effect on respiration is probably due to an inhibition of cytochrome oxidase (see page 487). The peroxide hypothesis has been discussed at length in previous papers (12, 14).

The slight increase of x-ray sensitivity obtained by cyanide and cupferron in nitrogen atmosphere is not consistent with the peroxide hypothesis, however. The conflict can be solved by assuming that in spite of all precautions, the system was not entirely oxygen-free during the irradiation. This seems to be very unlikely, however.

Another alternative, which does not involve respiration and which, therefore, also is applicable to EDTA, is that the enhancement of x-ray sensitivity at high oxygen pressures is due to complex formation between the compounds in question and metals localised in other sites than respiratory enzymes. For reasons mentioned previously (12), the metals involved are more likely to be heavy metals, such as iron, than calcium and/or magnesium as assumed by Wolff and Luippold (see page 479). It seems reasonable to assume that if this is the correct explanation, the metals involved occur inside the nucleus as components of either the chromosomes themselves or of the chromosome environment. The fact that iron occurs in cell nuclei and nucleoproteins is indicated by a number of recent studies (25, 3, 20) in addition to those previously mentioned (12).

A difficulty with this hypothesis is that, in contrast to the respiration hypothesis, it would seem to require that the enhancement is independent of oxygen concentration. Actually, the x-ray sensitivity in oxygen-free nitrogen is either not at all (EDTA) or only slightly (cupferron, KCN) increased. Since neither of the two hypotheses seems to fit all the facts obtained, it must be concluded that it has not been possible so far to explain satisfactorily the effect of cupferron (and cyanide) on x-ray sensitivity at high oxygen pressures.

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