EXPERIMENTALLY INDUCED CHROMOSOME ABERRATIONS IN PLANTS

II. The Effect of Cyanide and Other Heavy Metal Complexing Agents on the Production of Chromosome Aberrations by X-Rays

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INTRODUCTION

In their remarkable paper dealing with the production of chromosome aberrations by cyanide, Lilly and Thoday (9) also reported a cyanide effect in conjunction with x-ray treatments. In contrast to the production of aberrations by cyanide itself, the effect on x-ray breakage occurred only when the treatments were performed in the absence of oxygen. The cyanide effect consisted of an increased frequency of chromosome breakage by x-rays under anaerobic conditions (Lilly, personal communication).

Prior to the publication of these results by Lilly and Thoday, conflicting reports had been made about the effect of cyanide on the production of mutations and chromosome aberrations by x-rays. D'Amato and Gustafsson (2) found that pretreatments with low concentrations of potassium cyanide (10^{-4} to 10^{-3} M) increased the frequency of visible mutations produced by a given dose of x-rays in barley. High concentrations of potassium cyanide (10^{-2} M) decreased the mutation rate but increased the frequency of chromosome aberrations (as evidenced by decrease in F_1 fertility). Mikaelsen (10) reported that sodium cyanide in a concentration of 10^{-3} M decreased by 25 per cent the number of fragments per 100 cells produced by a continuous γ-irradiation in root-tip cells of *Tradescantia paludosa*. In *Drosophila*, Sobels (12) observed that pretreatments with potassium cyanide increased considerably the frequency of sex-linked lethals produced by subsequent x-radiations.

The so called cyanide effect has often been discussed in conjunction with the oxygen effect. As discovered by Thoday and Read (17) and subsequently confirmed by other workers, the frequency of aberrations produced by a given dose of x-rays is dependent on the oxygen tension prevailing at the time of the treatment. The x-ray effect is usually about three times higher in air (21 per cent oxygen) than in the absence of oxygen. A further increase in oxygen con-

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centration above 21 per cent has little influence on the x-ray effect. If the oxygen effect in the case of x-rays were connected with cytochrome respiration, cyanide, as a strong inhibitor of cytochrome oxidase, would be expected to have a protective effect similar to that of anoxia. On the other hand, if the oxygen effect is due to the fact that hydrogen peroxide is produced in aqueous solutions by x-rays only in the presence of oxygen (18), cyanide would be expected to increase the x-ray effect in air, since the peroxide-destroying enzymes, catalase, and peroxidase, are strongly inhibited by cyanide (19). In the latter case it is assumed "that hydrogen peroxide has some influence on the process involved in chromosome structural damage" (18).

Lilly's results do not appear to be in agreement with either of these hypotheses. The experiments described below support our belief that a new interpretation of the cyanide effect on x-ray breakage is necessary.

Materials and Methods

The experimental materials consisted of lateral roots of the broad bean, *Vicia faba* (English variety "Seville longpod"). The roots were grown and the treatments applied as described previously (5, 6). The chemicals tested were mainly the same as those used in the previous study in this series (7). As a rule, the x-radiations were given at 80 kv., filter 2 mm. Al., intensity 12 to 13 r/minute. The data on which Fig. 1 is based were, however, obtained after irradiation with non-filtered 60 kv. x-rays, intensity 16 r/minute. The anaerobic treatments were performed in sealed glass vials (5) after oxygen had been removed by bubbling nitrogen through the system for 10 minutes. The x-radiations were given at the end of ½ hour treatments with anoxia and/or chemicals so that the period of irradiation was included in the period of the other treatments.

Fixations were made in 3:1 alcohol-acetic acid, and the slides were prepared as Feulgen squashes. Two methods of scoring were adopted: either the number of anaphases containing fragments and bridges were scored, or the frequencies of isolocus breaks (16) and exchanges were analysed after a prefixation treatment in 0.05 per cent colchicine for 2½ to 4 hours.

RESULTS

Since we do not wish to anticipate the detailed report of the potassium cyanide (KCN) effect on x-ray breakage soon to be published by Lilly, we shall describe only those results which are of importance for our interpretation of the cyanide effect.

Our observations agree with those of Lilly and Thoday (9) in that there is an effect of cyanide on x-ray breakage under anaerobic conditions, but no effect in the presence of oxygen. The data in Table I and the curves in Fig. 1 show that, in the presence of cyanide, the effect of a given dose of x-rays in nitrogen is actually greater than the effect of the same dose in air. Cyanide exerts its effect on x-ray breakage in nitrogen in concentrations considerably lower than those effective in producing cyanide breakage ($>10^{-4}$ M); 5×10^{-5} M KCN being as effective as 10^{-3} M (the highest concentration tested) in increasing x-ray breakage, and 2×10^{-5} M still produced a substantial increase. At a concentration of 5×10^{-6} M, KCN was without effect on x-ray breakage.

It was important to decide, in the experiments combining x-rays and cyanide, whether the increased frequency of aberrations was the result of increased sensitivity to x-rays induced by cyanide, increased sensitivity to cyanide induced by x-rays, or that the increase was a summation of independent effects.

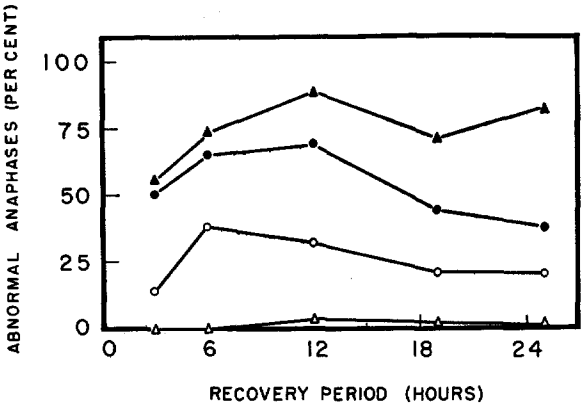

FIG. 1. The percentage of abnormal anaphases observed at different times after treatments with: 120 r x-ray, aerobic (●); 120 r x-ray, anaerobic (○); 10^{-3} M KCN, ½ hr. and 120 r x-ray, anaerobic (▲); 10^{-3} M KCN, ½ hr., anaerobic (△).

TABLE I

The Effect of Potassium Cyanide on X-Ray-Induced Aberrations in Air and in Nitrogen

Concentration of potassium cyanide M	Period of treatment	Treatment atmosphere	x-Ray dose	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
							Isolocus breaks	Exchanges
	hrs.		*r.*	*hrs.*		*per cent*		
—	—	air	100	24	100	55	46	24
5×10^{-5}	½	"	100	24	100	54	48	30
5×10^{-5}	½	"	—	24	100	0	0	0
—	—	nitrogen	100	24	100	27	21	5
5×10^{-6}	½	"	100	24	100	30	21	1
5×10^{-5}	½	"	100	24	100	69	76	32

Since the effect of cyanide in nitrogen is negligible, the latter explanation cannot be correct. We hoped that the distribution of breaks in *Vicia* in these experiments would provide an answer as to which of the first two possibilities is valid.

Table II shows the distribution of isolocus breaks in the long and short chromosomes following treatments with x-rays and cyanide, alone and in combination. The data indicate that the breaks are randomly distributed and that the breakage obtained after the combined treatments is of the x-ray type.

This is even more clearly shown by the distribution of breaks within the chromosomes. X-ray-induced breaks seem to be distributed at random along the arms of the chromosomes, and a similar distribution is obtained after the

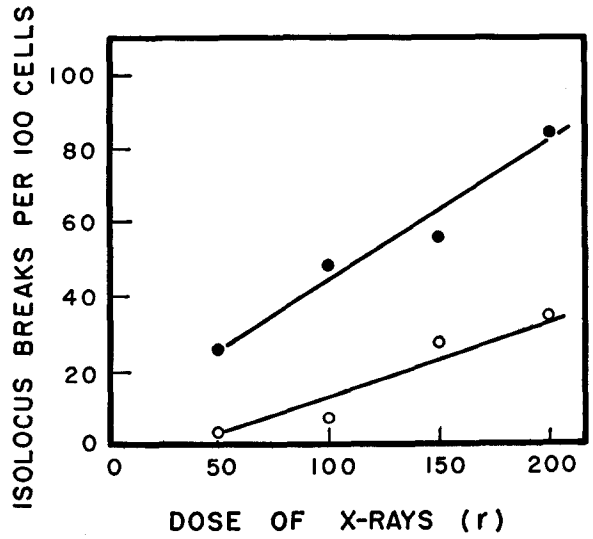

FIG. 2. The effect of potassium cyanide on x-ray sensitivity in anoxia. ○ ½ hr. anoxia (including the time of irradiation). ● ½ hr. 10^{-3} M KCN, anoxia (including the time of irradiation).

TABLE II

The Distribution of Isolocus Breaks between Long and Short Chromosomes in Vicia faba after Various Treatments

(Random distribution gives a ratio of approximately 2.5)

Treatment	No. of isolocus breaks counted			Ratio, short:long
	Total breaks	Breaks in short chromosomes	Breaks in long chromosomes	
x-Ray, air	291	215	76	2.8
x-Ray, nitrogen	372	266	106	2.5
x-Ray, nitrogen, KCN	710	505	205	2.5
KCN, air	735	562	173	3.2
KCN, nitrogen	26	20	6	3.3

combined treatments. Breaks induced by cyanide, on the other hand, tend to be concentrated in heterochromatic segments of the chromosomes, a phenomenon common to other chemical mutagens.

In Fig. 2, the frequencies of isolocus breaks obtained 24 hours after anaero-

bic x-ray treatments in the presence of cyanide are plotted against the x-ray dose. The results indicate that the sensitization of the chromosomes is a general one, not leading to a set increment of breaks irrespective of x-ray dose.

In addition to potassium cyanide, a number of other compounds were tested for their ability to increase the frequency of chromosome aberrations produced by x-radiation in nitrogen (or air). These compounds included the heavy metal

TABLE III

Comparison of the Effects of Potassium Cyanide and Cupferron Alone and in Combination with X-Rays

Chemical	Concentration M	Treatment atmosphere	Period of treatment (anoxia and/or chemical)	x-Ray dose	Recovery period	No. of cells analyzed	Abnormal metaphases	Aberrations per 100 cells	
								Isolocus breaks	Exchanges
			hrs.	*r.*	*hrs.*		*per cent*		
Potassium cyanide	10^{-3}	Nitrogen	½	—	24	100	6	2	3
" "	5×10^{-4}	"	4	—	24	100	11	6	9
Cupferron	10^{-3}	"	½	—	24	100	2	1	1
"	10^{-3}	"	2½	—	24	100	9	6	2
Potassium cyanide	10^{-3}	Air	½	—	24	100	32	31	9
" "	10^{-3}	"	1½	—	30	100	32	38	13
Cupferron	10^{-3}	"	2½	—	24	100	4	3	1
"	10^{-3}	"	5	—	30	100	16	13	5
—	—	Nitrogen	½	125	24	100	42	39	8
Potassium cyanide	10^{-3}	"	½	125	24	100	67	64	31
—	—	"	½	100	24	100	27	21	5
Cupferron	10^{-3}	"	½	100	24	100	55	42	34

complexing agents cupferron (the ammonium salt of N-nitrosophenyl hydroxylamine), sodium diethyldithiocarbamate, 8-hydroxyquinoline, carbon monoxide, sodium fluoride, and sodium azide, as well as the chemicals 8-ethoxycaffeine, maleic hydrazide, and *t*-butyl hydroperoxide. Of these compounds, only cupferron proved to be similar to cyanide in producing an increased x-ray breakage under anaerobic conditions. It has also been found (7) that, in anaerobic conditions, the radiomimetic effects of cupferron and potassium cyanide are quantitatively and qualitatively indistinguishable. After similar treatments with the two compounds, similar frequencies of aberrations were obtained, the breaks were similarly localized, and the interphase sensitivity was the same. Under aerobic conditions the effects of cupferron and cyanide

differ, however, cyanide being much the more effective mutagen. In Table III, the effects of KCN and cupferron, alone and in combination with x-rays, are compared.

DISCUSSION

The results described above show that when root-tip cells of *Vicia faba* are irradiated under anaerobic conditions in the presence of potassium cyanide, a frequency of aberrations is obtained which is even higher than that produced by the same x-ray dose in air. This finding is in agreement with the results of Lilly and Thoday (9), and Lilly (personal communication). In further agreement with Lilly and Thoday, no similar increase in sensitivity to x-rays was obtained when combination exposures were made in the presence of oxygen. Our results also show that increased sensitivity to x-radiation under anaerobic conditions is obtained with concentrations of potassium cyanide as low as 2×10^{-5} M, a concentration that is definitely lower than the threshold concentration for the radiomimetic effect of potassium cyanide in air, the latter being approximately 10^{-4} M (7). This fact, as well as the evidence that the distribution of breaks obtained is characteristic of x-radiation rather than of cyanide, indicates that it is the x-ray breakage and not the cyanide breakage which is increased. Furthermore, late interphase cells, which are unaffected by cyanide insofar as breakage is concerned (7), show the same increase in the frequencies of aberrations after the combined treatments as do cells at other interphase stages.

Sobels (12) observed that pretreatments with potassium cyanide or sodium azide significantly increased the frequency of sex-linked lethals in *Drosophila melanogaster* and tentatively ascribed these results to an increased production of hydrogen peroxide in the pretreated irradiated germ cells. Sobels thus supports an hypothesis which had been proposed previously by Wyss *et al.* in order to explain the mutagenic effect of azide on bacteria. The rationale of the hypothesis is that, "Azide, which is a general poison for iron-porphyrin type enzymes, will not only inhibit catalase, ... but will also inhibit the cytochrome system, thus forcing more of the aerobic respiration through the peroxide-producing spontaneous oxidation of the flavoprotein enzymes" (21). The hypothesis that an inhibition of cytochrome oxidase would result in an increased production of hydrogen peroxide was also adopted by King, Schneiderman, and Sax (8) to explain their finding that the frequency of x-ray–induced chromosome aberrations in *Tradescantia* microspores was increased when the irradiating was done in a gas mixture consisting of five atmospheres of carbon monoxide and one atmosphere of air. Recently Sobels (13) has found that the frequency of sex-linked lethals produced in *Drosophila* by a given dose of x-rays can be increased by dihydroxydimethyl peroxide as well as by cyanide and azide, and he considers that this observation supports his hypothesis that enzymatically produced peroxides are responsible for the increased effect of x-radiation on the mutation rate in *Drosophila* after pretreatments with azide and cyanide.

As has already been pointed out, it is exceedingly difficult to explain the

cyanide effect, discovered by Lilly and Thoday and confirmed by us, in terms of the peroxide hypothesis. Assuming that the peroxide hypothesis were valid, one would expect the effect of cyanide to occur in the presence of oxygen and not in its absence. The reverse is, however, true. The presence of cyanide during x-irradiation in anoxia results in a great increase of aberrations. In addition to these facts, results obtained from studies involving the use of heavy metal complexing agents do not confirm the assumptions inherent in the peroxide hypothesis. There is little correlation between the ability of an agent to increase x-ray breakage in anoxia and its ability to inhibit catalase and peroxidase (7). Sodium azide, which completely inhibits catalase, and sodium fluoride, which completely inhibits peroxidase, do not increase the aberration frequency induced by x-rays, either when they are used with x-rays separately, or in combination with each other. Cupferron, which does not inhibit peroxidase activity and inhibits catalase incompletely (7), increases the effect of x-rays in anoxia. Furthermore, considering the fact that *t*-butyl hydroperoxide does not influence the x-ray–induced frequency of aberrations, either in aerobic or anaerobic conditions, it does not seem feasible that the action of cyanide and cupferron on x-ray breakage in the absence of oxygen is associated in any way with the accumulation of peroxides, whether organic or inorganic.

An appraisal of the effects of x-radiation and chemical mutagens has led to a consideration of the role played by oxidative metabolism, and by oxidative phosphorylation in particular, in determining the degree of chromosomal damage caused by these agents. The work of Wolff and others (20, 15) supports the idea that metabolic systems in the cell are involved as part of a chain of reactions that begins with the introduction of the agent into the cell and leads to the observable effect. This idea is made more attractive by the discovery (1) of an oxidative system for the generation of energy-rich phosphate operative in isolated nuclei. The effect of cyanide and cupferron on x-ray breakage under anaerobic conditions cannot be explained as due to their effect on oxidative phosphorylation.

An hypothesis, developed more fully in the preceding companion paper (7), is therefore advanced to account for both the radiomimetic action of cyanide as well as the anaerobic effect of cyanide and cupferron on the enhancement of x-ray damage. This hypothesis, in effect, proposes that these two substances are effective because of the presence of iron and/or other heavy metals in the chromosome. The formation of complexes between a heavy metal, presumably iron, in the chromosome and cyanide or cupferron could account for both the radiomimetic action of these agents as well as the anaerobic sensitization of the chromosomes to subsequent doses of x-radiation. The chromosome-bound iron is believed to be autoxidizable and to function as an oxidation-reduction system, being largely in the ferric form in the presence of oxygen and in the ferrous form in its absence.

Assuming the proposed hypothesis to be correct there remains to be ex-

plained the results of the experiments with sodium diethyldithiocarbamate and 8-hydroxyquinoline. The fact that these heavy metal complexing agents do not increase sensitivity to x-rays in anoxia, although treatments with these substances alone result in breakage, does not invalidate, *per se*, the hypothesis that cyanide and cupferron produce their effects as a result of a complexing phenomenon. It is pertinent to mention that the distribution of breaks produced by DIECA and 8-hydroxyquinoline is quite different from that of cyanide and cupferron. This difference may well be the result of differential affinities of these compounds, and the affinity of each compound could conceivably be the determining factor in the qualitative differences of its effect. It is also pertinent to mention the possibility that organic iron complexes with different agents, may be different (and that therefore also their effects may be different). This assumption is supported by observations made during studies of the complexes formed between hematin compounds and various agents.

In viewing, in a somewhat larger sense, the data presented and the hypothesis offered to account for these data, it is of interest to consider whether they cast any light on, or provide for an alternative interpretation of, the oxygen effect in general as it applies to the problem of chromosomal breakage by ionizing radiations. Since Thoday and Read (17) first showed that the frequency of induced aberrations is, in part, oxygen-dependent, numerous interpretations of the oxygen effect have been advanced, and it is now generally agreed that a chain of events—physical, radiochemical, and metabolic (4, 20)—intervenes between the absorption of energy in the cell and the appearance of demonstrable damage. The work of Wolff and Luippold (20) indicates that energy-yielding metabolic events are operative before the rejoining of broken ends of chromosomes occurs, but there is no general agreement as to the mechanism leading to breakage. Thoday and Read (18) suggested that hydrogen peroxide was the active substance, but this concept is not supported by the present data and, indeed, was rejected in favor of the idea that the HO_2 radical was the active agent involved (3). Recently the significance of HO_2 as a mediator of radiation damage has been questioned (14). It may well be, therefore, that the postulated chain of events does not adequately encompass the actions taking place in the chromosome, and that the oxygen effect, insofar as breakage is concerned, stems from the fact that the chromosome is more fragile to ionizing radiations when the bound iron is in an oxidized state than in a reduced form. In ascending order from low to high sensitivity to ionizing radiations, one can therefore propose the following states of the chromosome in terms of its bound iron: ferrous, ferric-complexed, ferric, ferrous-complexed. The cyanide and cupferron experiments reported here are in agreement with this concept.

In the past, one of the main arguments against the view that the oxygen effect can be understood in terms of a changing sensitivity of the chromosome

was the observation that the frequency of aberrations induced by alpha particles, (which produce H_2O_2 in water independent of oxygen tension), is oxygen-independent. Giles (3) has expressed this by stating, "If the effect of oxygen were a metabolic one, there is no reason to suppose that the resulting modification in radiosensitivity would vary with different kinds of radiations." Muller (11), however, points out that the cogency of this argument is weakened if, with a densely ionizing radiation such as alpha particles, the oxygen influence is considered to be largely superfluous since the probability of breakage by an alpha track is close to one. We believe, therefore, that there is a need for a new interpretation of the oxygen effect and have tried to present one that is consistent with the observed phenomena.

SUMMARY

The discovery of Lilly and Thoday, that the presence of potassium cyanide (KCN) increases the production of chromosome aberrations by x-rays in anoxia, but has no effect on the production of chromosome aberrations by x-rays in air, was confirmed.

In the presence of cyanide, the effect of a given dose of x-rays in nitrogen was found to be even greater than the effect of the same dose of x-rays in air. The cyanide effect on x-ray breakage in nitrogen was obtained at cyanide concentrations as low as 2×10^{-5} M. The breakage obtained after the combined x-ray–cyanide treatments was of the x-ray type, as evidenced by the distribution of breaks within and between the chromosomes.

A number of other heavy metal complexing agents as well as some other compounds were tested for their ability to increase x-ray breakage in nitrogen and air. Of these compounds only cupferron proved to be effective.

The results are discussed and it is concluded that the increased x-ray breakage in the presence of cyanide or cupferron cannot be due to an accumulation of peroxides. Instead it is suggested that the cyanide effect may be due to a complex formation between the active agents and heavy metals, presumably iron, within the chromosomes. The consequences of this hypothesis on the concept of the "oxygen effect," are discussed.

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