EXPERIMENTALLY INDUCED CHROMOSOME ABERRATIONS IN PLANTS

I. The Production of Chromosome Aberrations by Cyanide and Other Heavy Metal Complexing Agents

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INTRODUCTION

The interest in the genetic and cytological effects of cyanide was greatly stimulated by the accumulation of data which indicated that hydrogen peroxide was involved in the production of mutations and chromosome aberrations by radiations (46, 43). If hydrogen peroxide is an important cause of mutation and chromosome breakage it would be expected that by inhibiting catalase and peroxidase (the enzymes which are responsible for the destruction of biologically formed peroxide) the natural frequencies of mutation and breakage would be increased.

Realizing this, Wyss *et al.* (46) tested the influence of subinhibitory concentrations of sodium azide on the rate of mutation to penicillin resistant and streptomycin resistant forms in the bacterium *Staphylococcus aureus* and observed a marked increase in the frequency of mutation. In *Neurospora*, Wagner *et al.* (45) observed similar results, using potassium cyanide instead of azide as an inhibitor of the peroxide-destroying enzymes. In contrast to this, Sobels (38) could find no mutagenic effect of these agents in *Drosophila.*

Although the cytological effect of cyanide alone or in combination with other treatments has been tested by several workers (*e.g.*, 28, 35, 19), the radiomimetic effect of this compound escaped detection until recently. The credit for having discovered that postassium cyanide actually is a radiomimetic agent goes to Lilly and Thoday (29) who found that treatments of roots of *Vicia faba* with cyanide resulted in a high frequency of chromosome aberrations, when the treatments were performed in oxygen. In the absence of oxygen, no effect was detected. Lilly and Thoday concluded that the chromosome aberrations observed after treatments with potassium cyanide, may actually have been produced by peroxides, accumulated in the cell as a result of the inhibitory action of cyanide on cytochrome oxidase and on peroxide-destroying enzymes.

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The fact that the frequency of chromosome aberrations produced by cyanide is influenced by oxygen tension is, of course, in agreement with the peroxide hypothesis. This is not the first instance, however, where the effect of a radiomimetic chemical has proved to be dependent on oxygen tension. Previously, oxygen has been found to influence the radiomimetic actions of 8-ethoxycaffeine (16) and maleic hydrazide (20) and in these instances the oxygen effect is correlated with energy-rich phosphate production (18–20), not peroxide accumulation. Therefore, it was necessary to examine the oxygen effect in connection with cyanide to determine the relationships.

If the peroxide hypothesis were correct, one would expect that in the case of other heavy metal complexing agents, there would be a correlation between ability to inhibit peroxide-destroying enzymes and radiomimetic activity. Therefore a comparative study of the radiomimetic effects of a number of heavy metal complexing agents and their ability to inhibit peroxidase and catalase has been made. Finally the cytological effects of hydrogen peroxide and organic peroxides (30) were reexamined and compared with the effect of potassium cyanide.

Materials and Methods

The experimental materials used in these experiments consisted of lateral roots of an English variety "Seville longpod" of the broad bean, *Vicia faba*. The roots were grown and the treatments performed as described previously (19, 20). In the anaerobic cyanide treatments, the treatment vials (19) were sealed after the oxygen had been removed by bubbling nitrogen through the solution. The vials were sealed because a prolonged bubbling of gas through the solution removed the cyanide. As a rule, the cyanide treatments were performed in solution buffered by M/150 sodium phosphate to pH 7 although no significant difference was detected between the effects of cyanide at pH 7 and the effect at pH 9 in an unbuffered solution.

The chemicals tested in this study were (formula or abbreviation within brackets): potassium cyanide (KCN), sodium diethyldithiocarbamate (DIECA), 8-hydroxyquinoline, 2.2-bipyridine, *o*-phenanthroline, cupferron (the ammonium salt of N-nitrosophenylhydroxylamine), potassium thiocyanate (KSCN), sodium fluoride (NaF), carbon monoxide (CO), hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide ($(CH_3)_3C(OOH)$),[1] 8-ethoxycaffeine (EOC), and 2.4-dinitrophenol (DNP).

The root tips were fixed in cold alcohol-acetic acid, 3:1, and the slides prepared as Feulgen squashes. Two methods of scoring were adopted: either the percentage of anaphases containing fragments and bridges was recorded, or the frequencies of isolocus breaks (42) and exchanges were analyzed after prefixation treatments in 0.05 per cent colchicine for 2½ to 3 hours.

RESULTS

A. Cyanide and Chromosome Breakage

Effect of Concentration and Period of Treatment.—Revell has shown that the effect of the radiomimetic chemical di(2.3-epoxypropyl) ether is proportional

[1] I thank Cadet Chemical Corporation, Burt, New York, for supplying the *t*-butyl hydroperoxide.

to dose, *i.e.*, to the product of treatment time and the concentration of the chemical (37). Thus, for instance, a 4-hour treatment with 2.5×10^{-4} M diepoxide produced the same effect as an 1-hour treatment with 10^{-3} M. Table I illustrates that there is little dose proportionality in the case of KCN. Whereas a considerable effect is obtained after a ½-hour treatment with 3×10^{-4} M KCN, a 1½-hour treatment with 10^{-4} M is without effect. In order

TABLE I

The Effect of Potassium Cyanide Treatments in Air, Varying the Concentrations and Treatment Times

Concentration of potassium cyanide	Period of treatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
					Isolocus breaks	Exchanges
	hrs.	*hrs.*		*per cent*		
0 (control)	—	—	500	0.2	0.2	0
10^{-4}	1½	24	100	2	1	0
		30	100	0	0	0
10^{-4}	5	24	100	21	22	4
		30	100	10	4	5
3×10^{-4}	½	24	100	23	22	5
		30	100	10	5	8
3×10^{-4}	1½	24	100	39	56	11
		30	100	28	36	8
10^{-3}	½	24	100	32	31	9
		30	100	20	25	13
10^{-3}	1½	24	80	22.5	22.5	6.3
		30	100	32	38	13

to get the same effect as that produced by the ½-hour treatment with 3×10^{-4} M, 5 hours in 10^{-4} M KCN are required. It seems apparent from Table I that after a certain effect has been reached, a further increase in concentration or treatment time is without influence on the frequency of aberrations. Practically the same effect is obtained after 1½ hour in 3×10^{-4} M KCN, ½ hour in 10^{-3} M KCN, and 1½ hour in 10^{-3} M KCN.

Interphase Sensitivity.—With the exception of EOC (17), radiomimetic chemicals seem to be unable to act on cells in late interphase (37, 34). This is indicated by the fact that no aberrations usually are obtained during the first 8 hours after the treatment. (In Vicia root tips, the cells which are dividing fastest complete one division cycle in about 20 hours at 21°C., mitosis

itself lasting about 2 hours (37).) Potassium cyanide proved to be no exception to this general rule. During the first 8 hours after treatment, some chromosome stickiness was observed and occasionally even a few isolocus breaks or exchanges, but a high frequency of aberrations was never observed earlier than 18 hours after the treatment, the maximum effect occurring between 24 and 30 hours after treatment. This is illustrated in the bottom section of Fig. 2, where the percentages of abnormal anaphases scored after a cyanide treatment are plotted against the recovery period.

TABLE II

The Influence of Temperature on the Effect Produced by 5 × 10^{-4} M KCN at pH 7

Treatment temperature	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
				Isolocus breaks	Exchanges
°C.	*hrs.*		*per cent*		
5	24	100	19	11	9
	30	100	31	28	18
12	24	100	28	35	5
	30	100	22	22	12
17	24	100	38	36	13
	30	100	25	27	8
26	24	100	25	28	2
	30	100	24	32	7

Localization of Breaks.—Whereas x-ray breaks seem to be distributed at random between and within the chromosomes (10, 37), breaks induced by chemicals are, as a rule, concentrated in certain chromosomes and chromosome segments. The breaks may have different localization patterns after treatments with different chemicals. Thus, EOC-induced breaks are concentrated in the nucleolar constriction (21, 19), maleic hydrazide breaks in a heterochromatic segment close to the centromere of the long chromosome (6, 34), and breaks induced by nitrogen mustard and di(2.3-epoxypropyl) ether mainly in heterochromatic segments in the middle of the short chromosomes (10, 37). When breaks are randomly distributed, the ratio obtained by dividing the total number of breaks in the short chromosomes by the total number of breaks in the long chromosomes (the S/L ratio) is about 2.5. Nitrogen mustard and diepoxide treatments give ratios considerably higher than 2.5, whereas EOC and maleic hydrazide give ratios below 1. The ratio calculated from 735 cyanide breaks was 3.2. This is so close to 2.5 that it seems to indicate a random distribution between chromosomes although breaks within chromosomes are

certainly not randomly distributed, being more highly concentrated in chromosome segments known to be heterochromatic.

Treatment Temperature.—In the cases so far investigated, the chemically induced chromosome damage was found to be drastically influenced by treatment temperature (20, 41). Surprisingly enough, the radiomimetic effect of cyanide proved to be practically independent of temperature during treatment (Table II). The reason for this is not understood at the present time.

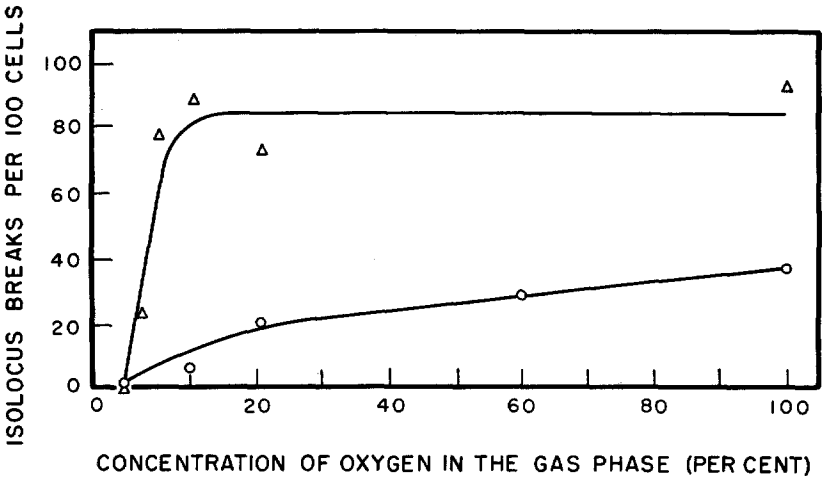

FIG. 1. The effect of oxygen tension on the frequencies of isolocus breaks observed after treatments with 8-ethoxycaffeine (△) and potassium cyanide (○).

The Effect of Oxygen Tension and the Influence of Pretreatments with DNP.—In order to analyze the influence of oxygen on the cyanide effect in more detail, the effect of KCN was tested at five different oxygen concentrations, *viz.*, zero (nitrogen), 10, 21 (air), 60, and 100 per cent. The results are expressed graphically in Fig. 1, where the frequencies of isolocus breaks are plotted against oxygen concentration. As a comparison, the corresponding curve for EOC is included in the graph. The two curves are entirely different. The EOC curve has the slope expected when an enzyme with high oxygen affinity is involved (*e.g.*, cytochrome oxidase) so that saturation is obtained at low oxygen concentrations. The KCN curve illustrating an increasing frequency of aberrations with increasing oxygen concentrations from zero to 100 per cent, is of the type expected if the enzyme involved either has a low affinity towards oxygen (*e.g.*, flavoproteins), or is embedded in a tissue resistant to the entry of oxygen (15).

Table III shows that KCN is not completely inactive in the absence of oxygen, although its effect is considerably less in nitrogen atmosphere than

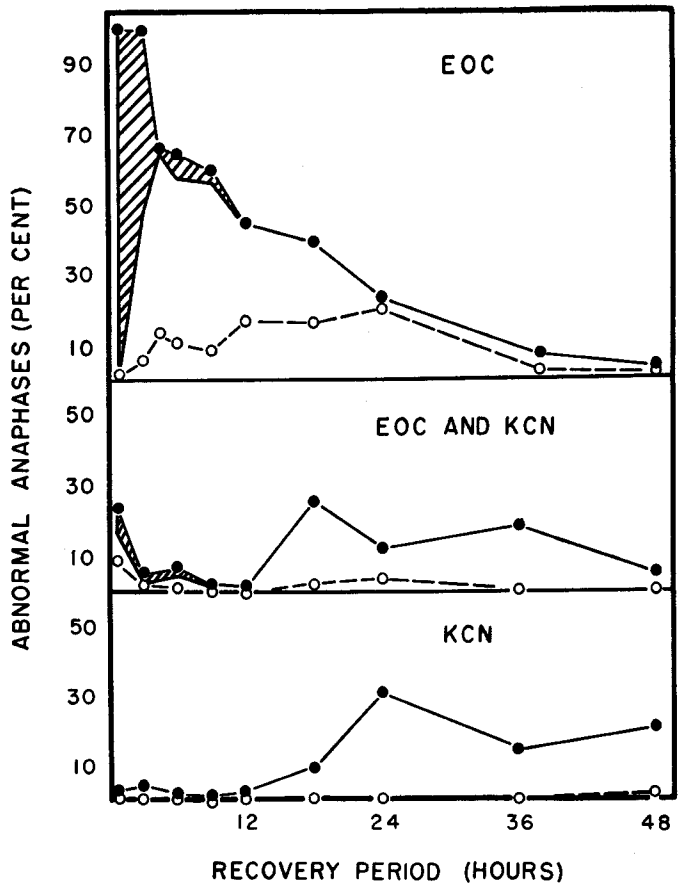

FIG. 2. The percentages of abnormal anaphases observed at different times after treatment with 8-ethoxycaffeine (EOC) and potassium cyanide (KCN) alone and in combination. ●—● = anaphases with sticky bridges and/or structural chromosome changes (total percentages of abnormal anaphases). O----O = anaphases with breaks localized in the nucleolar constriction. Striped area = anaphases with sticky bridges.

TABLE III

Comparison of the Effects of Potassium Cyanide in Air and in Nitrogen

Concentration M	Treatment atmosphere	Period of treatment	Recovery period	No. of cells analyzed	Abnormal metaphases	Aberrations per 100 cells	
						Isolocus breaks	Exchanges
		hrs.	*hrs.*		*per cent*		
10^{-3}	nitrogen	½	24	100	6	2	3
5×10^{-4}	"	4	24	100	11	6	9
10^{-3}	air	½	24	100	32	31	9
10^{-3}	"	1½	30	100	32	38	13

in air. To ascertain whether or not the effect observed after treatments in nitrogen is due to leakage of oxygen into the sealed treatment vial, parallel treatments were performed with EOC, whose effect, as illustrated by Fig. 1, is more sensitive to low concentrations of oxygen than that of KCN. EOC did not produce any breakage under the conditions employed.

The difference between the oxygen effects in the case of EOC and KCN is emphasized by the fact that the radiomimetic effect of KCN was not influenced by pretreatments with DNP as was the effect of EOC (19).

TABLE IV

The Influence of Potassium Cyanide (KCN) Pretreatments in Air and in Nitrogen on the Effect of Two-Hour Treatments with 10^{-2} M 8-Ethoxycaffeine at 12° C.

Pretreatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells			
				Isolocus breaks		Exchanges	
				Total	Localized in NC*	Total	Localized in NC
	hrs.		*per cent*				
—	6	100	46	35	10	28	0
	24	100	32	31	30	1	0
10^{-3} M KCN, ½ hr., air	6	100	2	0	0	1	0
	24	100	18	11	2	6	0
10^{-3} M KCN, ½ hr., nitrogen	6	100	10	8	1	3	0
	24	100	2	7	3	1	0

* NC = nucleolar constriction.

The Influence of Potassium Cyanide on the Effect of 8-Ethoxycaffeine.—In previous papers (18, 19) cyanide, like azide, was reported to produce a complete inhibition of the EOC effect. In these studies no breakage by cyanide itself was observed. In connection with the present study, the effect of combined KCN-EOC treatments was reexamined. Fortunately, it is possible after such combined treatments to distinguish between KCN and EOC breakage. The first 8 hours after the treatment, there is little, if any, KCN breakage, whereas the EOC effect is maximal at this time. Therefore, it can safely be assumed that almost all breakage occurring during the first 8 hours after the combined treatment is EOC breakage. Twenty-four hours after treatment, there is both KCN and EOC breakage, but the effects of the two chemicals can still be separated, since more than 90 per cent of the breakage obtained after 2-hour treatments with 10^{-2} M EOC is localized in the nucleolar constriction.

In Fig. 2 the effect of a 2-hour treatment at 17°C. with 2.5×10^{-4} M KCN and 10^{-2} M EOC is compared with the effects of the two chemicals alone. It

is evident both from the shape of the curve and from the frequencies of cells with breaks in the nucleolar constriction that practically all the breakage occurring after the combined treatment is KCN breakage. Table IV illustrates that pretreatments with KCN have a similar effect on the production of aberrations by EOC. When the KCN pretreatment is made in the absence of oxygen, the protection it affords against the EOC effect seems to be somewhat less.

In these experiments, there is clear evidence of KCN breakage. The reason that the radiomimetic effect of KCN was not detected in the earlier experiments is probably that the high KCN concentration and long treatment periods used resulted in such a strong inhibition of mitosis that the much delayed KCN breakage did not appear during the experimental period, which did not exceed 19 hours.

B. The Radiomimetic Effect of Other Heavy Metal Complexing Agents

In addition to potassium cyanide, a number of other heavy metal complexing agents were tested for radiomimetic activity. The following were found inactive: sodium fluoride (10^{-2} M), potassium thiocyanate (10^{-2} M), *o*-phenanthroline (10^{-3} M), 2.2-bipyridine (10^{-2} M), and carbon monoxide at 10°C. in saturated solution. One-hour treatments with sodium azide in concentrations from 5×10^{-4} to 5×10^{-3} M at pH 4 and 7 did not produce any chromosome aberrations. Some occasional chromosome aberrations were obtained after long periods of treatment with 5×10^{-4} M NaN_3 (Merz, unpublished). A 1-hour treatment with a mixture of 10^{-2} M NaF and 10^{-3} M NaN_3 proved to be without effect (Merz, unpublished).

Low but quite significant radiomimetic effects were obtained after treatments with cupferron, DIECA, and 8-hydroxyquinoline (Table V). Like potassium cyanide, these compounds acted mainly on cells in early interphase. Treatments with cupferron resulted in a distribution of breaks similar to that observed after treatments with KCN. Most of the breaks produced by DIECA and 8-hydroxyquinoline were, unlike cupferron and KCN, localized in the long chromosomes. 8-Hydroxyquinoline has previously been reported to be radiomimetic by Tjio and Levan (44).

The effect of cupferron was analyzed in more detail because it seemed to be so similar to that of KCN. In contrast to the effect of KCN, the effect of cupferron was unaffected by oxygen tension. Under anaerobic conditions the effects of the two compounds are indistinguishable and, as reported in detail in another paper (22), KCN and cupferron proved to have the same effect on the anaerobic production of chromosome aberrations by x-rays.

C. The Effect of Cyanide and Other Heavy Metal Complexing Agents on Catalase and Peroxidase Activity in Vicia

For these experiments, beans of *Vicia faba* were soaked for 24 hours and grown in vermiculate or with their root tips immersed in tap water. When the roots were about 2 cm. long,

they were excised and homogenized in ice cold 0.1 M phosphate buffer, pH 7. The homogenate was centrifuged and the supernatant used as a source for catalase and peroxidase. The catalase activity was determined according to the titration method of Feinstein (9), using perborate as a substrate. The peroxidase activity was determined according to the guaiacol test (5) in the laboratory of Dr. T. Sippel and under his guidance.

TABLE V

The Frequencies of Chromosome Aberrations Resulting from Treatments with Cupferron, DIECA, and 8-Hydroxyquinoline

Chemical	Concentration M	Period of treatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
						Isolocus breaks	Exchanges
		hrs.	*hrs.*		*per cent*		
Control	—	—	—	500	0.2	0.2	0
Cupferron	10^{-3}	1	26	100	9	7	2
"	10^{-3}	5	30	100	16	13	5
DIECA	2.5×10^{-3}	3	30	100	6	6	4
"	5×10^{-3}	1	30	100	11	14	4
8-Hydroxyquinoline	3×10^{-4}	1	26	200	9.5	6.5	2.5

TABLE VI

Production of Chromosome Aberrations and Inhibition of Catalase and Peroxidase Activity in Vicia Faba Root Tips by Known, Heavy Metal Complexing Agents

Chemical	Concentration tested M	Radiomimetic effect	Inhibition of enzyme activity	
			Peroxidase	Catalase
			per cent	*per cent*
Potassium cyanide	10^{-3}	++	100	100
Sodium azide	10^{-3}	(+)	0	100
	10^{-2}	Toxic	60	100
Sodium fluoride	10^{-2}	−	100	0
Potassium thiocyanate	10^{-2}	−	45	85
Cupferron	10^{-3}	+	0	85
8-Hydroxyquinoline	2.5×10^{-4}	+	40	10
o-Phenanthroline	10^{-3}	−	0	0

In Table VI, the ability of the compounds to inhibit catalase and peroxidase is compared with their ability to produce chromosome aberrations. Potassium cyanide, which has the strongest radiomimetic activity, inhibited both peroxidase and catalase. At a concentration of 10^{-3} M, NaN_3 affected only the activity of catalase. A 60 per cent inhibition of peroxidase was obtained with 10^{-2} M NaN_3. NaF (10^{-2} M) inhibited peroxidase completely but was without

effect on catalase. None of the other compounds tested produced a complete inhibition of catalase or peroxidase. DIECA could not be tested because it was decomposed in the reaction mixture. According to Galston (11), DIECA does not inhibit plant catalase.

The results indicate that in the cases analyzed there is little correlation between ability to inhibit peroxide-destroying enzymes and ability to produce chromosome aberrations. This conclusion is supported by the fact that a mixture of NaF and NaN_3, which would be expected to inhibit both catalase and peroxidase, proved to be non-radiomimetic (Merz, unpublished).

TABLE VII

The Frequencies of Aberrations Resulting from Treatments with Hydrogen Peroxide (H_2O_2) and t-Butyl Hydroperoxide ($(CH_3)_3C(OOH)$)

Treatment	Recovery period	No. of metaphases analyzed	Abnormal metaphases	Aberrations per 100 cells	
				Isolocus breaks	Exchanges
	hrs.		*per cent*		
5×10^{-3} M H_2O_2, 1¾ hrs.	5½	100	0	0	0
5×10^{-3} M H_2O_2, 1 hr.	24	100	0	0	0
10^{-4} M KCN ¼ hr. + 5×10^{-3} M H_2O_2, 10^{-4} M KCN, 1 hr.	24	100	6	4	2
10^{-3} M $(CH_3)_3$ C(OOH), 1 hr.	24	100	13	22	8

D. The Radiomimetic Effect of Peroxides

It appears from Table VII, that hydrogen peroxide does not produce chromosome aberrations in *Vicia faba*. This is in agreement with Loveless (30), who reported H_2O_2 to be non-radiomimetic in *Vicia*. When the roots were treated with KCN before and during the H_2O_2 treatment in order to prevent enzymatic destruction of peroxide, the effect obtained was not greater than that which KCN would have produced alone at high oxygen concentrations.

Loveless (30) found that in contrast to hydrogen peroxide, *t*-butyl hydroperoxide was active in producing breakage and rearrangement in *Vicia*. As appears in Table VII, this finding was confirmed in the present study. Revell (37) has determined the distribution of breaks after treatments with *t*-butyl hydroperoxide and found an S/L ratio of 3.6 (125 breaks). In the present study (130 breaks) an S/L ratio of 4.2 was observed. The breaks seem to be localized in the heterochromatin. Preliminary experiments indicated that the effect of *t*-butyl hydroperoxide is not influenced by oxygen tension.

An interesting effect of DNP pretreatments on the frequency of aberrations produced by *t*-butyl hydroperoxide was observed. In previous studies DNP

pretreatments were found to be either without effect on subsequent treatments with other agents, or they suppressed the production of chromosome aberrations. Pretreatments with DNP did not influence the effect of di(2.3-epoxypropyl) ether (31), x-rays (Merz, unpublished), or cyanide, and inhibited the effect of EOC (19), and maleic hydrazide (20, 41). The effect of *t*-butyl hydroperoxide was found to be greatly increased after pretreatments with DNP. These results will be reported in detail elsewhere.

DISCUSSION

As already indicated in the introduction, the main purpose of this investigation was to examine the validity of the hypothesis that chromosome breakage obtained after treatments with cyanide is produced by hydrogen peroxide, a chemical which is assumed to accumulate in the cell as a result of the inhibitory action of cyanide on the enzymes cytochrome oxidase, catalase, and peroxidase. It has been suggested that more H_2O_2 is formed as a result of the inhibitory action of cyanide on cytochrome oxidase, since this would force "more of the aerobic respiration through the peroxide producing spontaneous oxidation of the flavoprotein enzymes" (46). The inhibition of catalase (and peroxidase) would then result in the accumulation of hydrogen peroxide.

King *et al.* (25) pointed out that the flavoprotein enzymes have a low affinity towards oxygen. Their activity, therefore, increases with increasing oxygen tension up to 100 per cent oxygen. An increased activity would mean an increased production of H_2O_2, and more H_2O_2 would mean more chromosome breakage if this chemical is responsible for the structural chromosome changes observed after treatment with cyanide. Fig. 1 shows that the frequency of aberrations is increased when the oxygen concentration is increased from zero to 100 per cent.

Pretreatments with DNP, a chemical believed to be able to uncouple phosphorylation from respiration, did not influence the frequency of aberrations produced by potassium cyanide and therefore provided no evidence of oxidative phosphorylation being involved in the radiomimetic effect.

It seems that the analysis of the influence of oxygen on the effect of KCN is in agreement with the peroxide hypothesis. The picture is, however, complicated by the results of other experiments which are not compatible with this hypothesis.

According to Loveless (30), hydrogen peroxide does not produce chromosome aberrations in root tips of *Vicia faba*. Loveless' results have been confirmed by Kihlman in the present as well as in previous studies (19). When the hydrogen peroxide treatment was performed in the presence of potassium cyanide in order to prevent the decomposition of H_2O_2, the effect obtained was not greater than that which could be expected to be produced by KCN alone at high oxygen tensions. It may be mentioned in this connection that Kimball and his

coworkers (23, 24) have made extensive studies of the effects of hydrogen peroxide alone and in combination with x-rays on the frequency of mutations in *Paramecium aurelia*. In some of the experiments catalase activity was inhibited by KCN in order to prevent the destruction of H_2O_2. Kimball's results indicate that hydrogen peroxide is ineffective as a mutagen in *Paramecium* either by itself or in combination with x-rays.

An argument used by supporters of the hypothesis that hydrogen peroxide is a mediator of x-ray damage is that the concentration of H_2O_2 around, for example, an alpha particle track could be as high as one molar (12). The result would be that localized parts of the cell, *e.g.*, chromosomes, would be exposed to a concentration of H_2O_2 so high that it would be intolerable to the cell as a whole and therefore could not be externally applied. This argument is, however, not applicable where metabolically formed hydrogen peroxide is concerned. The concentration of H_2O_2, even at the enzyme surface, would probably not be very high, and since flavoproteins seem to be absent in cell nuclei (8, 3), the chemical would have to diffuse into the nucleus from its site of formation in the cytoplasm.

When all the evidence is examined in terms of the validity of the hydrogen peroxide hypothesis, it is difficult to accept hydrogen peroxide as an agent responsible for the structural chromosome changes observed after treatments with cyanide.

Another possibility to consider is that organic peroxides accumulate in the cell as a result of the cyanide treatments. Organic peroxides have been reported to produce chromosome aberrations in *Vicia* (30), as well as mutations in *Neurospora* (7) and *Drosophila* (4, 39). *t*-Butyl hydroperoxide, tested in the present study, produced an effect which as regards localization of breaks and interphase sensitivity was similar to that of potassium cyanide, although pretreatments with DNP influenced the radiomimetic effects of the two compounds differently. The difference could be explained, according to the hypothesis, as due to the fact that in one instance peroxide would be formed in the cell, and in the other must enter the cell from the outside.

Accepting this explanation for the differences observed, the experimental evidence, previously mentioned herein, certainly does not disprove the hypothesis that cyanide treatments result in an accumulation of organic peroxides, which in turn result in the radiomimetic effect. The fact that some aberrations occur after cyanide treatments in anoxia, when no peroxides can be expected to be formed, can be explained as due to the fact that these aberrations are produced in air after the treatment. Inherent in this explanation is the assumption that the peroxide-destroying enzymes do not immediately recover from the cyanide inhibition when the roots are transferred to water.

There is, however, other evidence to be considered in connection with the hypothesis that peroxides are the agents responsible for cyanide-induced

chromosome damage. This evidence comes from experiments with other heavy metal complexing agents. Assuming the hydrogen peroxide hypothesis is correct, one would expect a correlation between the radiomimetic effect of a compound and its ability to inhibit catalase and peroxidase. Assuming the organic peroxide hypothesis is correct, inhibition of peroxidase is by far the most important since organic peroxides are not decomposed by catalase (review of literature in Lemberg and Legge (27)). The results of the experiments with heavy metal complexing agents were not as one would have expected from any of the aforementioned peroxide hypotheses. There was in other words no correlation between radiomimetic effect and ability to inhibit peroxidase and catalase. A mixture of sodium azide and sodium fluoride was without effect (Merz, unpublished) though both enzymes should certainly have been inhibited. The fact that only in the case of potassium cyanide does oxygen tension influence the production of chromosome aberrations makes the argument against the peroxide hypothesis somewhat less convincing. The oxygen effect on cyanide-induced aberrations may be an indication of the fact that the mechanism by which potassium cyanide induces chromosome aberrations is different from that of the other heavy metal complexing agents tested, although the effect of cupferron is qualitatively and quantitatively indistinguishable from the effect of potassium cyanide in anoxia.

There remains one other effect of cyanide which has to be considered. Lilly and Thoday (29) have reported that cyanide increases the frequency of chromosome aberrations produced by x-rays in the absence of oxygen, but is without detectable effect in the presence of oxygen. Kihlman, Merz, and Swanson (22) confirmed this and observed that cupferron had the same effect. They concluded that the peroxide hypothesis could not account for the effect of cyanide and cupferron. Here then, we encounter a situation where the production of aberrations in the presence of oxygen might possibly be due to the formation of organic peroxides, but the increased anaerobic x-ray breakage could not be.

In order to account for all the effects of cyanide, a new hypothesis has been proposed. It is suggested that the cyanide effect on chromosomes is due to complex formation between cyanide and iron and/or other heavy metals localized within the chromosomes.

The idea that metals occur as parts of the chromosome structure has previously been suggested by Mazia (33). The results of his studies on the effects of the chelating agent versene on chromosome structure led him to propose a particulate organization of the chromosome where the units are linked together by bridges of divalent ions. Mazia assumed that the metals were calcium or magnesium or both, and this assumption was supported by the work of Steffensen (40) on breakage of plants grown on calcium-deficient medium. Mazia's observations do not indicate which metal is involved, and it is possible that

his results could as well be explained on the basis that the bridges were formed by iron. Steffensen's results could be due to a more indirect effect of the calcium deficiency than that considered by him. The acceptance of iron as a part of the chromosome structure, however, does not necessarily imply that the calcium (magnesium) hypothesis should be rejected. There is no reason why chromosomes could not contain several metals, perhaps differently bound and with different functions, but all necessary for the structural integrity of the chromosome. It is unlikely, however, that the observed effects of cyanide are due to an effect on calcium bridges, since the biological effects of cyanide in low concentrations ($< 10^{-3}$ M) seem to be connected with its ability to bind heavy metals (15). Similarly, the effects of cupferron, 8-hydroxyquinoline, and DIECA can hardly be explained as an effect on calcium bridges, since according to Albert and Gledhill (2) these agents do not form complexes with calcium and magnesium at pH 7. The same authors did not observe any reactions between cupferron and the heavy metals cobalt, manganese, and zinc. However, DIECA, cupferron, and 8-hydroxyquinoline, all form complexes with ferric and ferrous iron as well as with copper and the three agents have this ability to bind iron and copper in common with cyanide. Iron seems to be the heavy metal most likely to be connected with the observed radiomimetic effects, since it has been reported as a chromosome constituent (see below). It has been suggested that Ca and Mg form chelate bonds with the terminal phosphate groups of different DNA "species" (40). Iron may form similar bonds or it may be complexed with the guanine bases (1) of the nucleic acids. Finally it may be that iron is only absorbed on the chromosomes and influences their behaviour by affecting electron transfer within the chromosomes in the same way as iron is thought to affect electron transfer between the different oxidation-reduction elements of the electron transport system in mitochondria, a system which is believed by some authors to be a quasiconducting continuum (32, 13).

What is, then, the direct evidence for the existence of iron in the chromosomes?

In the early cytological literature, the occurrence of iron in chromatin was repeatedly reported, but Gulick, reviewing the literature up to 1940 concludes that "a considerable share of the . . . iron reactions in chromatin are quite probably due to outside contaminations . . . and for the remainder it is impossible to know the pre-mortem location of the iron, since the chromosomes are sure to take it over whenever it is released" (14).

Recently, autoradiographic studies of iron metabolism in the larvae of various species of *Drosophila*, have provided convincing evidence for the occurrence of iron in cell nuclei. Using the radioactive iron isotope Fe^{59} in their studies, Poulson and Bowen (36) reported that in some tissues the concentration of iron in the nucleus is considerably higher than in the cytoplasm. The occurrence of iron in nuclear con-

stituents is also indicated by a recent chemical study by Kirby (26), who reported the existence of metallic bonds linking deoxyribonucleic acid (DNA) to proteins in cell nuclei of mammalian tissues. Preliminary analyses of the metal present in the DNA-protein complex from rat liver indicated a relative increase in the amounts of iron and manganese over those present originally in the liver. The idea that chromosomes contain iron is thus supported by both chemical and cytological studies. The unique uncoiling effect which potassium cyanide exerts on chromosomes (28) could also be interpreted as indicating that heavy metals have an important function in maintaining chromosome structure.

It is believed that the proposed chromosome-bound iron is autoxidizable and functions as an oxidation-reduction system, being largely in the ferric state in the presence of oxygen and in the ferrous state under anaerobic conditions.

One might logically ask why all heavy metal complexing agents do not induce chromosome aberrations. The answer would be that all heavy metal complexing agents may not, because of steric hindrance, as well as other reasons, be able to form complexes with organically bound iron.

The fact that chromosome breakage by cyanide is enhanced by oxygen cannot at the present be explained entirely satisfactorily according to the chromosome iron hypothesis. It has to be assumed that cyanide forms different complexes with ferric and ferrous chromosome iron, and that it does not share this property with cupferron. Or it may be that in order to induce chromosome aberrations, the iron actually has to be removed from the chromosomes, and that this is more readily accomplished by cyanide at high oxygen tensions.

The oxygen effect in the case of KCN is, however, more in agreement with the organic peroxide hypothesis. But if the peroxide hypothesis were correct, it would mean that cyanide produces chromosome aberrations by one mechanism and enhances anaerobic x-ray breakage by another. Since the latter effect cannot be explained as a result of an accumulation of peroxides in the presence of cyanide a dual mode of the action of cyanide must be postulated, a mode of action which does not seem to be very likely although, of course, possible. The other alternative, the hypothesis involving chromosome iron, seems therefore preferable as an explanation of the production of chromosome aberrations by potassium cyanide.

SUMMARY

The finding of Lilly and Thoday that potassium cyanide produces structural chromosome changes in root tips of *Vicia faba* was confirmed. Like mustards, diepoxides, and maleic hydrazide, potassium cyanide seems to act on cells at early interphase. A tendency of cyanide breaks to be concentrated in heterochromatic segments of the chromosomes was evident. The production of chro-

mosome aberrations by cyanide proved to be practically unaffected by the temperature during treatment. In agreement with Lilly and Thoday, the effect of potassium cyanide was found to be dependent on oxygen tension during treatment. The effect of potassium cyanide increases with increasing oxygen concentration up to 100 per cent oxygen. In the absence of oxygen, potassium cyanide was not completely inactive, but produced a low, though significant frequency of aberrations. Pretreatments with 2.4-dinitrophenol did not influence the effect of potassium cyanide.

When bean roots were treated with potassium cyanide before a treatment with 8-ethoxycaffeine, or at the same time as they were treated with 8-ethoxycaffeine, the effect of 8-ethoxycaffeine was almost completely suppressed.

The effects of a number of other heavy metal complexing agents were also tested. Sodium fluoride, potassium thiocyanate, carbon monoxide, *o*-phenanthroline, 2.2-bipyridine, and sodium azide were without radiomimetic effect under the conditions employed, and so was a mixture of sodium azide and sodium fluoride. A low, but quite significant, radiomimetic effect was obtained after treatments with sodium diethyldithiocarbamate, cupferron, and 8-hydroxyquinoline. Under anaerobic conditions, the effects of cyanide and cupferron were both quantitatively and qualitatively indistinguishable. Unlike the effect of cyanide, the effect of cupferron was not enhanced by the presence of oxygen.

The effects of the same heavy metal complexing agents were tested on the activities of the enzymes catalase and peroxidase. The activities of both of these enzymes were found to be totally inhibited only by potassium cyanide. In the other cases, little correlation was found between ability to inhibit the activities of these enzymes and ability to produce chromosome aberrations.

In a number of experiments, hydrogen peroxide was found to be without radiomimetic effect, whether alone or in combination with potassium cyanide. *t*-Butyl hydroperoxide proved to be active. The effect of *t*-butyl hydroperoxide was substantially increased by pretreatments with 2.4.-dinitrophenol.

The results are discussed, and it is concluded that the observations made do not support the hypothesis that hydrogen peroxide is involved in the production of chromosome aberrations by potassium cyanide. The possibility that organic peroxides are involved cannot be excluded on the bases of the experimental results. As an alternative hypothesis, it is suggested that iron or other heavy metals are present in the chromosomes and that cyanide and other heavy metal complexing agents produce chromosome aberrations by reacting with these metals.

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